Novel Mechanisms for Controlled Antigen Expression (KODE™ CAE) on Erythrocyte Membranes.

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Abstract

Background: A major function of blood banking laboratories around the world is the ABO typing of blood from donors and recipients for the purposes of transfusion. Incorrect determination of donor and to a lesser degree recipients ABO blood types can result in a fatal outcome. Figures indicate that about 1:10,000 units of blood transfused is of incompatible group and death has been reported to ensue in 10% of cases. Some 33% of the mistakes that lead to these transfusion reactions have been attributed to breakdowns in blood banking procedure. Astoundingly, there is no standardised positive control in this field. A cell that weakly expressed blood group A and/or B antigens would be ideal for this purpose, but no natural weak subgroup cell is suitable as a control. The natural phenomenon by which glycolipids are able to be taken up by the RBC membrane was exploited to add blood group active A or B antigens and synthetic analogues to group O RBCs, thereby making them blood group A or B respectively. Control over the amount of antigen expressed is crucial.

Methods: Several methods for the transformation of cells to express low amounts of A and/or B antigen by insertion were evaluated; including insertion of natural, modified-natural, and specifically engineered synthetic glycolipids into group O cells. Alternative methods using enzymes were also investigated - using glycosidases to strip antigen from cells and glycosyltransferases to synthesise A and/or B antigens on group O cells.

Results: All the methods trialled were able to produce cells that showed weak expression of antigen against ABO blood grouping antisera. However, the synthetic glycolipid was found to offer significant advantages over the other methods. These features include: aqueous solubility, reactivity with all relevant active antibodies due to the generic presentation of the trisaccharide antigenic determinant, excellent batch to batch reproducibility, purity and homogeneity, capacity for production of unlimited quantity, superior insertion (compared to natural glycolipids both in efficiency and in low temperature insertion), and unmatched stability of transformed cells.

Conclusion: Following a successful field trial and further validation trials, synthetic glycolipids were used to prepare an ABO analytical control in the product known as Securacell®, which was launched onto the Australian market by CSL Biosciences. This thesis details the successful development of the world's first ABO blood grouping analytical sensitivity control system.

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Statement of Originality

I hereby declare that this submission is my own work, and that to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma at a University of other institution of higher learning, except where due acknowledgement is made.

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Auckland

November, 2005

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Abbreviations

A-6/14	Natural blood group A glycolipid sample extracted from RBCs
AC	(-$CO(CH_2)_5NH$-)
Ad	Adipate (-$CO(CH_2)_4CO$-)
AUT	Auckland University of Technology
Av	Avidin
$BioA_{tri}$-*x*	Biotinylated trisaccharide with blood group A determinant
$BioB_{tri}$-*x*	Biotinylated trisaccharide with blood group B determinant
BioG	Biotinylated gangliosides
BioIgG	Biotinylated Immunoglobulin G (gamma)
BioUEA	Biotinylated *Ulex europaeus* lectin
BSA	Bovine serum albumin
CAT	Column agglutination technology
CSL	CSL Immunohaematology group (a division of CSL Ltd), Melbourne, Australia
DBA	*Dolichos biflorus* Aureus lectin
DMSO	Dimethyl sulfoxide
DOG	Bi-lipid tail – rac-1,2-dioleoylglycerol
DOPE	Bi-lipid tail – 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine
DSPE	Bi-lipid tail – 1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine
FITC	Fluorescein isothiocyanate
Flu	Fluorescein
Fuc	L-Fucose
Gal	D-Galactose
GalNAc	*N*-acetyl-D-galactosamine
GEM	Glycosphingolipid-enriched microdomain
GlcNAc	*N*-acetyl-D-glucosamine
GTA	α3-*N*-acetylgalactosaminyltransferase
GTB	α3-galactosyltransferase

HPLC	High performance liquid chromatography
IgG	Immunoglobulin G (gamma)
IgM	Immunoglobulin M (mu)
Le^b-6	Natural glycolipid sample extracted from O Le(a-b+) RBCs and purified to Le^b-6 homogeneity
Lipo	Single lipid tail – octadecanoic acid
Mops	Morpholene propane-sulphonic acid
PBS	Phosphate buffered saline
R	Remaining part of a molecule
RBC	Red blood cell (erythrocyte)
sp	Spacer ($-O(CH_2)_3NH-$)
sp_2	Double spacer ($-O(CH_2)_3NHO(CH_2)_3NH-$)
Syn A	Synthetic glycolipid containing blood group A determinant attached to DOPE tail via the spacers sp and Ad.
Syn B	Synthetic glycolipid containing blood group B determinant attached to DOPE tail via the spacers sp and Ad.
tri	Trisaccharide
UEA	*Ulex europaeus* lectin

1 INTRODUCTION

1.1 ABO(H) Blood Groups in Humans

The ABO blood groups of human red blood cells (RBCs) were discovered by Landsteiner in 1901 (as reviewed by Landsteiner, 1931), leading to the chemical characterisation of the ABO(H) antigens in 1956 (as reviewed in Kabat, 1956). The ABO(H) antigens are oligosaccharides which adorn cell membranes as either glycolipids (glycosphingolipids) or glycoproteins i.e. the saccharide antigens are attached to lipids that sit in the outer leaflet of the lipid bi-layer or integral proteins that span the bi-layer (Schenkel-Brunner, 2000).

There are four major blood group types in humans. Cells of the blood group O type bear the H antigen, which, in blood group A individuals, is enzymatically modified to the A antigen. Likewise, the H antigen is modified to the B antigen in blood group B individuals, and blood group AB individuals have copies of both the A and B antigens. The expression of these antigens is determined by the inheritance of one allele from each parent, and the phenotype is expressed according to the Mendelian law of heredity (as reviewed in Race & Sanger, 1975). There are three clusters of alleles, namely A, B or O, with variation in each of these accounting for the subgroups of the A and B groups (see section 1.3.2). The 'O' alleles represent all mutations that result in non-functional transferases and sometimes incorrectly include transferases with activity so low that the antigenic end-products are serologically undetectable (Svensson *et al.*, 2005).

The ABO alleles encode glycosyltransferases, which are enzymes that catalyse the addition of a specific activated monosaccharide residue to the terminus of specific acceptor structures – the H antigens (section 1.1.1). The A enzyme is an *N*-acetylgalactosaminyltransferase, which catalyses an α-linkage between carbon 1 of an activated *N*-acetylgalactosamine (GalNAc) residue and the third carbon of the terminal galactose (Gal) of the H antigen. The B enzyme is a galactosyltransferase, which catayses

the formation of an α-linkage between carbon 1 of an activated Gal and the third carbon of the terminal Gal of the H antigen.

An important distinction of the ABO(H) system is that the carbohydrate blood group antigens present on the cell surface are the secondary products of a group of genes. The primary gene products are the glycosyltransferase enzymes.

ABO blood groups comprise the most clinically significant blood group system in humans. Characterised by the presence of pre-formed antibodies, fatal intravascular haemolysis and disseminated intravascular coagulation can result from the transfusion of blood cells bearing incompatible ABO antigen(s) (see section 1.3.4).

It is not known for what reason the immune system naturally forms such potent antibodies, but they are believed to arise through exposure to environmental A or B antigens early in life (as reviewed in Race & Sanger, 1975). Certainly, A and B antigens, and A- and B-like antigens, are known to exist universally in the microbial, plant and animal kingdoms (as reviewed in Race & Sanger, 1975).

The basic rule of thumb with ABO allo-antibodies is that individuals possess antibodies to antigens they do not themselves express. Thus, individuals of the O blood type, possessing only the H antigen, have antibodies to both the A and B antigens and the common glycotope shared by A and B antigens (anti-A,B). Similarly, individuals of group A have anti-B and B group individuals have anti-A, while group AB individuals have neither antibody. As group A, B, AB and O individuals all have the H antigen (due to incomplete utilisation of this precursor by GTA and/or GTB in blood groups A, B and AB), none of them have anti-H.

1.1.1 Blood Group Antigens

In the case of ABO(H) and Lewis, the term 'blood group antigens' is misleading. In fact, these antigens are also found in tissues (Clausen *et al.*, 1989; Szulman, 1960). As such, the antigens are best described as histo-blood group antigens (Clausen *et al.*, 1989).

Different glycoconjugate precursor chain structures have been identified and classified into six groups based on their terminal disaccharides (Oriol, 1995). These terminal disaccharide structures are shown in Table 1.

Table 1. Structures of glycoconjugate terminal disaccharides of the six classified precursor groups. Note that types 1 and 2 differ only in the location of the carbon on the GlcNAc involved in the bond, types 3 and 4 differ only in the anomeric configuration of the GalNAc (α vs β), and types 5 and 6 differ in the internal saccharide and the location of its carbon involved in the linkage. Type 5 has not been described in man.

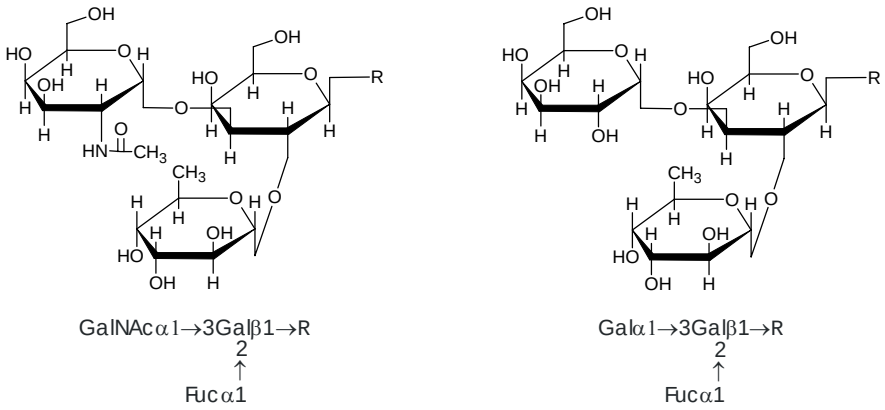
	Disaccharide Structure
Type 1	Galβ1-3GlcNAcβ1-R
Type 2	Galβ1-4GlcNAcβ1-R
Type 3	Galβ1-3GalNAcα1-R
Type 4	Galβ1-3GalNAcβ1-R
Type 5	Galβ1-3Galβ1-R
Type 6	Galβ1-4Glcβ1-R

Glycoprotein antigens predominate on the RBC membrane, with glycolipids carrying only 7% of all saccharides, but glycolipids are easier to isolate in pure form from tissues than glycoproteins, which are also often heterogeneously glycosylated. The blood group-related glycosphingolipids of the RBC membrane are predominantly type 2 (Schenkel-Brunner, 2000). These glycans, along with small amounts of type 3 and type 4 chains, are synthesised by the erythrocytes themselves in the bone marrow prior to RBC enucleation (Schenkel-Brunner, 2000). The type 2 ABO structures exhibit significant heterogeneity – ranging from simple, short glycans (six sugars) to long, complex polyglycosyl structures with multiple branches (>60 sugars) (Schenkel-Brunner, 2000).

Type 1 ABH antigens account for a small proportion of RBC glycosphingolipids and they are not synthesised by the RBCs themselves: instead they are synthesised in epithelial tissue and released into the serum, from where they are secondarily acquired by RBCs (Schenkel-Brunner, 2000). The Lewis glycosphingolipids are secondarily

acquired from the plasma in the same way as the type 1 ABH antigens (as reviewed in Henry *et al.*, 1995).

The A and B antigenic determinants for the human blood groups are trisaccharide structures that are different by only an *N*-acetyl group versus the hydroxyl group.



Blood group A trisaccharide Blood group B trisaccharide

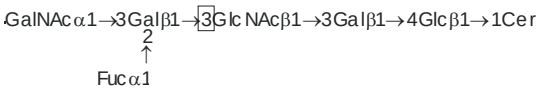
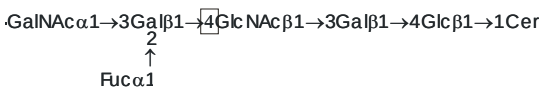
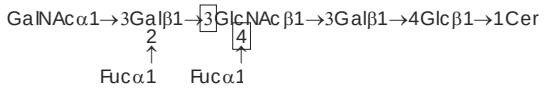
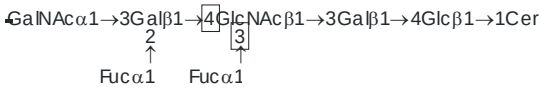
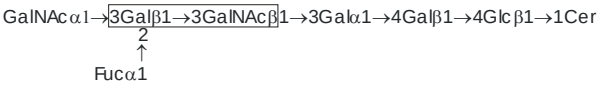
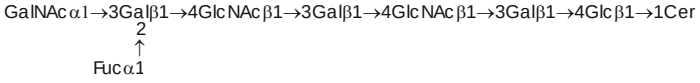
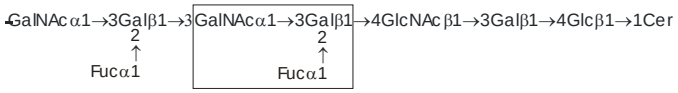
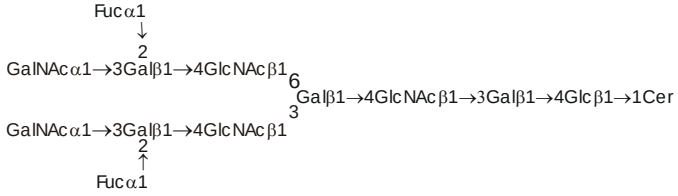
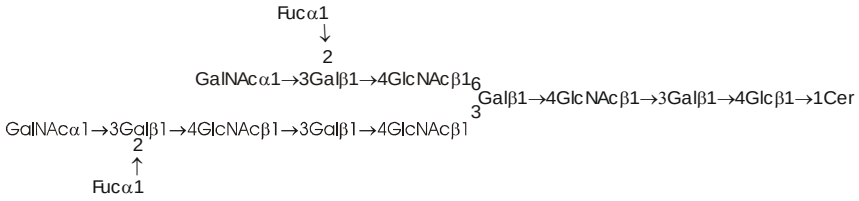
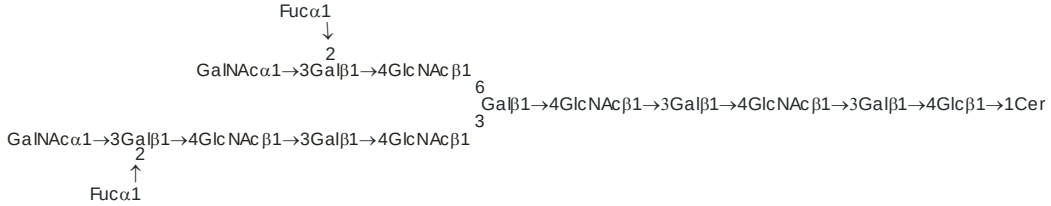
Figure 1: The terminal trisaccharide determinants of the blood group A and B antigens. Left – the blood group A trisaccharide determinant, and right – the blood group B trisaccharide determinant. Note that the A determinant has an *N*-acetyl galactosamine, while the B determinant has a galactose – this means that the difference between the two is an *N*-acetyl group (in box on left) versus the hydroxyl group (in box on right).

The RBCs of blood group A individuals bear the A trisaccharide glycotope on the end of many different types of carbohydrate chains. Examples of glycolipid A antigens are shown in Table 2. The B antigens of blood group B individuals generally have the same types of carbohydrate chain structures, with the exception of the A-9-3 structure.

The orientation of the blood group antigens in relation to the cell differs among the different chain types. The whole chain is almost perpendicular to the membrane in type 1, but is more parallel in types 2, 3 and 4. Furthermore, the α1-2 fucose of the A antigen faces the cell membrane when it is on type 2 chain, but faces out into the environment when it is on types 3 and 4. The huge conformational differences caused by these seemingly small variations may influence antigen-antibody reactivity, which is dependent on a correct 3D fit (Nyholm, 1992).

The ABO phenotypes and subgroup phenotypes are dealt with later in section 1.3.2.

Table 2. Structures of selected blood group A glycolipids. Compare linkage position in type 1 (molecule 1 – β1-3 in box) and in type 2 (molecule 2 – β1-4 in box). These same differences are seen between molecules 3 and 4, which changes the internal fucose linkage also. Molecule 5 is type 4 (box around type 4 disaccharide). Molecule 7 is type 3, which is extension of the chain from an A structure. Two nomenclatures are used in this table – Hakomori (Hakomori *et al.*, 1981) is A-6-1 and A-6-2 etc, while the ALe^b and ALe^y are from Holgersson (Holgersson *et al.*, 1992). Abbreviations: Cer – ceramide.

1	A-6-1		
2	A-6-2		
3	A-7-1	ALe^b	
4	A-7-2	ALe^y	
5	A-7-4		
6	A-8-2		
7	A-9-3		
8	A-12-2		
9	A-14-2		
10	A-16-2		

1.1.2 ABO(H) Glycosyltransferases

The ABO glycosyltransferases are the primary product of the ABO genes. The A and B alleles encode functional transferases, able to transfer UDP-α-GalNAc and UDP-α-Gal (the A and B immunodominant determinants) respectively to the acceptor molecule, the H antigen. The H antigen is essentially a terminal disaccharide with the structure, Fucα1-2Galαβ1-R (see Table 3). Inactive versions of both the A and B glycosyltransferases are very common and these are collectively known as the group O mutations. Persons carrying two alleles that have mutations preventing the transcription of active enzymes bear unmodified H on their red cells, and comprise the O blood group.

Table 3. Terminal disaccharide of the H antigen and the trisaccharides of H showing the difference between type 1 and type 2. Abbreviations: R – rest of the molecule.

H disaccharide	Galβ1→R 2 ↑ Fucα1
H type 1	Galβ1→3GlcNAcβ1→R 2 ↑ Fucα1
H type 2	Galβ1→4GlcNAcβ1→R 2 ↑ Fucα1

The wild-type A transferase can attain the specificity of the B transferase by the substitution of 4 amino acids; Arg176→Gly, Gly235→Ser, Leu266→Met, and Gly268→Ala (Seto *et al.*, 1999). Of the four critical amino acids, only those at positions 266 and 268 directly contact the donor. Leu/Met 266 is sited to contact the N-acetyl group of GalNAc or the OH group of Gal, and so is the one that distinguishes between the A or B donor (Patenaude *et al.*, 2002). When the leucine of GTA is in position 266, it admits the relatively large N-acetyl group of GalNAc, which could not be accommodated by the methionine of GTB. This is simply because leucine is the smaller of the two amino acids.

Possibly because of the similarity between the two enzymes, there is substantial cross-over in activity *in vitro*. It has been previously reported that wild-type GTA can transfer the B donor (UDP-Gal) with three times

greater efficiency than wild-type GTB can transfer the A donor (Seto *et al.*, 1999). This is in accordance with the mechanism discussed above – GTA, with its larger pocket designed to accommodate the large N-acetyl group on carbon 2 of GalNAc, can also accommodate and transfer Gal with its smaller hydroxyl group, but GalNAc cannot easily fit into the binding site of GTB, which is designed to accommodate only the smaller Gal. Despite this, there is no significant expression of A antigen in group B individuals or of B in A, however, exceptionally potent reagents have been claimed to detect A in some B individuals, the so-called B(A) phenomenon (Yamamoto *et al.*, 1993). Of interest, there is a mutation which is known as cisAB, where the transferase has both A and B mutations and can utilise A and B sugars equally (Madsen & Heisto, 1968; Seyfried *et al.*, 1964). Such a phenotype is very rare, and is distinguished from the normal AB individual who has both A and B transferases.

Mutation of the ABO alleles can cause variation in the efficiency of the resultant enzyme, and accounts for the quantitative differences between the subgroups (see section 1.3.2). Possible mutation-derived variation in the specificity of the enzyme may account for qualitative differences, although the existence of qualitative differences is still much debated. A recent paper from this group was able to show that significant quantitative and comparatively minor qualitative differences in glycolipid profile characterise the subgroups (Svensson *et al.*, 2005).

1.2 The RBC Membrane

All cell membranes are essentially lipid bilayers, where amphipathic lipids orient themselves in sheets 5 nm thick so that the hydrophobic hydrocarbon chains are isolated from the aqueous environment of the cytoplasm or extra-cellular milieu, as in the middle of a sandwich (Singer & Nicolson, 1972). The hydrophilic 'heads', which variously contain phosphorous, nitrogen and oxygen molecules, are in contact with the environment on either side of the membrane. In order for the hydrocarbon chains to be protected from the aqueous environment at the edges, the bilayer sheets spontaneously form sealed compartments, which can also re-form if they are disrupted (as reviewed in Alberts *et al.*, 1994).

The lipid profile of the plasma membrane varies by cell type. The function of the cell membrane is essentially to act as a barrier between the cell and the external milieu, through which the cell is able to regulate interaction.

The erythrocyte has a relatively static membrane. Being enucleated, it cannot synthesise new membrane components.

1.2.1 Glycerophospholipids

Most abundant of the lipids in the cell membrane are the glycerophospholipids, and they form the matrix or background of the bilayer. A sub-class of glycerolipid – two fatty acid tails joined together via glycerol – glycerophospholipids are amphipathic molecules composed of a hydrophobic bilipid tail and a hydrophilic head region containing a phosphate group. The acyl chains of these fatty acids are between 14 and 24 carbons long. An important feature of glycerophospholipids is that one of the fatty acids often has a *cis*-configured double bond that imparts a kink in the hydrocarbon chain (Alberts *et al.*, 1994).

1.2.1.1 Glycerophospholipid Asymmetry

All plasma membranes exhibit compositional differences between the inner and outer leaflets (Bretscher, 1972). This asymmetric lipid distribution is thought to arise through the action of translocator enzymes

(also termed flippases or translocases) that catalyse differential transmembrane movement (flip-flop). The precise distribution is thought to be maintained by specific interactions between the lipids and adjacent membrane proteins, in addition to ongoing translocase activity (as reviewed in Devaux, 1992). The net result is that choline-containing glycerophospholipids (phosphatidylcholine and sphingomyelin) predominate in the outer leaflet, while the amino glycerophospholipids (phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol) are found primarily in the inner leaflet.

Maintenance of this specific glycerophospholipid asymmetry in erythrocytes is of functional importance. For instance, there is evidence that the asymmetry of glycerophospholipids in the membrane helps to preserve the discocyte shape erythrocytes (Daleke & Huestis, 1989), although this function is predominantly carried out by the RBC skeleton.

There is evidence that one of the mechanisms for the identification and removal of worn-out RBCs from circulation is related to the specific distribution of phosphatidylserine in the membrane. Increasing the incidence of phosphatidylserine in the outer leaflet increases adherence of human RBCs to monocytes *in vitro* (Schwartz *et al.*, 1985), and also increases the phagocytosis of murine RBCs by macrophages *in vivo* (Schroit *et al.*, 1985).

1.2.2 Glycosphingolipids

Glycoconjugates are recognised as important tissue structures. The glycoconjugate carbohydrate chain has already been implicated in a diverse range of phenomena, such as the growth and differentiation of cells (Kojima & Hakomori, 1991), cell-cell recognition and attachment (Carson *et al.*, 1994; Fenderson *et al.*, 1986; Hakomori, 1990; Kimber, 2000), bacterial binding to cells (Karlsson, 1989), cancer (Hakomori, 1990; Lloyd, 2000), heart disease (Hein *et al.*, 1992) and other disease processes (Reid & Bird, 1990). Despite this seeming wealth of information, the biological significance of these molecules is still poorly understood, requiring further structural and functional research.

In comparison with the study of proteins, the study of carbohydrates is a small field, but it is growing. The expansion of this discipline is partly due to the sheer amount of information that can be imparted by the glycan structure. The variability in characteristics such as sugar type, linkage type, branch positioning, shape, charge, ring size, and epimeric and anomeric configuration results in more than 1.05×10^{12} possible structural combinations from a six-sugar glycan. This is compared with only 46,656 combinations possible with a chain made up of six amino acids, more than seven orders of magnitude lower than the diversity possible in glycans (Laine, 1994). It is unlikely that this wealth of variation evolved, and is maintained at significant energy cost, to no purpose. Thus, the potential importance of carbohydrates is beginning to be appreciated and the march is on to elucidate their biological functions.

Carbohydrate chains can be conjugated to protein or lipid molecules in biological systems; such molecules are called glycoproteins or glycolipids respectively. Glycosphingolipids are a class of glycolipids in which the carbohydrate chain is conjugated to a sphingolipid, the trivial name of which is ceramide. Glycosphingolipids constitute about 10.5% of the RBC membrane or 0.53 mg x 10^{-10} per cell (as reviewed in Schenkel-Brunner, 2000).

The study of glycosphingolipids has its beginnings in the early analysis of the brain, however, research into blood-group antigens did not begin until the turn of the 20th century when Karl Landsteiner discovered the ABO(H) system of human erythrocytes (Oriol, 1995).

1.2.2.1 Carbohydrate Moiety

Neutral blood group-related glycosphingolipids are composed primarily of six different monosaccharide residues, namely β-D-glucose (Glc), N-acetyl-β-D-glucosamine (GlcNAc), α-D-galactose (β-Gal), β-D-galactose (α-Gal), N-acetyl-α-D-galactosamine (GalNAc) and α-L-fucose (Fuc) (Figure 2). The sugars are linked by glycosidic bonds catalysed by a family of enzymes called glycosyltransferases. Biosynthesis of glycans is genetically controlled in terms of the presence or absence of the different types of glycosyltransferases, which are gene encoded. However, it is

unclear whether the order in which the stepwise addition of specific substrate residues to specific acceptor structures occurs is directly controlled or a matter of chance (see also section 1.2.5).

Glycosidic bonds are the result of a condensation reaction between the hydroxyl group of carbons 2, 3, 4 or 6 of one residue that is already part of the growing chain with the anomeric carbon hydroxyl group of a second sugar. The bond is considered to belong to the sugar whose anomeric carbon is involved, and is expressed as (1-2), (1-3), (1-4) or (1-6) depending on the point of attachment.

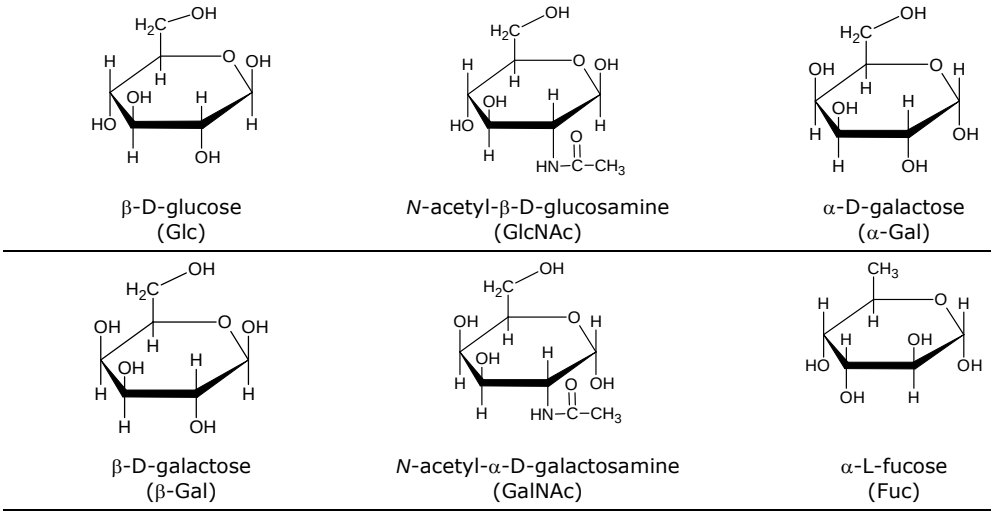
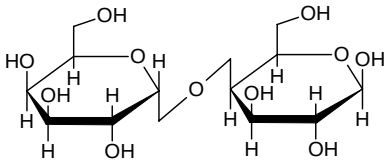
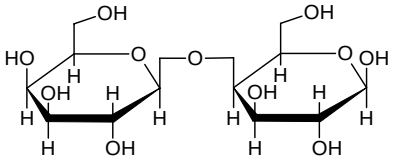


Figure 2. Haworth projection formulae of the sugar residues commonly found in blood group related glycoconjugates. The full and abbreviated (in parentheses) names are below the individual sugars.

Two types of glycosidic bond are possible; this depends on the configuration of the anomeric carbon hydroxyl group involved in the bond. The bond formed when the anomeric hydroxyl group is in the α configuration is designated an α bond (Figure 3). Likewise, a β bond will result when the anomeric hydroxyl is in the β configuration (Figure 3). The two monosaccharide cyclic hemiacetals - α and β - are in equilibrium with the open chain formation when in aqueous solution. However, when joined together by glycosidic linkages, the residues become fixed in the ring formation, with the exception of the reducing end residue, which can remain open (Pazur, 1994).



Galα1-4Gal



Galβ1-4Gal

Figure 3. The α and β linkage. Upper - an α1-4 linkage between galactoses. Lower - a β1-4 linkage between galactoses.

1.2.2.2 Lipid Moiety

Sphingolipids are composed of a long-chain base and a fatty acid.

Long-chain bases

The long-chain bases are amino alcohols attached to long, sometimes saturated, aliphatic hydrocarbon chains. About 60 long-chain bases have so far been characterised (Thompson & Tillack, 1985). The structure of long-chain bases can vary in four major areas:

a) *Number of carbon atoms in the molecule.* Long-chain bases vary between 12 and 22 carbon atoms in length including odd numbers (Karlsson, 1970).

b) *Straight or branched chains.* Some long-chain bases have a methyl branch. This branching occurs no further from the methyl end of the molecule than the third carbon (Karlsson, 1970).

c) *Number of double bonds and their configuration.* Up to two double bonds can be found in long-chain bases. Most commonly in monounsaturated molecules, the double bond is *trans*, and occurs at carbon four in the chain. In diunsaturated long-chain bases, the first double bond is always *trans*, found at carbon four. The second double bond is either *cis* – at (n-4), (n-9) or (n-13), or *trans* – at (n-10) (Karlsson, 1970).

d) *Number of hydroxyl groups present.* All of the long-chain bases have at least two hydroxyl groups, bonded to carbons one and three. Dihydroxy long-chain bases have two hydroxyl groups. In some cases a third hydroxyl group is located on the fourth carbon. These molecules are designated trihydroxy long-chain bases (Karlsson, 1970).

The most common long-chain base in human cells is sphingosine (d18:1^{4}), or *trans*-4-sphingenine (Degroote *et al.*, 2004), which is an 18 carbon molecule with one *trans*-double bond at carbon 4 to carbon 5, two hydroxyl groups on carbons 1 and 3, and the amino group at carbon 2. The *trans*-double bond does not alter the linearity of the hydrocarbon chain (Degroote *et al.*, 2004). Two other common bases are the saturated sphinganine (d18:0) and phytosphingosine (t18:0).

Fatty acids

Fatty acids (FA) are unbranched carboxylic acid chains between 16 and 28 carbons in length (Thompson & Tillack, 1985). They almost always have an even number of carbons and can either be fully saturated or have one or more double bonds – usually *cis*. Unlike the majority of FAs of intestinally derived sphingolipids, the FAs of RBC sphingolipids are not hydroxylated (Karlsson, 1970).

Sphingolipids

A sphingolipid – also called a ceramide – is a long-chain base joined to a fatty acid through an amide bond, which is formed in a condensation reaction between the long-chain base amino group and the fatty acid carboxylic acid group to yield a secondary amine (Figure 4).

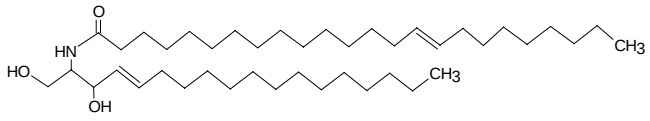


Figure 4. The structure of a sphingolipid incorporating the fatty acid nervonic acid (C24:1^{15}) and the long-chain base 4-sphingenine (d18:1^{4}).

Due to the abundance of both long-chain base and fatty acid species, there are numerous combinations of sphingolipid possible, however, not

all of the theoretical permutations occur with abundance in man (personal communication, S Henry).

Sphingolipids are the parent compounds of glycosphingolipids. The hydroxyl group on carbon 1 of the long-chain base is oxidised to form the β-linkage with the anomeric carbon of β-D-glucose to form glycosylceramide (Figure 5). Other linkages can occur between ceramide and a monosaccharide – β-D-galactose can be linked to the ceramide to form galactosylceramide (Thompson & Tillack, 1985).

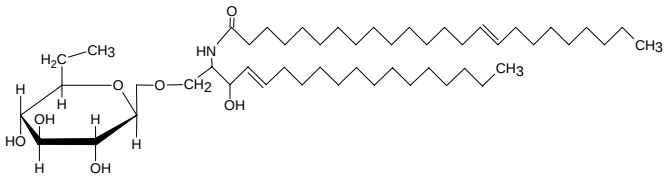


Figure 5. The structure of glycosylceramide (Glcβ1-1Cer) where the ceramide is composed of the fatty acid nervonic acid (C24:1[15]) and the long chain base 4-sphingenine (d18:1[4]).

1.2.3 Membrane Fluidity

Membranes are said to be fluid because lipid molecules are able to move laterally within their monolayer at a rate of up to 10^{-8} cm^2/sec (Alberts *et al.*, 1994). This equates to 3.6 x 10^{-4} cm^2/hour, or 3.6 μm^2/hour, which is just over half the 7.5 μm diameter of an average RBC (Bain, 1995). Lateral diffusion fluidity is critical to the membranes ability to modify the configuration of receptors and other domains in response to the presence of exogenous signal molecules. One important property of the bilayer is the ability to orient and cluster glycolipid species (as reviewed in Evans & MacKenzie, 1999).

As the basis of the fluid mosaic model of the cell membrane, glycerophospholipids (see section 1.2.1) contribute significantly to this most important of characteristics. Both the length and saturation of the glycerophospholipid acyl chains influence the fluidity of the membrane. A shorter length acyl chain has a reduced ability to complex with its neighbours, while the *cis* kink prevents the glycerophospholipid molecules from being tightly packed together. Accordingly, the fluidity of the

membrane is enhanced by glycerophospholipids with relatively short, unsaturated fatty acids.

Cholesterol makes up about 25% of the lipid fraction of RBC membranes (as reviewed in Schenkel-Brunner, 2000). It plays an important role in maintaining membrane fluidity, as well as stabilising the bilayer and preserving the membrane permeability barrier. The latter is achieved by decreasing the mobility of the glycerophospholipid acyl chains in the region closest to the hydrophilic head, thus inhibiting the passage of small polar molecules.

1.2.4 Lipid Microdomains

Critical to the function of the membrane is maintenance of the precise arrangement of certain constituents (Schenkel-Brunner, 2000) within the dynamically fluid membrane. Microdomains have various roles in nature – from the growth and differentiation of cells (Kojima & Hakomori, 1991), to cell-cell recognition and attachment (Hakomori, 2000; Harder & Simons, 1997) and signal transduction (Harder & Engelhardt, 2004).

In conjunction with lipid-engendered fluidity of the membrane, cells can confine certain lipids (and proteins) to specific locations within a leaflet (Simons & Ikonen, 1997). Due to interaction between molecular species, long-range lateral randomisation and/or equilibration does not occur – fluidity is restricted to compartments or domains by the biophysical forces involved. Segregation comes about because the structural differences of sphingolipids and glycerophospholipids render them immiscible (Holthius *et al.*, 2003). One of the main differences accounting for the immiscibility of these two classes of lipids is the length of the hydrocarbon chain. The fatty acids of the sphingolipids are commonly between 20 to 26 carbons in length, while the fatty acids of the glycerophospholipids are between 16 and 22 carbons long (Harder & Simons, 1997). The longer fatty acid chains of sphingolipids means these microdomains are thicker than the glycerophospholipid phase.

Cholesterol is a critical constituent of sphingolipid microdomains (as reviewed in Holthius *et al.*, 2003). The sphingolipid head group (amide group region) has a larger cross sectional area in the plane of the

membrane than the two attached hydrocarbon chains can occupy (Harder, 2003), and the resultant spaces between the sphingolipid molecules are filled by cholesterol. Cholesterol has flat rigid steroid rings that preferentially interface with the sphingolipid hydrocarbon chain through van der Waals interactions (Degroote *et al.*, 2004; Hakomori, 2000, 2003; Hakomori & Handa, 2002; Holthius *et al.*, 2003), and the third carbon hydroxyl group of cholesterol may hydrogen bond with the sphingolipid amide group (Harder & Simons, 1997). The long linear hydrocarbon chains of sphingolipids present a comparatively large surface for hydrogen bonding and van der Waals attractive forces with other sphingolipids and cholesterol. In contrast, glycerophospholipids are excluded because of their shorter, bent hydrocarbon chains, and carbonyl groups which function only as hydrogen bond acceptors. Sphingolipids are very tightly packed due to the linearity of the commonly saturated chain and the predominance of *trans* over *cis* double bonds in the infrequent unsaturated species (Alberts *et al.*, 1994). This increased packing density makes assemblies of sphingolipids more rigid than the glycerophospholipid phase, however, in sphingolipid domains the molecules are still capable of rotational and lateral movement.

One type of lipid microdomain is termed the lipid raft, the postulated role of which is the specific recruitment of one type of protein while excluding another (Harder & Simons, 1997) – possibly for the purpose of restricting or controlling the interactions of certain proteins (Harder & Engelhardt, 2004). The sheer number of different lipid species present in the plasma membrane indicates that there is probably a range of lipid phases, each with their own specific physico-chemical properties, which may be compatible with only one of a variety of proteins (Harder & Engelhardt, 2004). The sphingolipids in these domains are often glycosylated – labelled exogenous gangliosides have been observed in caveolae/rafts under immunoelectron microscopy (Mobius *et al.*, 1999). However, raft functions are not glycosylation dependent – the glycosphingolipids play only a mediatory role (Ostermeyer *et al.*, 1999). Furthermore, the carbohydrate moiety appears to be immaterial in the formation or maintenance of the raft assembly (Ostermeyer *et al.*, 1999).

Confinement of glycosphingolipids to specific domains in the outer leaflet of cell membranes has been well documented (Degroote *et al.*, 2004; Hakomori, 2000, 2003; Hakomori & Handa, 2002; Holthius *et al.*, 2003; Simons & Ikonen, 1997).

1.2.4.1 Glycosphingolipid-Enriched Microdomains

One specific type of lipid microdomain is composed solely of sphingolipids and much enriched with glycosphingolipids. Termed GEMs (glycosphingolipid-enriched microdomains), they are not functionally affected by cholesterol binding agents (in direct contrast to the effect on cholesterol-containing rafts), hence they do not contain cholesterol (Hakomori *et al.*, 1998). As with other microdomains, the functions of GEMs are recognition, attachment and signal transduction – but here carbohydrates are the effectors of all these functions: not simply mediators. The glycolipids are believed to interact either with lectins or other carbohydrate structures (Kojima *et al.*, 1992). Carbohydrate-carbohydrate interactions are thought to be the initial recognition/binding event, after which protein-based mechanisms originating from other membrane assemblies take over (Kojima & Hakomori, 1991).

In a glycosignalling domain (a GEM associated with signal transducer proteins of the inner leaflet), glycosphingolipids initiate signal transduction through stimulation or inhibition of the transducer molecules (Hakomori *et al.*, 1998).

Functionality is dependent on the molecules being clustered together (see section 1.3.1), which in turn depends on the physico-chemical properties of the sphingolipid – specifically the fatty acid (Hakomori *et al.*, 1998). In addition, the sugar chains of glycosphingolipids play a small role in the self-clustering into GEMs (Degroote *et al.*, 2004; Hakomori, 2003; Hakomori & Handa, 2002).

1.2.5 Critical Role of the Lipid

A deterministic role for the sphingolipid during carbohydrate biosynthesis has been suggested (Kannagi *et al.*, 1982; Kannagi *et al.*, 1983).

Globoside from human erythrocytes generally has a sphingolipid with a relatively long chain fatty acids, while gangliosides are associated with sphingolipids that contain short chain fatty acids (as reviewed in Kannagi *et al.*, 1982). It is possible that certain glycosyltransferases may associate with specific Golgi membrane domains depending on their physico-chemical properties (of both the enzyme and the domain) and thickness (of the membrane in relation to the length of the trans-membrane region of the enzyme) – both of which are influenced by lipid composition. Apply the same principle to the different sphingolipid species, and it becomes apparent how the activity of certain enzymes could be restricted to certain sphingolipids.

Carbohydrate chain structure differences that are specifically related to sphingolipid stucture are around the terminus and involve fucosyltransferases and sialyltransferases (Kannagi *et al.*, 1982). These particular glycosyltransferases have often been implicated in the prevention of chain elongation (Leppanen *et al.*, 1997; Mattila *et al.*, 1998; Sakamoto *et al.*, 1998). If the fatty acid has a deterministic role in the location of the sphingolipid next to a specific subset of glycosyltransferases, then it could also effect control of carbohydrate chain length.

In accordance with this proposition, it has been suggested that the absence of hybrid AB glycolipid structures (bearing both A and B determinants) on the RBCs of group AB individuals could be due to the differential localisation of the A and B transferases in the Golgi (Kushi *et al.*, 1995). However, other papers have found such a high level of homology in the amino acid sequences of these two particular enzymes (see section 1.1.2), that it seems unlikely that the transmembrane sequences could be different enough to bring about their segregation.

There are further effects that may be related to the identity of the fatty acid in the ceramide. Ligand binding may be dependent on the orientation of antigens at the cell surface (Kannagi *et al.*, 1982), which may be affected by the ceramide – specifically the fatty acid (Urdal & Hakomori, 1983). The physicochemical properties of each species of glycolipid, for which the definitive determinant may be the fatty acid

(Kannagi *et al.*, 1983; Urdal & Hakomori, 1983), may dictate its location in the membrane and hence the other species of molecules it is adjacent to. This mechanism could play a role in the regulation of crypticity – where a glycolipid antigen cannot be detected at the cell surface but can be detected in cell extracts. The antigen may be obscured by other larger structures around it, or orientated in such a way that the glycotope is inaccessible to the antibody or lectin.

Ceramides may also influence clustering ability. Comparison of the membrane localisation of Forssman and asialo-G_{M1} showed differential clustering in phosphatidylcholine bilayers (Rock *et al.*, 1990). Forssman was present in groups of no more than a few molecules, while asialo-G_{M1} associated in groups of 15 or so. The explanation for these molecules adopting different layouts may lie in the fatty acid composition of the ceramide. Forssman glycolipid had a very heterogeneous mix of ceramides. The predominating long chain base was C18 at >95%, but the predominating fatty acid was only at 21%, with quite a few other species close to that frequency. Forssman glycolipids thus had a large range of physicochemical properties. Clustering occurs between molecular species that have similar physicochemical properties, which possibly provides an explanation for the many small groups. In contrast, asialo-G_{M1} had a much more homogeneous ceramide grouping – the long chain bases were C18 at 70% and C20 at 30%, and the predominating fatty acid was C18:0 at 81%. This describes a group of molecules with a much narrower range of physicochemical properties, which means they would be likely to form larger clusters.

The studies presented above provide evidence for the ceramide, and specifically the fatty acid, having a deterministic role in defining the structure of the carbohydrate moiety, the ultimate membrane location and the organisation of clustering.

1.2.6 Membrane Proteins

Proteins can be associated with the membrane in a variety of ways, but are divided into two general groups based on their membrane association. Those that are only loosely associated with the membrane are termed

extrinsic membrane proteins, while the remainder, those whose link to the membrane is strong, are called intrinsic membrane proteins (Singer & Nicolson, 1972).

The majority of RBC proteins are extrinsic and associated with the inner leaflet. However, the protein with the most relevance to ABO(H) blood group antigens is an intrinsic one – glycophorin. Glycophorin is an amphipathic protein which spans the bilayer, and exists as four distinct species – designated A, B, C and D – that carry different blood group antigens. These molecules are generously glycosylated, with carbohydrate comprising 60% of their mass. Between 30-45% of RBC glycosylation and 90% of the negative charge of RBC membranes (conferred by the saccharide sialic acid) is associated with glycophorin (Schenkel-Brunner, 2000).

1.2.7 Insertion

Modification of the RBC membrane, or indeed any cell membrane, using glycolipids or glycolipid-like molecules is possible because of the unique properties of membranes. The plasma membrane is a dynamic structure where the lipids of the bilayer are capable of rearrangement (see sections under section 1.2). This characteristic, along with the amphipathic properties of glycolipids, allows the spontaneous incorporation of glycolipids from the surrounding milieu into the membranes of RBCs (and other cells). This phenomenon of insertion, as it is called, was first identified by Stormont when bovine J-negative erythrocytes acquired the J antigen upon incubation with J-positive serum (Stormont, 1949). This finding was substantiated by work undertaken in sheep with regard to the R antigen (Rendel *et al.*, 1954).

Sneath and Sneath (Sneath & Sneath, 1955) then investigated this phenomenon in humans, investigating patients who had been transfused with Lewis and Rh incompatible blood. A recipient of Le(a-b+) type received Le(a+b-) blood, and when the cells were separated according to their Rh status by differential agglutination, the donors Le(a+b-) cells had become Le(a+b+). This result was explained in terms of the donor RBCs having acquired Le^b from the plasma of the recipient.

In vitro experiments were also conducted by Sneath and Sneath using one part packed RBCs and 10 parts plasma incubated at 37°C for three days, with daily plasma refreshment. In summary, the results indicated that Le(a-) RBCs acquired Le^a from Le(a+) plasma and Le(b-) cells acquired Le^b from Le(b+) plasma. The acquisition of these antigens was effected in 24 hours.

It is now well established that Le^a and Le^b are glycolipid antigens that are synthesised and secreted (and expressed) by epithelial cells and acquired by RBCs from the plasma. Owing to the successful methodology employed by Sneath and Sneath in their *in vitro* work, most subsequent insertion experiments used plasma as the glycolipid carrier medium.

At this stage, the structural identity of the antigens that could be acquired from plasma was unknown. Mäkelä (Makela & Makela, 1956) ruled out glycoproteins when Le(b+) saliva was unable to transform Le(b-) RBCs. Similarly, Lewis and co-workers failed to show transformation of RBCs with group A or B antigens from the saliva of secretors (Lewis *et al.*, 1960). Glycosphingolipids were finally identified as the effectors of transformation by Marcus and Cass in 1969 (Marcus & Cass, 1969). Until this time, the Le^a and Le^b antigens in plasma were assumed to be glycoproteins, as in saliva. However, lipoprotein fractions were found to be strongly antigenic, and the removal of lipoproteins from plasma effectively prevented erythrocyte transformation. Subsequent isolation of glycosphingolipids from plasma lipoproteins resulted in a sample that could successfully transform RBCs.

Tilley and co-workers (Tilley *et al.*, 1975) found that the A, B and ALe^b antigens that could be taken up by RBCs were also glycosphingolipid in composition, but not before the uptake of A and B antigens by cells *in vivo* was described by Renton and Hancock (Renton & Hancock, 1962). Group O cells given to blood group A or B recipients were found to be agglutinable with certain group O serum (that which presumably had high-titre anti-A,B). It was concluded that the transfused group O cells had acquired the A and/or B antigens present in the plasma of the recipient. Renton and Hancock also found that *in vivo* transformation of

transfused RBCs appears to take about a fortnight to reach a steady level (although this is debatable).

Tilley and co-workers (Tilley *et al.*, 1975) made the interesting observation that the sera of O individuals was more effective at agglutinating *in vitro* transformed cells than that of either group A or B individuals. They postulated that the anti-A,B of group O sera was of the IgM class, implying that the reason anti-A of B sera and anti-B of A sera did not often work is that they were predominantly IgG. The sugar chains of glycolipids that can be extracted from RBCs, plasma or intestine/faeces tend to be shorter than the sugar chains of the RBCs own innate glycoproteins. This makes agglutination of an O cell transformed with glycolipids less likely to occur when against IgG (see section 1.3.1.1 for more explanation of IgM and IgG).

It is now clear that there is free exchange of glycolipids between plasma and RBC membranes, and the glycolipids are thought to be divided between the membrane and plasma (in the lipoprotein fraction) compartments at an equilibrium of one third to two thirds respectively (Schwarzmann, 2001; Wilchek & Bayer, 1987).

Scrutiny of the mechanism of ganglioside (charged glycolipids) insertion has revealed that assemblies of these molecules (usually micelles or lipoprotein particles) are able to interact with the cell membrane in three ways (as reviewed by Mobius *et al.*, 1999). Some assemblies loosely associate with the outside of the cell membrane and can be easily washed away by serum. Other assemblies bind more strongly to membrane structures such that they can only be removed with trypsin. As trypsin is a proteolytic enzyme, the membrane structures to which the ganglioside assemblies bind must either be protein or themselves bound to protein. Because gangliosides are positively charged glycolipids, this interaction may be influenced by their charge. No equivalent phenomenon has been reported for the neutral blood group glycolipids. The final mechanism of interaction is stable incorporation into the membrane, impervious to proteolytic attack.

1.2.7.1 Insertion Dynamics

Previous experiments have established some parameters for the variables of glycolipid concentration, cell and glycolipid ratios, and incubation time and temperature.

In appendix 2, the most efficient ratio of RBCs to natural glycolipids in plasma for insertion into RBC membranes was found to be 3 parts packed RBCs to 1 part transformation solution. At this ratio, maximal 4+ agglutination was produced, and reduction of the amount of RBCs maintained this maximal agglutination result. An increase in the amount of RBCs to glycolipid (4:1) became suboptimal because a weaker agglutination was produced.

An unpublished study (Appendix 1) indicated that natural glycolipids do not significantly insert into RBCs at refrigeration temperature (2-8°C). *In vitro* transformation of human Le(a-b-) red cells was carried out with natural Le^b-6 glycolipid added to Le(b-) plasma. The reaction was performed with a final glycolipid concentration of 2 mg/mL and a final cell suspension of 10%. The cells were incubated for 8 hours at temperatures of 37°C, 22°C and 4°C, and samples were tested at hourly intervals. Flow cytometry showed minimal insertion – below serological detection level – after 8 hours at 4°C (see Appendix 1). That the rate of insertion decreased with temperature was demonstrated as a level of insertion that corresponded with serological detection achieved after the first hour at 37°C, did not occur until after the fifth hour at 22°C.

However, Callies and coworkers (Callies *et al.*, 1977) found that very low quantities of exogenous gangliosides diluted in Eagle's medium (without added calf serum because the authors believed it to interfere with cell accumulation of ganglioside) were able to be taken up by different cell types at temperatures between 0°C and 4°C. Of the three cell types used (murine Cl-1-D fibroblasts, chicken embryo fibroblasts and chicken erythrocytes), the erythrocytes bound the lowest quantity of ganglioside at low temperatures and at 37°C. In these cells, the amount of associated ganglioside was seven times lower after low temperature incubation than when incubated at 37°C.

1.2.7.2 Other Methods of Modifying ABO Group

Glycosidases

Seroconversion of erythrocytes can also be accomplished with enzymes. Much work has been conducted using specific glycosidases, which can hydrolyse glycosidic bonds, to remove the immunodominant A (GalNAc) and B (Gal) sugars from the H determinant.

Lenny and co-workers (Lenny *et al.*, 1994) were able to convert group B RBCs to group O with a green coffee bean enzyme, such that upon transfusion of 160-200 mL into healthy blood group A and O volunteers, their life-span was normal. There was transient and benign elevation in the titre of anti-B, especially in the blood group O individuals, indicating that removal of the B active sugar was not absolute, and indeed, the anti-B titre was most elevated upon transfusion of RBCs treated with the lowest amounts of enzyme.

Other studies confirm that the action of α-galactosidase on B RBCs, while seroconverting them to group O, does not alter cell functionality (Phillips & Smith, 1996; Zhu *et al.*, 1996).

Seroconversion of blood group A RBCs to group O has encountered more hurdles. Attempts to obtain an appropriate enzyme have been hampered by issues related to purification (Levy & Aminoff, 1980) and enzyme pH optima outside the physiological range (Phillips *et al.*, 1995). However, isolation of an α3-N-acetylgalactosaminidase from a bacterial strain found in the human intestinal tract has been reported (Falk *et al.*, 1991; Hoskins *et al.*, 1997). This enzyme is active at physiological pH and can hydrolyse the α1-3 bond between the terminal GalNAc and sub-terminal Gal of A glycotopes.

Glycosyltransferases

Research as above has focussed on the creation of universal O RBCs from surplus blood group A and B cells using glycosidases. However, using glycosyltransferases, group O RBCs have been converted into A (Schenkel-Brunner & Tuppy, 1973).

In addition, there are several examples of the use of glycosyltransferases to modify carbohydrate expression on cells through constructive addition of saccharides. Srivastava and co-workers (Srivastava *et al.*, 1992) used a Lewis α3/4-fucosyltransferase to transfer a preassembled B trisaccharide that was covalently bound to the fucose donor, to type 1 or type 2 precursor structures on OLe(a-b-) RBCs. The authors noted that as this structure was unnatural, the resultant cell was not suitable (nor intended) for use *in vivo*.

In other experiments, an α3-galactosyltransferase was used to construct the Galili antigen on RBCs by adding α-Gal to de-sialylated LacNAcs, as a prospective method for rendering tumor cells more immunogenic for the purposes of autologous immunisation (Galili & Anaraki, 1995; La Temple *et al.*, 1996).

1.3 Blood Group Determination

A major function of blood banking laboratories around the world is the ABO typing of blood from donors and recipients for the purposes of transfusion. The existence of potent pre-formed ABO antibodies means it is imperative that blood types are correctly determined.

Routine blood typing of samples of unknown group is resolved by forward grouping and the result confirmed with reverse grouping. In forward grouping, RBCs are tested against antisera of known reactivity. In reverse grouping, serum is tested against reagent RBCs that have a known antigenic profile. In both cases a positive reaction is indicated by agglutination of the RBCs. Agglutination in ABO grouping occurs through crosslinking of cells by (mainly) IgM antibodies as they bind with the appropriate antigens on more than one cell, causing them to clump together.

1.3.1 Antigen-Antibody Interactions

Antibody binding of antigen is a complex event that is subject to many forces. It can be described in terms of avidity and affinity. Antibody affinity is the strength of a single binding event – binding of a single monovalent Fab fragment to a single antigenic determinant. Antibody

avidity is the strength of all the interactions between a single antibody possessing multiple binding sites (monovalent Fab fragments) and a multivalent antigen. Avidity is influenced by the valence of both the antigen and the antibody, and therefore describes the stability of the entire antibody-antigen complex.

ABO antigens are carbohydrate in nature, while antibodies are protein. Antibodies contact the sugar residues of the glycotope mainly through aromatic amino acids, whose steroid rings orient alongside the hydrophobic sugar rings. The binding pockets are lined with polar side chains which can form hydrogen bonds with the hydroxyl and other polar groups of the glycotope (as reviewed by Evans & MacKenzie, 1999).

Monovalent interactions between carbohydrates and proteins have poor affinity, and must be compensated for by the simultaneous formation of multiple protein-carbohydrate associations. To facilitate these concurrent interactions, carbohydrate antigens and protein antibodies need to ensure they are present in sufficient numbers and that they have their binding sites and epitopes spaced to complement each other. One way of doing this is to implement mechanisms to achieve multivalency. Pentameric IgM antibodies have 10 antigen binding sites, and some IgG molecules can form clusters through Fc-Fc interactions or homophilic binding (as reviewed by Evans & MacKenzie, 1999). Additionally, glycolipid antigens are mobile in the RBC membrane (Namork, 1994) and tend to cluster, which promotes antibody binding (as reviewed by Bovin, 2002; Feizi, 2001; as reviewed by Hakomori & Kannagi, 1983; Kannagi *et al.*, 1983; Nores *et al.*, 1987) and there is a density threshold below which antibody binding does not occur (Hakomori *et al.*, 1998).

There has been some suggestion that clustering might actually be a function of antigen composition (Namork, 1994). Glycoproteins – most of which are firmly bound to spectrin – would not be expected to cluster in the same manner as glycolipids. This appeared to be borne out in fact by the different labelling patterns exhibited by different subgroup cells. Clustering was less in evidence on A_1 cells compared with A_x, and Namork thus concluded that fewer glycolipid-based A antigens may have been carried by A_1 cells (Namork, 1994). An alternative explanation might

have been that the antibody may have had a lower affinity for the A_1 cell antigens than the A_x cell antigens, thus inducing less clustering. A further explanation is that the antigens on the A_1 cells may be too closely spaced to allow the simultaneous binding of two antigens by both arms of the antibody (cross-linking) which stimulates clustering.

Modelling of the trisaccharide globotrioasylceramide revealed that the bond linking the sphingolipid and the first sugar residue, glucose, can adopt three different conformers when in a membrane (as reviewed by Evans & MacKenzie, 1999). In one of them, the glucose residue extends perpendicular to the membrane, while in the other two conformers, it lies flat against the surface of the membrane. This finding emphasises the direct influence of the membrane on the presentation of glycolipid antigens.

The identity of the fatty acid also may play a role in the reactivity of the carbohydrate antigen it bears (Evans & MacKenzie, 1999; Kannagi *et al.*, 1983; Stewart & Boggs, 1993). In carbohydrate-carbohydrate binding experiments in liposomes, glycolipids whose fatty acid moieties were long (24-26 carbons) and fully saturated demonstrated increased binding ability (Stewart & Boggs, 1993). It was proposed that those with longer fatty acid chains relative to the bilayer matrix were more exposed to the extracellular environment, and the more rigid saturated chains were able to avoid shortening their effective length by bending within the membrane. Interdigitation in plasma membranes could possibly compensate for the increased fatty acid chain length, and it is unclear whether this interdigitation occurs in liposomes (Stewart & Boggs, 1993).

The composition of the local membrane bilayer may be a key factor in protein-glycolipid recognition (see subsections under section 1.2). The fluidity of the bilayer is also believed to affect the geometric positioning of the carbohydrate epitope relative to the binding sites of antibodies (as reviewed by Evans & MacKenzie, 1999). Furthermore, the lipid tail of the glycolipid in combination with the physicochemistry of the local membrane may alter the environment around the glycotope that is encountered by the antibody during the recognition process (as reviewed by Evans & MacKenzie, 1999). Some antibodies are also believed to

recognise portions of the aglycone in order to bind to the glycotope, and preferentially bind to sphingolipid-based structures as opposed to glycerolipids (as reviewed by Evans & MacKenzie, 1999). However, it is unlikely that ABO(H) antibodies bind any part of the glycosphingolipid other than the glycotope – the importance of the aglycone moiety in terms of membrane location is probably contained solely in its contribution to the overall physicochemical properties of the molecule.

Subtle changes in the microenvironment of the membrane, and specifically the GEM, can modulate glycotope presentation and alter the balance of the equilibrium controlling antigen-antibody reactivity (as reviewed by Evans & MacKenzie, 1999). It becomes clear that the recognition of the carbohydrate epitope by a passing antibody is an event that finely balances the complex variables of the relative fluidities of the membrane matrix and the GEM, along with the nature of the interaction between the physicochemical properties of each species of glycolipid and its local environment.

1.3.1.1 Erythrocyte Agglutination

The reaction between RBC antigens and antibodies is quite easily distinguishable by eye. Called agglutination, the cells adhere together, forming a range of reactions from a solid disc in strong reactions, to a collection of small soft clumps at the weaker end of the scale.

Agglutination is contingent on several factors, including the relative amounts of antigen and antibody present. Equilibrium between antigen and antibody will produce optimal agglutination, while excess antibody can obstruct the RBC antigens and prevent crosslinking and agglutination, causing a false negative reaction. This phenomenon, called prozone, is the reason that antibody concentration can be important when testing for small amounts of antigen. It is however of note that low concentrations of ABO antigens (e.g. A_x) actually require high concentration antibody to be detectable (see section 1.3.2).

Agglutination also requires that antibodies are able to bridge the gap between RBCs. Depending on the antibody subtype and the nature and location of the RBC antigen they recognise, this may not be possible.

RBCs maintain a minimum distance between them due to repulsive forces. Electrostatic repulsion is generated by the considerable amounts of ionised sialic acid present on RBC glycoproteins and glycolipids, causing RBCs to become surrounded by a space-filling shell of hydration, which induces polar repulsion (Alberts *et al.*, 1994).

IgG antibodies are monomeric, and can span a distance of 12-14 nm (Van Oss, 1994). Under physiological conditions of pH and salinity, the minimum distance between the membrane surfaces of two RBCs is 15-16 nm, which is greater than the IgG span distance. Thus IgG antibodies cannot crosslink the RBC membrane surface antigens of different RBCs, such as the D antigen. In this case, an antibody which can crosslink the Fc regions of IgG is required. In contrast, the glycocalyx layers of two RBCs can approach to 4-5 nm, and thus antigenic epitopes at this location, such as ABO, can cause agglutination with IgG.

Pentameric IgM antibodies have a spanning distance of about 27-28 nm (Van Oss, 1994) and can thus agglutinate RBCs whether the epitopes are at the membrane surface or at the glycocalyx.

There are some methods to reduce the electronegativity and hydrophilicity of the RBC surface to allow surface antigen agglutination with IgG. Enzyme treatment can remove proteins (many of which are generously glycosylated and contain large amounts of sialic acid) or the sialic acid specifically. The addition of plurivalent cations, such as polybrene, and potentiators, such as albumin, also reduces the net negative charge of the RBC. These methods result in smaller shells of hydration around each RBC and allows cells to approach each other more closely (Van Oss, 1994). The drawback of these methods is that they have the potential to alter the antigenic profile of the cell.

Modern ABO antibodies are predominantly IgM monoclonals, which means that they are the product of a single cell line producing antibodies to a single antigenic structure. Commercial ABO blood grouping reagents are often blends of two or more IgM clones and designed to optimise reactivity with weak expressing A/B cells at RT. There is also a vast array

of MAbs (not available commercially) that are directed to a large range of antigenic structures, and they are useful for research purposes.

1.3.2 ABO Subgroups

The ABO(H) blood group system probably developed 13 million years ago from a 400 million year old ancestral gene (Saitou & Yamamoto, 1997). The first subgroup of A – named A_2 – was described in 1911 (Von Dungern & Hirszfeld, 1911). The known subgroups are now well defined serologically, but differences in carbohydrate structures present in the subgroups are still not fully characterised. The subgroups are thought to have arisen through spontaneous mutation of the A or B allele so that activity of the resultant enzyme is altered in relation to the enzyme encoded by the original gene.

Historically, the subgroups were arbitrarily classified on serological characteristics and the presence/absence of secretory antigens. Today we know that a single subgroup may consist of individuals whose transferases have a similar reduced efficiency, but are the result of many different mutations. These mutations appear to have all arisen independently (as reviewed in Race & Sanger, 1975). In addition, some subgroups may be the result of the action of other genes (e.g. para-Bombay phenotype) or the environment (i.e. through the effects of disease or microbial infection e.g. acquired B) on normal ABO antigens (as reviewed in Race & Sanger, 1975).

Quantitative differences between the subgroups are well established. Table 4 shows the results of serum absorption of radiolabelled antibodies with respect to A and B antigens (Economidou *et al.*, 1967).

As shown in Table 4, A_1 cells are believed to have more than 10^6 A antigens, whereas A_2 cells have only about 300,000. The A_2 cells have instead more unconverted H structure due to the action of a less efficient A transferase. The existence of qualitative differences between some or all of the subgroups is a subject of ongoing investigative scrutiny. Previous work has identified novel antigens in some of the subgroups. Clausen and co-workers proposed that globo-A (Clausen *et al.*, 1984) and type 3 chain A (Clausen *et al.*, 1986) formed the basis of qualitative

differentiation of the A_1 and A_2 subgroups, but unpublished data has refuted this in terms of the type 3 chain A structure (S Henry, personal communication).

Table 4. Results from serum absorption quantitation of A and B antigens. Reproduced from (Economidou *et al.*, 1967).

Blood group	A antigens (x 10^6)	B antigens (x 10^6)
A_1	0.98 – 1.17	–
A_2	0.24 – 0.29	–
B	–	0.62 – 0.83
A_1B	0.46 – 0.85	0.31 – 0.56
A_2B	0.12	n.d.

The serum of some A_2 individuals (1-8%) contains anti-A_1 (Issitt & Anstee, 1998), which does not agglutinate A_2 RBCs. This antibody is thought to define the existence of a different structure on the RBCs of A_1 individuals that A_2 individuals do not possess, and is now believed to be anti-globo A alone (Svensson, unpublished).

The commonly known weak subgroups of A are A_3, A_{bantu}, A_{end}, A_{finn}, A_m, A_x, A_y and A_{el}. The serological characteristics of these subgroups are varied, however, they are generally typified by reduced expression of the A antigen and strong expression of the H antigen on the RBCs and in plasma. A_x individuals may give a negative reaction with anti-A in the forward group and may show strong anti-A_1 in their serum in the reverse group, and so may be mistyped as blood group O (Race & Sanger, 1975). The sera of individuals belonging to some of these weak subgroups contains anti-A_1. Agglutination with anti-A and anti-A,B is weak or not detectable, however the RBCs of all the subgroups can adsorb anti-A.

In theory, the weak subgroups of B are the same as those of A, but have been less fully investigated (Schenkel-Brunner, 2000).

1.3.3 Quality control of ABO Blood Banking

ABO subgroups having less than 5000 antigens per cell are unable to effectively support agglutination *in vitro* (and are not generally of clinical significance either) although this does depend on several other factors, such as antigen clustering, mobility and size (Schenkel-Brunner, 2000).

Detection of subgroups depends largely on antisera quality and reactivity – there is a range of antisera performance. Surprisingly, the most critical component is the concentration of the antibody (mg/mL), with minor dilution often resulting in loss of activity against cells expressing low levels of antigen, and the prozone phenomenon seems not to occur with potent IgM reagents (Le Pendu & Henry, 2002).

Modern monoclonal reagents are often actually blends of monoclonal antibody cell lines. They are so potent that even a small amount of cross-contamination can lead to erroneous agglutination results. For example, if a dropper from anti-A is accidentally replaced on an anti-B reagent, group A cells could type as AB.

High quality antisera are critical to the effective performance of blood grouping laboratories. Antisera can deteriorate for a variety of reasons including sub-optimal conditions during transport and storage. Other causes of error are inappropriate use, bacterial contamination or cross-contamination with other reagents (as described above).

In some studies, blood banking practice has been implicated in fatal transfusion events in about 33% of cases (18% clerical or management system failure in a blood bank and 15% technical error in a blood bank – (as reviewed by Issitt & Anstee, 1998)). Therefore, routine quality control and quality assurance must be undertaken to safeguard the performance of blood grouping procedures and should encompass the management of the testing process to ensure that tests are reproducible, accurate and sensitive.

Quality control of reagents should involve testing of avidity, specificity and sensitivity. There should always be controls for provision of negative and positive reactions. Specificity can be assured by the inclusion of all four common ABO blood groups. The use of a weak positive control can detect a loss of sensitivity against weak subgroup cells that would not show up against a strong positive control.

It is not only the performance of antisera that can result in mistyping of samples: errors may also occur at many other points in the system. Human error is a big factor, which automation has reduced to a significant

degree in all aspects of testing, specifically the transcription of scores and tracking/labelling of samples and determination of reaction. However, full automation is available only in some laboratories. Most have partial automation and some still have no automation, even in first world countries.

In order to test the performance of the entire process, the controls should be tested alongside, and no differently than, patient samples. These controls should therefore simulate patient samples, and be designed to uncover any deviation from established methodology throughout the entire testing procedure. This is especially pertinent with automated systems, which can often be set up to run process controls differently from the normal process, entirely defeating the purpose. This is not to say that manual techniques are immune to this type of strategy. It is not unknown for staff members to change a negative result because they 'know' that it is a positive control. Routine use may also assist in the detection of transcription errors.

Due to the subjective nature of agglutinate score assignment in manual serology (see Appendix 7), continual monitoring is necessary to ensure standardisation. Grading weaker reactions (seen with weak subgroups) is especially dependent on the skill of the individual, and due to the increasing reliance on automation, especially in larger centres, not many Medical Laboratory staff have the experience to recognise weak reactions. It also needs to be mentioned that weak subgroups do not frequently occur in blood grouping, and so staff do not have a lot of experience with the weaker types of agglutination reactions. Moreover, the performance of modern monoclonal reagents may lead staff to expect the clear negative or positive result that is usually seen. They may overlook weak positivity because it resembles the more familiar negative result. In the hands of experienced serologists, manual serology can often out-perform many automated technologies (personal communication, S Henry), but when the operator has not had a lot of experience scoring weak reactions, the results can be variable, with a lesser degree of reliability and reproducibility.

Most of the procedures for quality controlling of antisera and the testing systems that they form part of are indirect, and also do not examine all the stages of the blood testing system where errors can occur. The most common method of testing antibody potency is titration, where the antibody is diluted and tested against normal cells to find the point at which it fails to detect antigen. This type of testing thus provides only an indirect and extrapolated comparison, as the 'true' test should be the detection of diluted antigen (as in subgroup cells) by undiluted reagent. Antibody titre testing can indirectly test the potency of antisera, but does not also take account of the possibility of errors occurring in recording the results, for example. A further drawback to antibody titre testing is that the multiple monoclonal antibodies in the blend may not be of the same concentration, and thus one may fail before the other.

Some laboratories use natural weak subgroup cells when they can acquire them to test the performance of their systems, but a reliable supply of cells can be hard to obtain. Group A_2B is often used because it is the easiest to come by, but the levels of both A and B antigen expression are too high for it to really function as a weak positive control for either anti-A or anti-B. Also, there is great variation between individuals within a single defined subgroup, which may mean variation from one batch to the next. A_x are the generally accepted benchmark for anti-A analytical sensitivity (Appendix 11.2), but again the antigen levels are widely variable and the cells are even more rare than A_2B. Furthermore, unless the internally manufactured standard is characterised by an external accredited laboratory, it will not necessarily be able to detect any errors inherent in the laboratories procedures. This is because the standards were determined with their own techniques, which may be internally flawed.

As the matter stood at the beginning of 2005, there existed no satisfactory quality ABO control system that was able to assure the maintenance of standards across all blood accreditation laboratories.

1.3.4 The Consequences of Getting it Wrong

There are many types of transfusion reactions, which is the term used to cover all types of unfavourable response by a patient upon receipt of

transfused blood. The most severe involves immediate destruction of the transfused cells in the blood vessel – otherwise known as intravascular destruction.

The incidence of transfusion reactions is dependent on a number of factors; including the amount of incompatible blood transfused, the amount of antigens present on the transfused cells, the potency of the recipients antibodies and the health status of the recipient. As most recipients of blood transfusions are significantly unwell, it is essential to correctly type subgroups. The subgroups essentially represent an ever-decreasing amount of antigen expression (see section 1.3.2). It is generally accepted that A_x is at the clinical threshold for donation in blood transfusion, as it has caused a haemolytic transfusion reaction in a recipient who had high titre anti-A (as reviewed by Mollison *et al.*, 1997). Weaker subgroups like A_{el} have often been transfused as group O blood without consequence (as reviewed by Mollison *et al.*, 1997).

Briefly, the attachment of ABO antibodies to incompatible RBCs can induce their destruction through two different mechanisms. The cells can be lysed through the activation of complement – a cascade reaction comprised of polypeptide factors that lead to intravascular lysis of RBCs or they can be engulfed by phagocytes (extravascular). Intravascular destruction of a large number of transfused RBCs and also innocent bystander self cells induces severe shock, which, along with the ensuing disseminated intravascular coagulation and renal failure, may be fatal. Extravascular destruction of RBCs can result in anaemia, but is usually not too serious.

Issitt and Anstee cite several articles reporting the incidence of ABO-incompatible transfusion (as reviewed by Issitt & Anstee, 1998). From these reports and taking into account supposed under-reporting or under-detection of the less serious events, they conclude that about 1:10,000 units transfused is of incompatible group. Death has been reported to ensue in 10% of cases, which translates to an incidence of 1:100,000. This figure is corroborated by the NIH (USA), and they further estimate that 1:4,000 units transfused causes a non-fatal transfusion reaction (NIH, 1988).

1.4 Summary

The ABO blood groups of human RBCs were discovered by Landsteiner in 1901 (as reviewed by Landsteiner, 1931). There are four major blood group types in humans; A, B, AB and O. ABO blood groups comprise the most clinically significant blood group system in humans. Characterised by the presence of pre-formed antibodies, transfusion of blood cells bearing incompatible ABO antigen(s) can result in death.

As the ABO blood group system evolved over time, mutation lead to the development of weak subgroups, which essentially represent an ever-decreasing amount of antigen expression. It is generally accepted that A_x is at the clinical threshold for donation in blood transfusion, as it has been responsible for the death of a recipient who had high titre anti-A (as reviewed by Mollison *et al.*, 1997). Detection of subgroups depends largely on antisera quality and reactivity – there is a range of antisera performance, and deterioration can occur due to a variety of reasons including sub-optimal conditions during transport and storage.

In blood banking, the incorrect determination of donor and to a lesser degree recipient ABO blood types can result in a fatal outcome. Incompatible transfusions are believed to occur in 1:10,000 units transfused, and death is the outcome in 10% of cases (as reviewed by Issitt & Anstee, 1998) or 1:100,000. Some 33% of these errors have been attributed to breakdowns in blood banking procedure (as reviewed by Issitt & Anstee, 1998).

Astoundingly, there is no standardised positive control in this field, and quality control of the systems used in blood group determination is haphazard, and varies in both form and standard from laboratory to laboratory.

There is a need for a reliable supply of a standardised and reproducible positive control RBCs for routine use in blood banks. A cell that weakly expressed blood group A and/or B antigens would be ideal for this purpose, but no natural weak subgroup cell fulfils all that is required. If a cell could be made in which the antigen strength equated to that of the

natural A_x cell, which defines the clinical threshold, then a negative reaction with that control cell would indicate system failure.

The unique properties of membranes allow the spontaneous incorporation of glycosphingolipids from the surrounding milieu. This natural phenomenon can be utilised to add blood group active A or B glycolipids to group O RBCs, thereby making them blood group A or B respectively. Control over the amount of antigen incorporated, in line with the clinical threshold antigen level for transfusion purposes, is crucial for success.

1.5 Aims

The primary aim of this research is to evaluate the methods for the creation of RBCs that express controlled amounts of ABO blood group antigens (controlled antigen expression - CAE).

Two approaches were taken. In the first, the principles of glycolipid insertion were established and then synthetic analogues created, inserted and evaluated.

In the second approach, the potential alternatives of stripping blood group A/B cells down using glycosidases or synthesising A/B antigens on O cells using glycosyltransferases were evaluated.

The cells created to express controlled levels of ABO antigen were evaluated for their effectiveness as ABO analytical controls.

Results

The results are presented in five sections related to the preparation of ABO quality control cells:

Section 2	Natural glycolipids
Section 3	Modified natural glycolipids
Section 4	Synthetic glycolipids
Section 5	Glycosidase enzymes
Section 6	Glycosyltransferase enzymes

2 RESULTS - NATURAL GLYCOLIPIDS

Glycolipids (also called glycosphingolipids) are a group of molecules in which a carbohydrate chain is conjugated to a sphingolipid, the trivial name of which is ceramide (see sections 1.2.2.1 and 1.2.2.2).

Blood group active glycolipids were used in this research, the focus of which was to produce RBCs expressing a controlled weak expression of blood group A and/or B antigens. The structure of a common RBC six-sugar blood group A and blood group B glycolipid are shown in Figure 6 to demonstrate the general features of this molecule. RBCs express predominantly type 2 glycans.

A-6-2

B-6-2

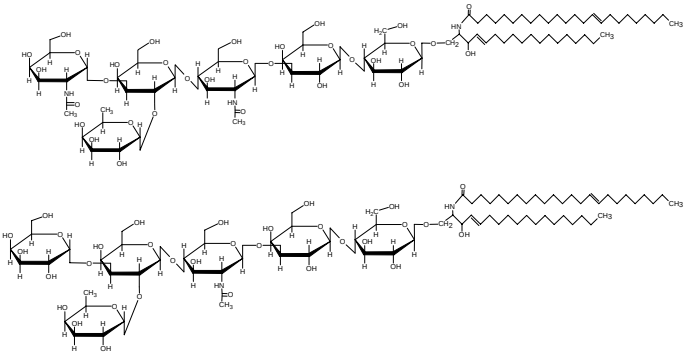


Figure 6. The structure of blood group active glycolipids. **Upper**: blood group A glycolipid, six sugars in length and type 2 (A-6-2). **Lower**: blood group B glycolipid, six sugars in length and type 2 (B-6-2). In both cases the ceramide/sphingolipid is composed of the fatty acid, nervonic acid ($C24:1^{15}$), and the long chain base, 4-sphingenine ($d18:1^{4}$).

Natural blood group glycolipids can be obtained from biological sources such as RBCs, intestinal scrapings or faeces. Using a method based on that of Karlsson (Karlsson, 1987), blood group active glycolipids were extracted from RBCs for this work.

Following solvent extraction and some chromatographic purification (Karlsson, 1987), HPLC purification was carried out – the methodology for which was refined during this project (not shown).

Short chain natural glycolipids are usually only soluble in organic solvents. For transformation of RBCs, they must be able to be solubilised in aqueous isotonic solutions (protocol 1.1). The natural glycolipids were

dried onto salts before being redissolved in de-ionised water, with sonication and heat being used to maximise solubility.

The variables of glycolipid concentration, incubation temperature, incubation duration, diluent and ratio of cells to glycolipid were examined for their effect on transformation as assessed by serological agglutination. Stability trials were also carried out over extended time periods as a blood grouping sensitivity control cell would be required to have an 8 week shelf life (two weeks for delivery and six weeks with the customer). Cell health after transformation is an implicit requirement and was assessed visually by haemolysis and antigen expression.

2.1 Glycolipid Concentration

Experiments were carried out to determine the relationship between natural glycolipid concentration and the strength of transformation and cell health over a 62 day time period.

Initial transformation experiments were carried out with an HPLC highly purified (>95%) natural Le^b-6 glycolipid sample (Le^b-6) and a less pure (≈70-80%) natural blood group A glycolipid sample ranging from 6 to 14 sugars in length (A-6/14). The results in Table 5 show that higher concentrations of glycolipid in the transformation solution produce stronger agglutination results. For example, on day 1 Le^b-6 at 5 mg/mL gave a 4+ (maximal agglutination – see Appendix 7 for scoring details), whereas 0.01 mg/mL generated a 2+. The same trend can be seen for A-6/14, although not in the same concentration range. A-6/14 needs to be approximately 100-fold higher in concentration than Le^b-6 to generate equivalent agglutination i.e. A-6/14 at 10 mg/mL gives a 3+, which is achieved by Le^b-6 at 0.1 mg/mL. The A-6/14 sample was pigmented brown, and this was due to contaminating impurities of unknown identity.

The scores for all the cells decreased over the 62 day time period. Le^b-6 gave a 4+ at 5 mg/mL on day 1, which reduced to 2-3+ after 62 days. A slight strengthening was seen with A-6/14 at 1 and 2 mg/mL between day 1 and day 25. This effect was not evident for any of the other cells.

Table 5. Stability of RBCs transformed with A-6/14 and Le^b-6 glycolipid over 62 days.

Glycolipid (mg/mL)	Serology*					
	Anti-Le^b			Anti-A		
	d 1	d 25	d 62	d 1	d 25	d 62
10	4		2-3		2	ǂ
5	4		2-3	2	2	w
2	3		1-2	0	1	0
1	4		2	0	1	0
0.1	3	2	0	0		
0.01	2	2	0	0		
0.001	2	2	0	0		
0.0001	2	0	0	0		
0	0	0	0	0	0	0

* Serology in tube (protocol 3.1), scoring (appendix 7).
Cells were transformed at 37°C for 1.5 hours (protocol 2.1, v1) and stored in CellStab. The cells were tested against CSL anti-Le^b and Seraclone anti-A.

Insertion of glycolipids into the RBC membrane may be damaging or even bring about the complete rupture (haemolysis) of the membrane. The extent of haemolysis could be determined by the amount of red colouring in the supernatant.

This experiment evaluated whether insertion had any effect on the integrity of the RBC membrane or the long-term survivability of the cells.

From Table 6 it can be seen that minimal haemolysis was present after transformation took place on day 1 and this does not appear to correlate with the concentration of the glycolipid. For example, the cells transformed with Le^b-6 at 10 mg/mL were less haemolysed than those transformed at 5 mg/mL. The cells transformed with A-6/14 at the concentration of 10 mg/mL were not more haemolysed than the cells transformed at 1 mg/mL – in fact 10, 5, 2 and 1 mg/mL were equivalent.

After 62 days, there was greater haemolysis across the board, but again little correlation with concentration. The controls were as affected by haemolysis as the cells treated with between 5 and 1 mg/mL Le^b-6 and 1 mg/mL A-6/14. However, the cells transformed with Le^b-6 at concentrations below 1 mg/mL were less haemolysed than the control, which cannot be explained. Also perplexing is the mild haemolysis of the

cells transformed with 10 mg/mL Le^b-6, weaker than that seen when cells were transformed with lower concentrations.

The cells transformed with 10 mg/mL A-6/14 appeared brown and completely haemolysed, but this finding may be explained by the presence of microbial contamination (visibly observed) in the sample. There was significant haemolysis in the samples transformed with 2 and 1 mg/mL A-6/14, and slightly less in the 5 mg/mL sample.

Table 6. Visual assessment of haemolysis in A-6/14 and Le^b-6 transformed cells over 62 days. Day 1 – in the supernatant of the first wash after transformation, days 25 and 62 – in the cell preservative solution of the sedimented cells.

Glycolipid (mg/mL)	Haemolysis*					
	Le^b-6			A-6/14		
	d 1	d 25	d 62	d 1	d 25	d 62
10	1h	0	1h	1h	1h	n.r.
5	2h	0	3h	w	0	2h
2	w	0	3h	w	0	4h
1	w	0	3h	1h	0	3h
0.1			1h			
0.01			2h			
0.001			1h			
0.0001			1h			
0 (control)	1h	0	3h	1h	1h	3h

* Haemolysis scoring scale is analogous to the 4+ to 0 agglutination scale: 4h – severely haemolysed, 3h – very haemolysed, 2h – moderately haemolysed, 1h – mildly haemolysed, w - faintly haemolysed and 0 – no haemolysis seen. Legend: n.r. – cells not read because they were brown.
The cells were transformed at 37°C for 1.5 hours (protocol 2.1, v1) and stored in CellStab.

Because there was no correlation between glycolipid concentration and haemolysis, these results suggest that observed cell haemolysis is not caused by the insertion of glycolipids. Instead, the transformation process itself may be damaging to cells. This is supported by the observation that haemolysis increased by a similar degree for most cells over the 62 day period, including the controls. This leads to the conclusion that the factors affecting the health of transformed cells are the same as those affecting untransformed cells, and the most significant of those may simply be time. Later experiments using cell preservative solutions during

transformation were able to overcome the storage haemolysis issues (see section 2.6).

2.2 Duration of Incubation

In order to find the optimal incubation period for insertion at 37°C, cells were transformed for 1 and 2 hours (Table 7). A natural blood group B glycolipid sample with 6 to 14 sugar chains (B-6/14) was used along with the A-6/14 sample. At each given glycolipid concentration, the score after 2 hours was the same as or less than the score obtained after 1 hour (Table 7). These results suggest that increasing the duration of incubation during insertion of natural glycolipids at 37°C does not enhance transformation.

Table 7. Optimisation of the incubation time for transformation with A-6/14 and B-6/14 glycolipids at 37°C.

Glycolipid	Concentration (mg/mL)	Serology*			
		Albaclone		Bioclone	
		1 h	2 h	1 h	2 h
A	10	4	4	4	4
	5	4	4	4	2
	2	4	3	3	2
	1	3	2	2	2
	0.5	2	2	1	w
B	10	3	2	4	1
	5	3	2	3	2
	2	2	2	2	1
	1	1	w	1	w
	0.5	1	w	w	w

* Serology in tube (protocol 3.1), scoring (appendix 7).
Cells were transformed at 37°C (protocol 2.1, v2), and tested against Albaclone and Bioclone anti-A and anti-B.

It is possible that insertion reached a plateau between 1 and 2 hours, entering into equilibrium with the transformation medium. An exchange of glycolipids back into solution may explain the reduction in apparent insertion. It should, however, be noted that serological experiments measure agglutination, not insertion. Therefore, changes of membrane

shape induced by prolonged incubation may also influence the serological result obtained.

Another important factor is that these cells were transformed with no nourishment or support such as would be gained from the use of a cell preservative solution. The transformation diluent was PBS, and the temperatures required to transfer natural glycolipids over from organic to aqueous solvents are too high for cell preservative solutions. Later transformation experiments with synthetic glycolipids were performed in cell preservative solution.

2.3 Temperature

Previous insertion experiments had been carried out predominantly at 37°C after initial work set the precedent (Sneath & Sneath, 1955; Stormont, 1949). Later work had queried the efficiency of transformation at lower temperatures with natural glycolipids (appendix 1; Callies *et al.*, 1977).

In order to learn more about the effect of temperature on natural glycolipid insertion *in vitro*, transformation experiments were conducted at other temperatures. RT (25°C) and refrigeration temperature (2-8°C) were the obvious choices for simplicity and with a view to manufacturing scale (see section 4.3).

Previous data (appendix 1) had indicated that reduction of the incubation temperature would slow down the rate of insertion, requiring the use of longer incubation periods in this study to achieve equivalent serology.

Interval testing was used to establish 4 hours as the optimal incubation time at RT (results not shown). There appeared to be some strengthening of the agglutination reaction between 4 and 6 hours, but this difference did not appear to be significant after the first week.

Validation testing of RT transformation using the established 4 hour incubation period was then carried out in comparison with the previous method using 37°C. It can be seen in Table 8 that cells transformed with 10 mg/mL Le^b-6 at 37°C/1.5 hours and 25°C/4 hours both gave a 2+ agglutination score. At 1 mg/mL Le^b-6, there was no significant difference

(see Appendix 7 for the definition of significant score differences in serology) in scores – 1+ for 37°C/1.5 hours and w+ for 25°C/4 hours. Accordingly, transformation with natural glycolipids at 25°C for 4 hours appears to generate equivalent serology to the method where reactants are incubated at 37°C for 1.5 hours.

Also in Table 8, the affect of temperature on cell health was examined by rating haemolysis. At both concentrations of Le^b, 10 mg/mL and 1 mg/mL, the 37°C/1.5 hour incubation resulted in moderate haemolysis (2h on a 4h scale), whereas the 25°C/4 hour method produced only faint haemolysis (w).

Table 8. Comparison of RT and 37°C for effect on haemolysis and agglutination† during insertion of Le^b-6.

Leb-6 (mg/mL)	Haemolysis*		Anti-Le^b	
	RT	37 °C	RT	37 °C
10	w	2h	2	2
1	w	2h	1	w

* Haemolysis was scored visually as per Table 6.
† Serology in tube (protocol 3.1), scoring (appendix 7).
Cells were transformed at 37°C for 1.5 hours or at 25°C for 4 hours (protocol 2.1, v4).
Transformation was assessed by agglutination against CSL anti-Le^b.

Consequently, RT appears to be less harmful to RBCs than 37°C, while generating equivalent serology. The combination of these two factors means that RT is preferable to 37°C in the method for transforming RBCs with natural glycolipids. Although later experiments used cell preservative solutions instead of PBS, these results are still valid as they evaluate an extreme condition.

Natural glycolipid insertion was investigated at 2°C after the synthetic glycolipids showed improved transformation at this temperature (see section 4.3). The results presented here show that natural glycolipids can insert at refrigeration temperatures (see Tables 9 and 10), although they do not show the same improvement in efficiency over the 37°C method as occurred with the synthetics (section 4.3). The rate of transformation at 2°C is slower for both A-6/14 and B-6/14, as can be seen by the negative agglutination scores after 1 hour at all concentrations. At 37°C, a serology score of 3+ is accomplished at 10 mg/mL in the same 1 hour interval. A-6/14 is just starting to show some positivity (1-2+) after

8 hours incubation, but B-6/14 serology only reaches 1+ after 24 hours and 2+ after 48 hours (see Tables 9 and 10). A-6/14 insertion at 2°C has essentially reached the same level as the 37°C transformation after 48 hours and has plateaued after this time, while B-6/14 insertion at 2°C does not really even come close to the 37°C method (Table 10). B-6/14 seems to have reached maximal insertion at 48 hours at these concentrations – it does not improve with 24 hours further incubation.

Table 9. Comparison of 2°C and 37°C for insertion of A-6/14.

Temp	Time (hours)	Anti-A*				
		A-6/14 (mg/mL)				
		10	5	2	1	0
37°C	1	3	3	2-3	2	0
2°C	1	0	0	0	0	0
	4	0	0	0	0	0
	8	1-2	0	0	0	0
	24	2-3	2	1-2	0	0
	48	3	2-3	2-3	0	0
	72	3-4	3	2	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour or at 2°C for varying intervals (protocol 2.1, v5), and tested against Bioclone anti-A.

Table 10. Comparison of 2°C and 37°C for insertion of B-6/14.

Temp	Time (hours)	Anti-B*				
		B-6/14 (mg/mL)				
		10	5	2	1	0
37°C	1	3	2-3	2	0	0
2°C	1	0	0	0	0	0
	4	0	0	0	0	0
	8	0	0	0	0	0
	24	1	0	0	0	0
	48	2	1-2	0	0	0
	72	2	1	0	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour or at 2°C for varying intervals (protocol 2.1, v5), and tested against Bioclone anti-B.

Overall, the picture is complex: A-6/14 glycolipid insertion at 2°C achieves equivalent serology to that of 37°C/1 hour after 48 hours, while B-6/14 glycolipid insertion at 2°C does not, even after 72 hours.

The difference between the agglutination performance of A-6/14 glycolipids and B-6/14 glycolipids at 2°C is difficult to explain. It is possible to speculate that the difference may be due to slight differences in sample profile. However, given that this phenomenon was seen in subsequent experiments with both the biotinylated trisaccharides and the synthetic glycolipids, it is more likely to be related to serology. Accordingly, anti-B is known to have a lower affinity for the B antigen than anti-A does for the A antigen (see discussion).

Stability trials on cells transformed at 25°C and 2°C were not performed because at this time the synthetic molecules had become the primary focus of this work as they were outperforming the natural molecules in all respects. The synthetic samples were clean, pure, and had better solubility, and the glycotope could be engineered to have no chain type (see section 1.1) and thus react with many different antibody clones.

A stability trial of cells transformed at 37°C had been carried out before the synthetics had been evaluated (see section 2.6). Further evaluation of temperature effects on natural glycolipid insertion, although of interest, was not warranted in this project.

2.4 Ratio of Cells to Natural Glycolipid

The optimal ratio of cells and natural glycolipids for transformation is one that balances the potency of transformation (amount of molecules inserted as assessed by agglutination) with economy of glycolipid consumption – in other words, maximises efficiency. The ratio of RBCs to natural glycolipid for insertion had been established in previous work as 3:1 (three parts RBCs to one part natural glycolipid) (appendix 2), but this matter was revisited for natural glycolipids after it was found that the optimal ratio for the synthetic glycolipids was 1:1 (see section 4.5).

The RBC to natural glycolipid ratios of 1:5, 1:2, 1:1, 2:1, 3:1, 4:1 and 5:1 were chosen because of the data from previous work (appendix 3) and for synthetic glycolipids (section 4.5).

For A-6/14 glycolipids (Table 11), there is variation of less than a score between each ratio and its neighbours (2-3+, 2-3+, 2-3+, 2+, 1-2+ and 1+). Combined with the fact that there appears to be no observable point at which the score trend suddenly changes, this makes it difficult to select one ratio over another. The 1:1 ratio showed only a slightly higher agglutination score (2-3+) than the reactions where less glycolipid was added (2:1 generated a 2+ and 3:1 a 1-2+), and this score difference (from 2-3+ to 1-2+) is well within the margin of error for agglutination. Looking at the problem from this perspective, it is hard to argue that the 1:1 ratio has any case for selection over the 2:1 and 3:1 ratios. However, the 1:1 ratio appears to be the most efficient in terms of insertion quantity (as measured by agglutination) because it produces the same serology as the reactions where more glycolipid is added (1:2 and 1:5). From this point of view, the 1:1 ratio is the least wasteful choice. As the data does not clearly establish which ratio produces the best agglutination, the demonstration of economy is the only criteria that can be assessed. On this basis, the 1:1 ratio appears to be the best option.

Table 11. The effect on transformation of varying the amount of RBCs with respect to a finite amount of A-6/14 glycolipid solution. The A-6/14 was at a concentration of 2 mg/mL and 10 μL was added to each transformation tube – the RBC amount was varied in relation to this.

	Anti-A*						
	RBC:A glycolipid ratio						
	1:5	1:2	1:1	2:1	3:1	4:1	5:1
Score	2-3	2-3	2-3	2	1-2	1	w

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour (protocol 2.1), and tested against Bioclone anti-A.

For B-6/14, the ratios of 1:2 to 3:1 produced agglutination scores of 2+ (Table 12), while the ratios of 4:1 and 5:1 produced no detectable agglutination at all. This means that use of more glycolipid than in the 3:1 ratio (such as in the ratios of 1:2 through to 2:1) was uneconomical because it did not result in stronger agglutination, and adding less

(4:1 or 5:1) did not effect discernible transformation. Thus from these results, the optimal ratio for B-6/14 would seem to be 3:1.

Table 12. The effect on transformation of varying the amount of RBCs with respect to a finite amount of B-6/14 solution. The B-6/14 was at a concentration of 2 mg/mL and 10 μL was added to each transformation tube – the RBC amount was varied in relation to this.

	Anti-B*						
	RBC:B glycolipid ratio						
	1:5	1:2	1:1	2:1	3:1	4:1	5:1
Score	1	2	2	2	2	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour (protocol 2.1), and tested against Bioclone anti-B.

2.5 Volume Correction

This experiment examined the effect of increasing the volume of non-reactants on the efficiency of insertion. It was considered possible that dilution may affect glycolipid micelle size, which in turn may affect insertion.

In order to compare each ratio with and without added diluent, parallel tubes were set up in the following way. Within each pair of tubes, the ratio of cells to natural glycolipid were kept constant (and the amount of glycolipid was always 10 μL at 2 mg/mL), but extra diluent was added to one of the tubes to make the total volume up to 60 μL. For example, the 2:1 tubes contained 20 μL packed RBCs and 10 μL glycolipid solution, and one of the tubes had 30 μL working strength PBS added.

The greatest dilution effect occurs in the 1:5 tube – this tube contained 2 μL RBCs, 48 μL working strength PBS and 10 μL A-6/14 or B-6/14 (in contrast to the 4:1 tube for example, which contained 40 μL RBCs, 10 μL working strength PBS and 10 μL A-6/14 or B-6/14). As the ratio of cells increased (through 1:2, 1:1 and so on), the factor of dilution decreased until it equated with packed cells in the 5:1 tube, to which no working strength PBS was added. In each set of tubes at a given ratio, the amount of glycolipid present in the reaction in relation to the amount of cells does not change.

The significance of this experiment is in the comparison of the agglutination score obtained from the volume corrected tube with that obtained from packed cells at each given ratio. Comparing thus, no significant difference is seen between the volume corrected and the packed cell tubes at the ratios of 1:2, 1:1, 2:1, 3:1, 4:1 or 5:1 for A-6/14 glycolipids (Table 13).

Table 13. The effect of increasing the volume of non-reactants on transformation with A-6/14 glycolipids. A-6/14 was at a concentration of 2 mg/mL and 10 μL was added to each transformation tube. The amount of cells added was varied as shown to provide the stated ratio. In the volume corrected tubes, working strength PBS was the diluent.

Ratio	RBC (μL)	Diluent (μL)	Total (μL)	Anti-A*
1:5	2	-	2	2-3
	2	48	50	1-2
1:2	5	-	5	2-3
	5	45	50	2-3
1:1	10	-	10	2-3
	10	40	50	2-3
2:1	20	-	20	2
	20	30	50	2
3:1	30	-	30	1-2
	30	20	50	1-2
4:1	40	-	40	1
	40	10	50	1-2
5:1	50	-	50	w
	50	-	50	1

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 37°C for 1 hour (protocol 2.1), and tested against Bioclone anti-A.

For B-6/14 (Table 14), the greatest difference between the volume corrected and packed cell agglutination scores occurs in the 3:1 tubes. The variance is insignificant at all the other ratios.

Table 14. The effect of increasing the volume of non-reactants on transformation with B-6/14 glycolipids. B-6/14 was at a concentration of 2 mg/mL and 10 μL was added to each transformation tube. The amount of cells added was varied as shown to provide the stated ratio. In the volume corrected tubes, working strength PBS was the diluent.

Ratio	RBC (μL)	Diluent (μL)	Total (μL)	Anti-B*
1:5	2	-	2	1
	2	48	50	0
1:2	5	-	5	2
	5	45	50	1-2
1:1	10	-	10	2
	10	40	50	1-2
2:1	20	-	20	2
	20	30	50	1
3:1	30	-	30	2
	30	20	50	vw
4:1	40	-	40	0
	40	10	50	0
5:1	50	-	50	0
	50	-	50	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 37°C for 1 hour (protocol 2.1), and tested against Bioclone anti-B.

Taken together, these results indicate that increasing the volume of non-reactants in the assay does not affect insertion efficiency.

Previous unpublished work had demonstrated that RBCs are less efficiently transformed when in a low suspension than they are in a higher suspension (see appendix 3). For example, cells incubated for 36 hours in a 10% suspension with 1.5 μg/mL Le^b-6 natural glycolipid resulted in negative agglutination, whereas the samples at 25% and 50% under the same conditions gave 2+ scores. Increase in the volume of non-reactants (PBS) in experiments with natural glycolipids in the present research did not show a similar reduction in insertion strength (see section 2.5).

This difference may possibly be explained by the different diluents – serum in the previous unpublished work, and PBS or cell preservative in the current research. These diluents may differently affect natural

glycolipid micelle size, which may change insertion characteristics and effectiveness.

2.6 Stability Trials

In order to produce useful blood grouping sensitivity controls, transformed cells must be stable for an appropriate length of time. RBCs can be maintained for a limited time at 2-8°C, and Reagent RBCs in the market generally have an 8 week shelf life. The purpose of the stability trial was to establish the stability of RBCs transformed with A-6/14 and B-6/14 glycolipid solutions of varying concentrations when stored in two different cell preservative solutions – CellStab (Diamed) and Alsevers (standard serology solution). Stability for less than the 8 weeks required would make the cells unsuitable for commercialisation.

New A-6/14 and B-6/14 blood group glycolipid samples were prepared to a higher purity than those used in previous experiments (subjected to an extra HPLC stage). Based on the results of concentration finding experiments (results not shown), concentrations of 10, 5, 2, and 1 mg/mL were selected for stability trialling with A-6/14 and B-6/14 glycolipids. In this experiment, the effect of leaving the transformation solution in with the cells overnight was also investigated with one of the concentrations (2 mg/mL). The cells in these tubes were washed just prior to the first agglutination test (day 2), while the cells of all the other tubes were washed immediately after the 1 hour incubation (before overnight storage in CellStab). This highlights the less obvious difference between these two treatment groups – the unwashed cells spent an extra 12-18 hours in PBS, while the washed cells were in the cell preservative solution.

All cells were tested using the standard tube serology platform (protocol 3.1) up to 42 days, at which time the cell agglutination reactions had become too difficult to score manually (see Tables 15 and 17). Diamed gel-card testing (protocol 3.2) was carried out to day 56 for the Alsevers stored cells (discontinued at day 63 due to fungal contamination – although still returning positive scores). The CellStab stored cells continued to be tested up to day 70, and were still viable at this point (Table 16, Table 18, Figure 8 and Figure 9).

Table 15. 42 day stability trial of A-6/14 glycolipid transformed cells stored in either CellStab or Alsevers.

Day	Cell storage solution	Anti-A*									
		Albaclone					Bioclone				
		Transformation Solution (mg/mL)									
		10	5	2	2†	1	10	5	2	2†	1
2	AL	4	3	2	1	w	3	3	1	1	0
	CS	4	4	3	1	1	3	3	2	1	0
8	AL	4	4	2	1	1	2	2	1	1	0
	CS	4	4	3	2	1	3	3	2	w	0
14	AL	4	3	2	2	w	2	1	w	vw	0
	CS	4	3	3	2	w	3	2	w	vw	0
21	AL	3	2	2	2	1	2	2	2	1	0
	CS	3	3	2	1	‡	2	‡	‡	‡	0
28	AL	2	2	1	1	0	2	2	1	1	0
	CS	2″	2″	‡	‡	0	1	w	0	0	0
36	AL	3	2	2	2	1	3	3	2	1	1
	CS	3″	2″	‡	‡	‡	3″	‡	‡	‡	‡
42	AL	3	3	1	w	0	2	2	2	1	1
	CS	4″	4″	‡	‡	‡	‡	‡	‡	‡	0

* Serology in tube (protocol 3.1), scoring (appendix 7), † transformation solution (containing glycolipids) was left in over night and washed out the next day, ‡ score assignment impossible, ″ score assignment difficult (positive cell button, but cells stream off as if negative). Abbreviations: AL - Alsevers, CS – CellStab.
The cells were transformed at 37°C for 1 hour (protocol 2.1, v6).

Up to 14 days, A-6/14 transformed cells maintained scores within one score level of their initial result (Table 15). After 21 days, some of the CellStab stored cells became impossible to score in manual serology. This effect was more pronounced against Bioclone anti-A and occurred over the three lower A-6/14 concentrations (5, 2 and 1 mg/mL). Surprisingly, some of the cells that had been unreadable subsequently became readable again. By day 42, all CellStab stored A-6/14 transformed cells were unreadable against Bioclone anti-A, while only the 2 and 1 mg/mL A-6/14 transformed cells were thus against Albaclone anti-A. The results for the 10 mg/mL A-6/14 transformed cells stayed within two score levels

from day 1 to day 42 against both antibodies. Of the cells that remained readable, there appeared to be no significant difference between the 2 mg/mL A-6/14 transformed cells in which the glycolipid was washed out after transformation and those cells in which the glycolipid remained. In addition, the serology of the unwashed cells did not indicate that they were negatively affected by their extra 12-18 hours in PBS instead of CellStab.

The most significant point about these results is, however, that the concentrations of A-6/14 producing serology in the range required – the 2+ to 3+ – suffered from deterioration leading in some cases to the cells being unreadable in manual systems.

Figure 7 shows a comparison of the relative abilities of Alsevers and CellStab to support cells transformed with A-6/14 glycolipids at 5 mg/mL. The cells stored in CellStab show as good or better agglutination against Albaclone anti-A than the Alsevers stored cells. However, the score difference is not significant in any of the three instances that it occurs (days 2, 21 and 42).

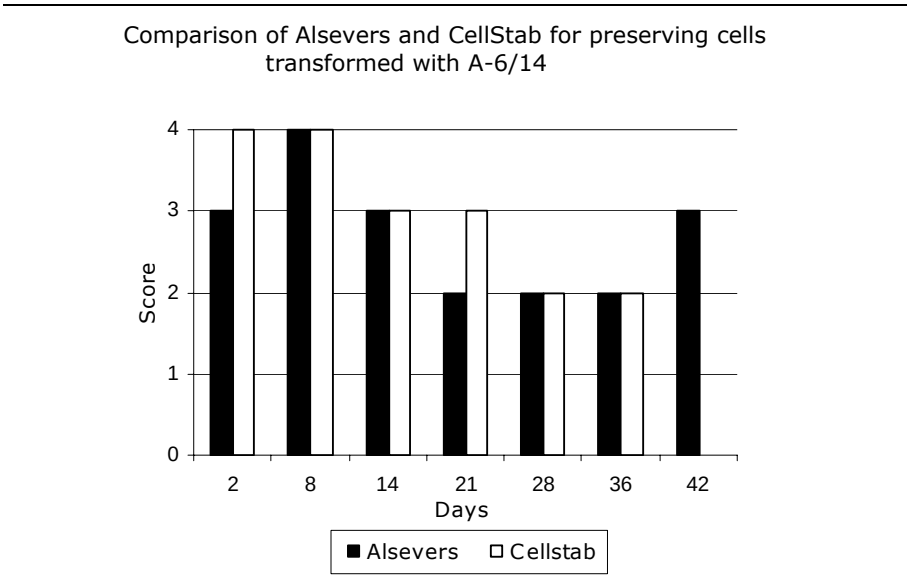


Figure 7. Comparison of Alsevers and CellStab as storage solutions for cells transformed with A-6/14 at 5 mg/mL. The cells were transformed at 37°C for 1 hour (protocol 2.1, v5), and then stored in either Alsevers or CellStab. Testing against Albaclone anti-A was carried out on the days shown. The day 42 result for the cells stored in CellStab is not shown because, although recorded as a 4+ (see Table 15), it was difficult to score and therefore not a true 4+. Score – serology in tube (protocol 3.1), scoring (appendix 7).

Parallel column agglutination testing was carried out and Table 16 shows the gel-card agglutination results over 70 days. Figure 8 shows images of the day 2 and days 70 Diamed gel-cards. The 10 mg/mL and 5 mg/mL A-6/14 transformed cells stored in Alsevers or CellStab gave 3+ and 4+ results over the 70 day period (although testing of the Alsevers stored cells was discontinued at day 63 due to fungal contamination) against Albaclone anti-A, and 4+ agglutination against Bioclone anti-A. The 2 mg/mL transformed cells stored in Alsevers or CellStab, both washed and unwashed, began at 3+ or 2-3+ and stayed around this score, with one instance of weakening to 2+ against Albaclone anti-A (the Alsevers stored washed sample at day 36). The final score for these cells was 2-3+ (on day 56 for the Alsevers stored cells and day 70 for the CellStab stored cells). Against Bioclone anti-A, these cells started and finished at 3+ (on day 56 for the Alsevers stored cells and day 70 for the CellStab stored cells), with the scores in between ranging from 3-4+ at the highest to 2-3+ at the lowest. Thus, there was no significant difference between the 2 mg/mL A-6/14 glycolipid transformed cells in which the glycolipid was washed out after transformation and those cells that received a further 12-18 hours incubation with the glycolipid solution PBS based) at 2°C. The 1 mg/mL transformed cells stored in Alsevers started at 2+ and weakened to negative at the midpoint (day 36) but ended on 1+ at day 70 against Albaclone anti-A. Against Bioclone anti-A, 1 mg/mL transformed cells stored in Alsevers began on 2+, but weakened to 1+ at day 14 through to day 56. The 1 mg/mL CellStab stored cells began on 2+ against both antisera, but Albaclone gave weaker scores through the trial, falling to a final score of 1+ at day 70. Bioclone anti-A was able to maintain the 2+ score with the exception of a 1+ at day 36.

These results show that the concentrations of 10, 5 and 2 mg/mL have good stability when tested using column technology – each concentration exhibited a spread of only one score level over the 70 day trial. The 1 mg/mL transformed cells were also stable against Bioclone anti-A, but varied by two full score levels against Albaclone anti-A. The cells all gave a definite score at each time point in column, which was not the case using manual tube serology.

Table 16. 70 day stability trial of A-6/14 glycolipid transformend cells stored in either CellStab or Alsevers.

Day	Pres soln	Anti-A*									
		Albaclone					Bioclone				
		Transformation Solution (mg/mL)									
		10	5	2	2†	1	10	5	2	2†	1
2	AL	3	3	3	2-3	2	4	4	3	3	2
	CS	3	3	2-3	2-3	2	4	4	3	3	2
8	AL	4	3	2-3	2-3	1	4	3	3	3	2
	CS	3-4	3-4	2-3	2-3	1-2	4	4	3	3	2-3
14	AL	4	3	2-3	2-3	1	4	4	3	3	1
	CS	4	4	3	3	2	4	4	3-4	3-4	2-3
21	AL	4	3	2-3	2-3	1	4	4	3	3	1
	CS	4	4	3	3	2	4	4	3-4	3-4	2-3
28	AL	3	3	2-3	2-3	w	4	4	3	3	1
	CS	4	4	3	3	1-2	4	4	3	3	2
36	AL	3	3	2	2-3	0	4	4	2-3	2-3	1
	CS	4	4	2-3	2-3	1	4	4	3	3	1
42	AL	4	3	2-3	2-3	w	4	4	3	3	1
	CS	4	3	2-3	2-3	1-2	4	4	3	3	2
49	AL	4	4	2-3	2-3	w	4	4	3	3	1
	CS	4	4	3	3	1	4	4	3	3	2
56	AL	4	4	2-3	2-3	w	4	4	2-3	3	1
	CS	4	4	2-3	2-3	1	4	4	3	3	2
63	AL										
	CS	4	4	2	2	1	4	4	3	3	2
70	AL										
	CS	4	4	2-3	2-3	1	4	4	3	3	2

* Diamed gel-cards (protocol 3.2), scoring (appendix 7), † transformation solution (containing glycolipids) was left in over night and washed out the next day. Abbreviations: AL - Alsevers, CS - CellStab.
The cells were transformed at 37°C for 1 hour (protocol 2.1, v6).

Furthermore, over this time, the cells appeared to maintain a good standard of health. Figure 8 shows images of the Diamed gel-cards at days 1 and 70, and there is no evidence of haemolysis in the tops of the gel-card wells. As the cells were not washed prior to being introduced into the cards, any haemolysis that occurred over the course of the trial would be seen as discolouration of the antisera above the gel.

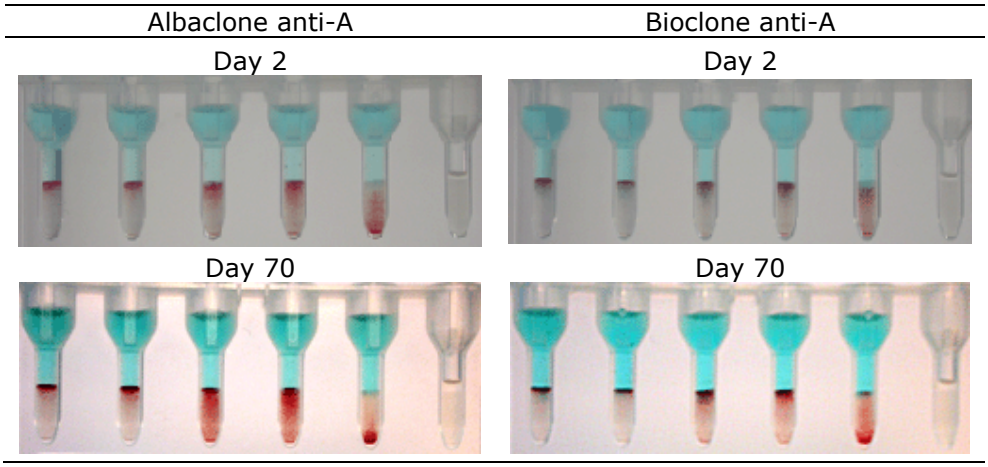


Figure 8. Day 2 and 70 Diamed gel-card images of CellStab stored cells transformed by A-6/14 glycolipid. The wells contain (L to R): A-6/14 glycolipid transformation solution at 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL (in which the transformation solution was left in over night and washed out the next day) and 1 mg/mL.

The cells were transformed at 37°C for 1 hour (protocol 2.1, v5).

The manual serology results of the experiment in which B-6/14 glycolipid was used to transform cells are shown in Table 17. Testing was discontinued at day 42 due to fungal contamination (visibly detected) in the Alsevers stored cells and the fact that the CellStab stored cells did not consistently return scores after day 14. By days 36 and 42, the CellStab stored cells were all impossible to score with either anti-B, and this meant that there was no meaningful data for the stability of CellStab stored cells.

The Alsevers stored cells showed a different pattern of deterioration to the A-6/14 glycolipid transformed cells. At day 21 the cells transformed with all concentrations of B-6/14 were impossible to score against Albaclone anti-B, as were the 2 and 1 mg/mL B-6/14 transformed cells against Bioclone anti-B. This is in contrast to the A-6/14 trial, in which Alsevers had been able to maintain transformed cells so that they could be scored through to day 42. However, after day 21, the Alsevers stored cells recovered and were able to be scored to day 42 to provide meaningful data. The 10 mg/mL B-6/14 transformed cells stored in Alsevers reduced

by only one score level – from 3+ to 2+ – against Albaclone anti-B over the 42 day trial. Against Bioclone anti-B, they started at 2+, but dropped to negative for two weeks before recovering to end on 2+. The 5 mg/mL B-6/14 transformed cells started at 3+, and weakened through to 2+, with some fluctuation to 1+ against Albaclone anti-B.

Table 17. 42 day stability trial of cells transformed with varying concentrations of B-6/14 and stored in either CellStab or Alsevers.

Day	Cell storage solution	Anti-B*									
		Albaclone					Bioclone				
		Transformation Solution (mg/mL)									
		10	5	2	2†	1	10	5	2	2†	1
2	AL	3	3	1	1	1	2	1	1	1	0
	CS	3	3	2	2	1	2	2	2	1	w
8	AL	1	1	w	0	0	0	0	0	0	0
	CS	2	1		w	0	1	1	w	0	0
14	AL	2	2	0	w	0	0	1	1	2	0
	CS	1	w	0	0	0	2	2	w	1	1
21	AL	‡	‡	‡	‡	‡	1	1	‡	‡	‡
	CS	‡	‡	‡	‡	‡	1	1	1	‡	‡
28	AL	2	1	w	0	0	2	1	2	0	0
	CS	‡	‡	‡	0	0	‡	0	‡	‡	0
36	AL	2	2	2	1	1	2	2	2	1	1
	CS	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡
42	AL	2	2	2	2	w	2	2	1	w	w
	CS	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡

* Serology in tube (protocol 3.1), scoring (appendix 7), † transformation solution (containing glycolipids) was left in over night and washed out the next day, ‡ score assignment impossible, ” score assignment difficult (positive cell button, but cells stream off as if negative). Abbreviations: AL - Alsevers, CS – CellStab.
The cells were transformed at 37°C for 1 hour (protocol 2.1, v6).

Against Bioclone anti-B, these cells started at 1+ – unexpectedly low – before weakening to negative and then strengthening to 2+ at day 36 to 42. The 2 mg/mL transformed cells all started out at 1+ against both antisera. All these cells at different points weakened to negative, but recovered to end on 2+ against Albaclone anti-B, and 1+ and w+ for the

washed and unwashed cells respectively against Bioclone anti-B.Parallel Diamed gel-card serology results are shown in Table 18. The 10 mg/mL and 5 mg/mL B-6/14 glycolipid transformed cells gave very similar scores over the 70 day trial. The cells stored in Alsevers started out at 2-3+, and ranged down to w+ before finishing at 1-2+ at day 56, at which time testing was discontinued on these cells at day 63 due to fungal contamination.

Against Albaclone anti-B, the starting score was 2-3+ for the 10 mg/mL and 5 mg/mL transformed cells. However, the Alsevers stored cells reduced to w+ by day 36, recovering slightly to end on 1-2+ on day 42, at which point testing was discontinued due to fungal contamination. The CellStab stored cells maintained a 2-3+ or 2+ score through to day 70. Against Bioclone anti-B, these concentrations started on 3-4+ and ended on 2+ or 2-3+ (at day 42 for the Alsevers stored cells and day 70 for the CellStab stored cells).

The cells transformed with 2 mg/mL B-6/14 glycolipid and stored in Alsevers started at 1-2+ against Albaclone anti-B, and 2+ against Bioclone anti-B. Against Albaclone anti-B, the Alsevers stored cells cells weakened to the lowest point of negative around day 36, and finished on w+ on day 42. Against Bioclone anti-B, the washed cells weakened through w+ to finish on 2+, while the unwashed cells weakened to 1+ at day 28 and maintained that score through to day 42.

The CellStab stored cells transformed with 2 mg/mL B-6/14 started at 2+ against Albaclone anti-B and the washed cells weakened to negative over the course of the trial, while the unwashed cells weakened to w+. Against Bioclone anti-B, the starting score was 2-3+ for both the washed and unwashed cells, and they mirrored each other in weakening to 2+ from day 36 to day 56. After this point their scores differed when the washed cells weakened to 1+ at day 70 while the unwashed cells finished on 2+.

The 1 mg/mL B-6/14 transformed cells were negative against Albaclone anti-B, and vw to w+ against Bioclone anti-B for the first weeks, but becoming negative by day 36.

Table 18. 70 day stability trial of B-6/14 transformend cells stored in either CellStab or Alsevers.

Day	Pres soln	Anti-B*									
		Albaclone					Bioclone				
		Transformation Solution (mg/mL)									
		10	5	2	2†	1	10	5	2	2†	1
2	AL	2-3	2-3	1-2	1-2	0	3-4	3-4	2	2	vw
	CS	2-3	2-3	2	2	0	3-4	3	2-3	2-3	vw
8	AL	2	2	w	w	0	2-3	2-3	2	1-2	0
	CS	2-3	2-3	2	2	0	3	3	2-3	2-3	0
14	AL	2-3	2	w	w	0	2-3	2-3	2	2	0
	CS	2-3	2-3	2	2	0	3	3	2-3	2-3	w
21	AL	2	2	w	1	0	2-3	2-3	2	2	0
	CS	2-3	2-3	2	2	0	3	3	2-3	2-3	vw
28	AL	1	1	0	vw	0	2	2	w	1	0
	CS	2	2-3	2	2	0	3	3	2-3	2-3	vw
36	AL	w	w	0	0	0	2	2	w	1	0
	CS	1-2	2	w	1	0	2	2-3	2	2	0
42	AL	1-2	1	w	1	0	2	2	w	1	0
	CS	2-3	2-3	2	2	0	2-3	2-3	2-3	2-3	0
49	AL	1	1	0	vw	0	2	2	w	1	0
	CS	2	2	1	1-2	0	2-3	2-3	2	2	0
56	AL	1-2	1-2	w	w	0	2	2	2	1	0
	CS	2-3	2-3	1	2	0	2-3	2-3	2	2	0
63	AL										
	CS	2	2	0	1	0	2	2	1	1-2	0
70	AL										
	CS	2	2	0	w	0	2-3	2	1	2	0

* Serology in tube (protocol 3.1), scoring (appendix 7), † transformation solution (containing glycolipids) was left in over night and washed out the next day. Abbreviations: AL - Alsevers, CS - CellStab.
The cells were transformed at 37°C for 1 hour (protocol 2.1, v6).

Bioclone anti-B appeared to give stronger results than Albaclone anti-B overall – at day 2, the scores against Bioclone were about one score level higher than the Albaclone results. For example, at 10 mg/mL B-6/14, the day 2 score against Bioclone anti-B was 3-4+, whereas against Albaclone anti-B it was 2-3+.

As with the A-6/14 transformed cells, there appeared to be no significant difference between the 2 mg/mL B-6/14 transformed cells in which the glycolipid was washed out after transformation and those cells in which that did not occur. Also in agreement with the results from A-6/14 transformed cells, B-6/14 transformed samples that were impossible to score in tube gave rational results using the gel-card platform.

Figure 9 shows the agglutination results at days 2 and 70 of cells transformed with B-6/14. The slight discolouration in the wells of the 10 mg/mL and 5 mg/mL transformed cells at day 70 is not haemolysis, it is a layer of cells adhering to the plastic.

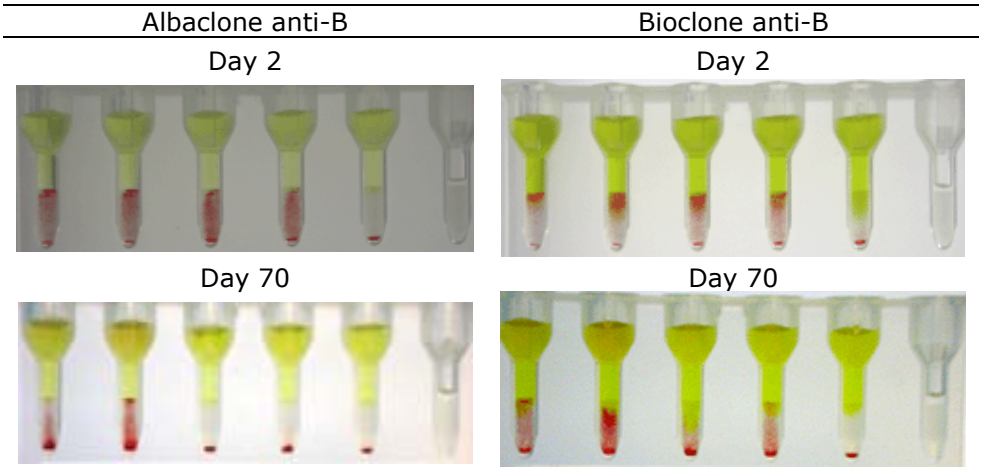


Figure 9. Day 2 and 70 Diamed gel-card images of CellStab stored cells transformed by B-6/14 glycolipid. The wells contain (L to R): B-6/14 glycolipid transformation solution at 10 mg/mL, 5 mg/mL, 2 mg/mL, 2 mg/mL (in which the transformation solution was left in over night and washed out the next day) and 1 mg/mL.

The cells were transformed at 37°C for 1 hour (protocol 2.1, v5).

It was interesting that both the A-6/14 and B-6/14 glycolipid transformed cells became impossible to score in the tube method. In a further stability trial carried out on cells transformed at 25°C for 4 hours, the cells could be scored over 80 days (Table 19). The collective results of Tables 15, 17 and 19 indicate that antigen loss or loss of detectability occurred universally, but the deterioration that rendered transformed cells

impossible to score by manual serology could have been related to the insertion methodology. It is possible that an hour's incubation at 37°C damaged the RBC membrane and affected the preservation or presentation of antigen in the long term.

The overall weakening of agglutination scores during storage suggests a reduction in antigen-antibody complex formation. This could come about through two mechanisms; loss of antigen, which could occur through passive loss of the molecules into the external environment; or loss of the antigen's ability to be recognised by antibody (probably through antigen presentation issues). The fact that some of the cells that had been impossible to score subsequently recovered indicate that the mode of antigen loss was due to some form of membrane effect. During storage, the RBC membrane is known to undergo changes in conformation, forming poikilocytes, or irregularly shaped erythrocytes. Associated membrane rearrangement may have caused some of the inserted natural glycolipid antigens to become masked or, more likely, to change their presentation so as to be undetectable with antibodies. The end result of this experiment is that cells modified in this way are unsuitable for use in manual serology assays.

In column, the cells appeared to have quite good stability, dropping their agglutination scores by two score levels at most over 70 days and showing negligible haemolysis.

The above results indicate that CellStab is an effective preservative for A-6/14 and B-6/14 glycolipid transformed cells, allowing them to perform well in column testing for 10 weeks.

The CellStab stored cells contained no contamination that was visible to the naked eye after 70 days, whereas the Alsevers stored cells became fungally and possibly bacterially contaminated (first noted at about day 42). CellStab contains antibiotic and anti-fungal agents, while Alsevers does not, which probably accounts for these results. Preparation of transformed cells was not conducted in a sterile manner, so fungal spores and bacteria could be introduced during this time and at each of the testing intervals. While still returning positive scores, but looking

almost black, the Alsevers stored cells had to be discarded after 56 days (8 weeks). Even if prepared in a sterile manner, it is doubtful that this cell storage solution could maintain uncontaminated cell suspensions for the required 8 weeks of daily use.

Celpresol is the cell storage solution manufactured by CSL, and its ability to support transformed cells was compared with that of CellStab.

Table 19 shows the results of a trial to compare the performance of Celpresol with that of CellStab. The cells transformed with 10 mg/mL A-6/14 and stored in CellStab started out with 3+ serology in tube, with erratic variation over the 80 day testing period, losing two to three score levels (see day 73 – vw), to finally settle at 2+, one level below the initial score. The cells stored in Celpresol also started out on 3+ and exhibited inconsistent scores to end on 1+ after 80 days. This is a drop of two score levels.

Table 19. Comparison of cell storage solutions CellStab and Celpresol on agglutination of 10 mg/mL A-6/14 glycolipid transformed cells.

Day	Anti-A*	
	Cell Preservative Solution	
	CellStab	Celpresol
2	3	3
8	1	2
15	1	2
22	3	3
28	3	2
36	2	1
43	3	2
51	1	2
59	2	0
66	2	w
73	vw	1
80	2	1

* Serology in tube (protocol 3.1), scoring (appendix 7).
The cells were transformed at 25°C for 4 hours (protocol 2.1, v3) and tested against Seraclone anti-A to day 15 and thereafter against Albaclone anti-A.

The greatest variation between storage solutions occurred at day 59, when the 10 mg/mL A-6/14 transformed cells in CellStab are 2+, while

the corresponding cells in Celpresol were negative, and day 62, when the scores were 2+ and w+ respectively. Other than this, the scores remained within one level of each other, which is within the range of accuracy of manual serology.

The similarity of the agglutination scores means that the two solutions performed equally to maintain cells transformed with natural glycolipid.

Significantly, the above cells did not become impossible to score in tube over the 80 day testing period, as did the cells in the previous stability trial (see Table 15). It is possible that the revised transformation method may have had some contribution. The above cells were transformed at 25°C, whereas the previous stability trial was conducted at 37°C. This suggests that temperatures above 24°C may compromise RBC function and survival in some way – a concept supported by others (Hobbs *et al.*, 1995). It is thus possible that an hour's incubation at 37°C (as the cells in Table 15 underwent) caused some kind of damage to the RBC membrane that had negative consequences with regard to long term antigen presentation.

When compared to Table 15, the agglutination scores in Table 19 appear to be lower, e.g. in Table 15, 10 mg/mL A-6/14 insertion produces sustained maximal agglutination, but in Table 19, the same concentration of A-6/14 only gives 3+ agglutination, which promptly drops to 1+ for the next two weeks. This would seem to indicate that the 25°C/4 hour insertion method is sub-optimal in this case, and that less glycolipids had inserted into the RBC membrane. The lower amount of glycolipids in the membrane would not account for the absence of deterioration, because the lower concentrations of A-6/14 used to transform cells at 37°C (Table 15), in which it could be expected that fewer glycolipids had inserted into the cell membrane, exhibited the most pronounced deterioration. Equally, it was possible that the antiserum used had deteriorated and changing to a new reagent restored expected score values.

Summary

In conclusion, the 37°C transformation method using natural glycolipids resulted in cells that deteriorated to the point that they could not be scored in manual tube serology. This deterioration appeared after just 3 weeks, and became widespread. In contrast, the 25°C transformation method produced cells that did not appear to deteriorate and which could be scored in tube over an 80 day testing period. However, both methods showed some variability in the scores from week to week in tube. As the variation was up to two levels for the 37°C method and three levels for the 25°C method, it was significant and due to more than the subjectivity of this scoring method – methodological variation was probably the cause. The 25°C transformation method produced lower agglutination scores by manual serology than the 37°C method, indicating that insertion at 25°C was suboptimal for natural glycolipids in this instance.

Diamed gel-card score variation was only one score level for A-6/14 transformed cells, but up to 2 score levels for the B-6/14 transformed cells. These cells were all transformed at 37°C.

These results show that A-6/14 glycolipid produced transformed cells with good stability when tested using column technology, but not when the testing method was manual tube serology. Furthermore, over this time, the cells appeared to maintain a good standard of health.

Despite these limitations, it was clear that the natural glycolipids are suitable to prepare quality control cells expressing reduced amounts of ABO antigen.

3 RESULTS - MODIFIED NATURAL GLYCOLIPIDS

Biotin and avidin have long been used as a coupling system in many applications. Gangliosides are glycolipids that have one or more sialic acid residues in the carbohydrate chain. Sialic acid is a nine carbon monosaccharide with a highly reactive carboxyl group on carbon 1 that can be used for the attachment of biotin (Wilchek & Bayer, 1987). It is usually a terminal sugar and carbohydrate chains can bear multiple units. Biotin and avidin comprise a well known coupling system, and are used in this research to link the functional molecule (biotinylated saccharide epitope) to the inserted membrane anchor (biotinylated ganglioside). Gangliosides are a heterogeneous mix of different sized carbohydrate chains and physicochemical properties. They form micelles in aqueous media and can insert into the RBC membrane (Schwarzmann *et al.*, 1983) much like that of neutral glycolipids (Schwarzmann, 2001). However, the charge possessed by gangliosides may cause them to behave differently to neutral glycolipids, for example, the association of gangliosides with trypsin on the cell membrane (see section 1.2.7) has not been reported for neutral glycolipids. The biotinylated synthetic trisaccharides comprise the A glycotope (see Figure 1 for structure of the A trisaccharide glycotope) joined to biotin through a polyacrylamide (PAA) linker.

Biotinylated gangliosides (BioG) were prepared using a modified procedure described by Wilchek and Bayer (Wilchek & Bayer, 1987), and formulated for experimental work as presented in protocol 1.2.

The biotin-avidin transformation system involves three sequential stages. The sphingolipid tail of the biotinylated gangliosides (BioG) can insert as an anchor into the lipid bilayer of the RBC membrane. In this way, cells can be decorated with biotin, which function as avidin attachment points. The concentration of the BioG affects the final outcome as it determines the number of attachment points on the cell. It must be used at a concentration that is high enough to result in positive transformation following completion of the final step, but not so high that agglutination is too strong or that the cell membrane is damaged by excess insertion.

The second step in the process is the attachment of avidin to the biotin molecules. Excess avidin is used to ensure that every biotin is bound by an avidin molecule and to minimise/prevent crosslinking of two or more adjacent BioG molecules present in different red cells – i.e. prevent agglutination.

The final step is the addition of the biotinylated trisaccharide to utilise some or all three of the remaining biotin binding sites of each avidin molecule.

The number of BioG molecules that can be inserted into RBC membranes can be manipulated because insertion is concentration, time and temperature dependent. The attachment of the biotinylated trisaccharide is believed to occur almost instantly and is dependent on concentration but not temperature. It is also limited by the number of avidin molecules present. Thus, the transformation of RBCs using the biotin-avidin system can be controlled at two stages – the insertion of BioG and the attachment of the biotinylated trisaccharides.

In order to determine optimal concentrations of each constituent, block titres comparing multiple concentrations of both BioG and biotinylated trisaccharide were carried out.

3.1 Biotinylated Trisaccharides

Several biotinylated trisaccharide molecules of different structure (see Table 20) were trialled. $BioA_{tri}$ 1a and 1b, although different structures, were not identified as such by the supplier and were tested separately, and results attributed without knowledge of which molecule was which. It is believed that one was A_{tri}-sp-biotin, while the other was A_{tri}-sp-AC-biotin. The four $BioA_{tri}$ molecules (molecules $BioA_{tri}$-1a, $BioA_{tri}$-1b, $BioA_{tri}$-2 and $BioA_{tri}$-3 from Table 20) were assessed for transformation by agglutination (see Table 21) to determine the best molecules to give positive serology.

Table 20. Biotinylated saccharide ($BioA_{tri}$) molecules.

$BioA_{tri}$-1a $BioA_{tri}$-1b	(A_{tri}-sp-biotin/A_{tri}-sp-AC-biotin)
$BioA_{tri}$-2	A_{tri}-sp-AC_2-biotin
$BioA_{tri}$-3	A-PAA-biotin
$BioB_{tri(1)}$-3	B-PAA-biotin

Table 21 shows the results of the initial trial of the four $BioA_{tri}$ candidates. $BioA_{tri}$-2 gave a negative reaction with anti-A, as did one of the $BioA_{tri}$-1a or $BioA_{tri}$-1b molecules, while the other gave a weak positive reaction.

$BioA_{tri}$-3 gave the best agglutination reaction, and thus was selected for further investigation.

To determine the optimal concentration of BioG, excess $BioA_{tri}$-3 must be present to fully saturate all the free biotin binding sites on avidin. If the biotinylated trisaccharide was not added in excess quantities, the biotin binding sites may not all be utilised, and the agglutination reaction would not be a true reflection of the amount of BioG inserted. Once the excess $BioA_{tri}$-3 concentration is determined (based on agglutination results), the BioG optimal concentration can be inferred from the agglutination results. The threshold where the 4+ score is just changing to 3+ is the highest BioG concentration that should be used. Concentrations above this will cause unnecessary transformation – there is nothing to be gained from inserting more BioG than required to generate the 4+ reaction.

Table 21. Assessment of the four $BioA_{tri}$ molecules by agglutination.

BioG (μg/mL)	Anti-A*		
	$BioA_{tri}$ molecules (100 μg/mL)		
	1a/1b	2	3
30	0/2	0	4

* - Serology in tube (protocol 3.1), scoring (appendix 7).
Cells were prepared using the method outlined in protocol 2.2, v1, and tested against Seraclone anti-A.

A combination of several block titre experiments (results averaged) with BioG and $BioA_{tri}$-3 is shown in Table 22. From these results, 10 μg/mL appears to be an appropriate excess concentration for $BioA_{tri}$-3, being the point at which an increase in concentration did not show stronger agglutinations. The 50 μg/mL concentration did not appear to be generating stronger agglutination than the 10 μg/mL, and lower concentrations of $BioA_{tri}$-3 started to show reduced agglutination at the same BioG concentrations, e.g. at 500 μg/mL BioG, 10 μg/mL $BioA_{tri}$ produced a 4+, while 2.5 μg/mL produced a 3+ and 1.2 μg/mL a 2+. These scores were probably decreasing because the available biotin binding sites were not fully saturated.

Looking at the 10 μg/mL $BioA_{tri}$-3, it can be seen that BioG concentrations above 30 μg/mL generated maximal agglutination, which began to lessen

below this concentration (Table 22). The BioG concentration of 30 μg/mL appears to define the critical point. Weak A antigen expressing cells, producing agglutination results in the region of 2+ to 3+, would require a BioG concentration of less than 30 μg/mL combined with a $BioA_{tri}$-3 concentration in the range of 1.2 to 2.5 μg/mL.

Table 22. Averaged agglutination scores from a number of BioG-Avidin-$BioA_{tri}$-3 transformation experiments.

$BioA_{tri}$-3 (μg/mL)	Anti-A*										
	BioG (μg/mL)										
	500	250	120	60	50	30	20	10	5	2.5	1.6
50					4				0.5	0	0
10	4	4	4	4	4	4	3.5	3	1	0	0
5	4	4	4	3	4	4	4	3	0	0	0
2.5	3	2	2	3	0	3	2.5	2	0	0	0
1.2	2	1	2	2		2	2.5	1	0	0	0
0.6						1	1.5	2			

* Serology in tube (protocol 3.1), scoring (appendix 7).
The cells were transformed using equivalent methodology, and tested against Seraclone anti-A.

The B-active molecule that corresponded to $BioA_{tri}$-3, called $BioB_{tri(1)}$-3, was also assessed. Table 23 presents the results of a block titre of $BioB_{tri(1)}$-3 and BioG. Earlier experiments had shown that $BioB_{tri(1)}$-3 produced significantly lower agglutination results at biotinylated trisaccharide and BioG concentrations that were appropriate for $BioA_{tri}$-3 (results not shown). In other words, as with the natural glycolipids (and later synthetics), the B antigen need to be expressed at higher concentrations to achieve the same agglutination result as seen with A.

The concentration of $BioB_{tri(1)}$-3 that was used in the experiment presented in Table 23 was about 10-fold higher than that used for $BioA_{tri}$-3 (Table 22), and still did not achieve a 4+ result. The BioG concentration range was up to 500 μg/mL, but probably needed to go higher, as indicated by the plateaued 2+ agglutination scores for 100 and 500 μg/mL $BioB_{tri(1)}$-3, which suggests that $BioB_{tri(1)}$-3 was in excess for 500 μg/mL BioG. Later

(unrelated) experiments indicated that cells transformed with 5000 μg/mL BioG became auto-agglutinable – sticking to one another without the presence of antibodies (results not shown). The "stickiness phenomenon" probably occurred at BioG concentrations lower than 5000 μg/mL, which could have introduced an artefact into the agglutination results obtained with BioG transformed cells. However, no auto-agglutination was noted using BioG concentrations up to 500 μg/mL (Table 22). Because the BioG-avidin-biotinylated trisaccharide system had been superseded by the synthetic glycolipids when BioG-inserted RBC auto-agglutination was noted, the issue was not further investigated.

These results indicate that 500 μg/mL BioG combined with 100 μg/mL $BioB_{tri(1)}$-3 gives a similar serological result to that produced by 10 μg/mL BioG and 2.5 μg/mL $BioA_{tri}$-3.

Table 23. Agglutination results for BioG-avidin-$BioB_{tri(1)}$-3 transformed RBCs.

$BioB_{tri(1)}$-3 (μg/mL)	Platform	Anti-B*			
		BioG (μg/mL)			
		500	250	100	0
500	Tube	2	1	0	0
	Column	2	0		
100	Tube	2	1-2	0	0
	Column	2	0		
50	Tube	1	w	0	0
	Column	2	0		
10	Tube	1	vw	0	0
	Column	2	0		
0	Tube	0	0	0	0
	Column	0	0		

* Serology in tube (protocol 3.1) and Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were prepared using the method in protocol 2.2, v1, and tested against Bioclone anti-B.

The difference in agglutination scores is believed to be due to the poorer performance of B antisera (see Discussion). This trend was also seen with the natural and synthetic glycolipids (see section 2 and section 4 respectively).

3.2 Stability Trials

Transformed cells must be stable for an appropriate length of time at 2-8°C for them to function as useful blood grouping sensitivity controls.

A 10 week trial of BioG-avidin-BioA$_{tri}$-3 cells was conducted to establish stability and performance characteristics. The results are presented in Table 24. At the lowest concentration of BioG (50 μg/mL), the agglutination score decreased from 4+ at day 1 to 1-2+ by day 72. This is a significant reduction in agglutinability. The 4+ reaction was maintained for 22+ days – by day 28 the agglutination reaction had dropped to 2+.

The higher concentrations of BioG (500, 250 and 120 μg/mL) remained at 4+ over the entire 72 day trial. It is important to appreciate that serology cannot detect a reduction in antigen numbers above the 4+ threshold, therefore, a reduction in antigen reactivity (large or small) may not be detectable.

Table 24. Stability trial of cells with BioA$_{tri}$-3 at 10 μg/mL and BioG at varying concentrations.

Day	Anti-A*			
	BioG (μg/mL)			
	500	250	120	50
1	4	4	4	4
8	4	4	4	4
16	4	4	4	4
22	4	4	4	4
28	4	4	4	2
37	4	4	4	2
44	4	4	4	3
48	4	4	4	4
58	4	3	3	2
65	4	4	4	3
72	4	4	4	1-2

* Serology in tube (protocol 3.1), scoring (appendix 7).
Cells were made using the method of protocol 2.2, v2, and tested with Albaclone anti-A.

The cells were transformed with concentrations of BioG ranging from 500 μg/mL to 50 μg/mL, and with the one $BioA_{tri}$-3 concentration of 10 μg/mL. The cells transformed with 500 μg/mL BioG produced 4+ agglutination scores throughout the entire 72 day testing period. The cells transformed with 250 μg/mL and 120 μg/mL BioG produced relatively consistent 4+ scores, with the one exception of a single 3+ score at day 58. BioG 50 μg/mL transformed cells exhibited significant variation over the testing period. Maximal agglutination was seen until day 28, when the score dropped to 2+, and held at this to day 44. At this time, the score strengthened to 3+, and then to 4+ at day 48 before dropping again to reach 1-2+ by day 72. Thus the BioG 50 μg/mL transformed cells dropped in score by two and a half score levels – a significant reduction in agglutinability.

Cells that started out at 4+ decreased to 1-2+ over 72 days, a drop of two and a half score levels. The results thus indicate that cells transformed with BioG-avidin- $BioA_{tri}$-3 are not stable over 10 weeks.

The desired agglutination score for a weak sensitivity control cell is in the range of 2+ to 3+. Application of the drop of two and a half score levels to fictional transformed cells that had a starting score of 2+ could see the score drop to negative, or from 3+ to 1+ over 10 weeks.

The mechanism for this instability may be that BioG does not remain in the membrane, or that over time the BioG-avidin-$(BioA_{tri}\text{-}3)_3$ construct may assume a presentation at the membrane that is unfavourable to antibody binding. Alternatively, there may be some change in the membrane itself that reduces antibody recognition of the inserted antigen.

To further investigate this phenomenon, BioG-avidin cells were prepared and the $BioA_{tri}$-3 molecule was conjugated on each testing day. These cells were trialled in parallel with cells prepared in the conventional manner (protocol 2.2, v2). The concentrations of BioG and $BioA_{tri}$-3 were considerably lower than previous testing to try and obtain a starting score in the region of 2+ to 3+. The results in Table 22 show that this level of score was obtained with BioG at 20 μg/mL and $BioA_{tri}$-3 at 2.5 μg/mL.

Table 25 shows that the agglutination score on day 1 for the cells transformed with 2.5 μg/mL $BioA_{tri}$-3 was in line with previous testing (see Table 22). However, both treatment sets of cells became negative by day 8.

Table 25. Comparison of BioG-avidin-$BioA_{tri}$-3 transformation methods. BioG-avidin-$BioA_{tri}$-3 cells (protocol 2.2, v2) were compared with BioG-avidin cells where the $BioA_{tri}$-3 was conjugated on the day of testing (protocol 2.2, v3).

		Anti-A*		
BioG (μg/mL)	$BioA_{tri}$-3 (μg/mL)	Conventional method		$BioA_{tri}$-3 later method
		Day 1	Day 8	Day 8
20	2.5	3	0	0

* Serology in tube (protocol 3.1), scoring (appendix 7).
The cells were stored in CellStab and tested against Seraclone anti-A.

These cells were prepared at lower concentrations of both BioG and $BioA_{tri}$-3 than previous experiments, but nevertheless confirmed the reduction in antigen detection. The fact that cells not possessing the $BioA_{tri}$-3 molecule were no more stable than fully transformed cells indicates that the mechanism for the reduction of antigen recognition is independent of the $BioA_{tri}$-3 molecule. Therefore, it seems most likely that the phenomenon is related to the BioG molecule or the BioG-Avidin complex. The BioG itself may be unable to remain in the membrane, or in an appropriate domain or it may be unable to retain a configuration that adequately presents the glycotope for antibody recognition. This effect could be related to the fact that Avidin is quite a large molecule, at around 55 kDa in size, which could change the insertion characteristics of the BioG that it complexes with.

3.3 Antisera Comparison

A panel of several monoclonal and polyclonal antisera (appendix 10) were used to assay cells transformed with the BioG-avidin-biotinylated trisaccharide system. Historical reagents were used in order to test the ability of the cells to be agglutinated by a wide range of antibody clones, and because they are likely to have reduced performance – the very characteristic that a sensitivity control system must be able to detect. Antisera from different manufacturers (and even the same manufacturer

over different time periods) can react differently because of individual formulation. Antiserum formulation can differ in terms of the identity of the clone or blend of clones used, and in the titre of each antibody in the blend, and they also deteriorate with age. These cells need to perform well against a range of antisera, but could also highlight unsatisfactory threshold performance of certain reagents.

A panel of cells transformed with BioG concentrations of 500 to 50 μg/mL and $BioA_{tri}$-3 concentrations of 7.5 and 5 μg/mL was prepared for testing against the antisera. All the anti-A reagents were able to generate a 4+ reaction against natural group A cells (Table 26). The best performing reagents were the A-I and A-XX, giving predominantly 4+ scores against cells transformed with 250 μg/mL BioG. It is very interesting to note that these same antisera gave weaker reactions against the cells transformed with 500 μg/mL, which could be expected to give stronger scores than the 250 μg/mL BioG cells.

Table 26. BioG-avidin-$BioA_{tri}$-3 transformed RBCs against a panel of expired A antisera. Cells were prepared with a range of BioG and $BioA_{tri}$-3 concentrations.

BioG (μg/mL)	$BioA_{tri}$-3 (μg/mL)	Serology*									
		A antisera†									
		I	IV	VII	VIII	XII	XIII	XIV	XVI	XVII	XX
500	7.5	3	2	1	2	2	2	w	0	1	4
	5	3	3	1	1	0	0	0	w	2	2
250	7.5	3	2	0	1	0	0	0	0	1	4
	5	4	2	0	w	0	0	0	0	w	4
120	7.5	1	1	0	2	0	0	0	0	0	3
	5	w	w	0	0	0	0	0	0	0	w
50	7.5	2	w	0	0	0	0	0	0	0	2
	5	w	0	0	0	0	0	0	0	0	1
Group O cells		0	0	0	0	0	0	0	0	0	0
Group A cells		4	4	4	4	4	4	4	4	4	4

* Serology in tube (protocol 3.1), scoring (appendix 7), † reagent descriptions (appendix 10).
Cells were prepared by the method outlined in protocol 2.2, v2.

Seven out of the ten of the historical antibodies could not detect the cells transformed with a BioG concentration of 250 μg/mL or less. This

highlights an important facet of antisera deterioration. Underperforming reagents can still detect the majority of cells and produce a normal strength agglutination reaction.

Expired anti-B reagents were also tested. All the anti-B reagents showed that they could detect the natural group B cells with a 4+ reaction (Table 27). The B-X reagent detected the transformed cells the most strongly, followed by B-IV. Below the BioG concentration of 250 μg/mL, only the B-X reagent detects the transformed cells (apart from a w+ reaction from the B-IV reagent).

Table 27. BioG-avidin-$BioB_{tri(1)}$-3 transformed RBCs against a panel of expired B antisera. Cells were prepared with a range of BioG and $BioB_{tri(1)}$-3 concentrations.

BioG (μg/mL)	$BioB_{tri(1)}$-3 (μg/mL)	Serology* B antisera† I	II	III	IV	V	VI	VIII	X
500	7.5	0	0	0	2	0	0	0	0
	5	0	0	0	1	0	0	0	2
250	7.5	0	0	0	0	0	0	0	0
	5	0	0	0	1	0	0	0	2
120	7.5	0	0	0	0	0	0	0	1
	5	0	0	0	w	0	0	0	2
50	7.5	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	w
Group O cells		0	0	0	0	0	0	0	0
Group B cells		4	4	4	4	4	4	4	4

* Serology in tube (protocol 3.1), scoring (appendix 7), † reagent description (appendix 10). Cells were prepared by the method outlined in protocol 2.2, v2.

The other six anti-B reagents failed to detect the transformed cells, while effectively detecting the natural B cells. The most interesting of these are B-V and B-VI (CSL). These reagents do not react with glycolipids, yet react well with the trisaccharide (see also Syn B – appendix 8a and 8b). However, here B-VI failed to react with the same trisaccharide (albeit biotinylated) when attached to BioG-avidin. This result suggests that perhaps the biotin-avidin is causing some interference, or at least hindering antigen antibody binding. The trisaccharide of the $BioB_{tri(1)}$-3 is

identical to that of the Syn B molecule, but it is possible that the configuration of the PAA linker somehow presents the $BioB_{tri(1)}$-3 epitope incorrectly for the CSL anti-B to bind.

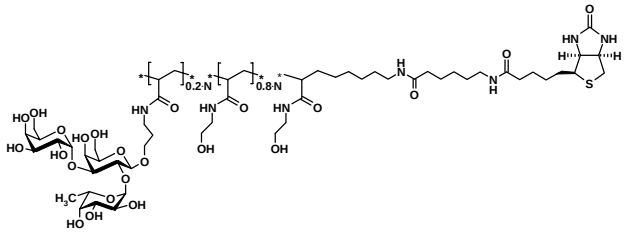
As expected, the transformed cells generated vastly different agglutination results against the panel of historical ABO blood grouping reagents. In contrast to natural ABO cells expressing the normal amount of blood group antigen, the transformed cells generate weak agglutination reactions.

3.4 Biotinylated Multivalent B

In order to try and address the low performance characteristics of anti-B and B transformation, a multivalent B molecule was designed. The three variants of the biotinylated multivalent B molecule (Table 28) have multiple copies of the B_{tri} glycotope (the same glycotope as the $BioB_{tri(1)}$-3 molecule) spaced along the PAA backbone, with the substitution rate being 20 mol %. The three molecules contain the same approximate B-glycotope content as a ratio of their mass but are different in their average molecular masses, which means the three molecules thus contain different total amounts of the B trisaccharide glycotope. Molecule 1 in Table 28 is the largest molecule, and is projected to have about thirty B trisaccharide glycotopes. Molecule 2 is about half the size of molecule 1, and thus has about fifteen B glycotopes. Molecule 3 is about one third of the size of molecule 1, and has about ten B glycotopes. All the molecules contain one biotin as an end-chain group. Because these molecules have more B glycotopes than the single $BioB_{tri(1)}$-3, and the prospect that they may assume a more favourable spacing for the antibody than occurs between multiple $BioB_{tri(1)}$-3 molecules, it is possible that less BioG may be required.

Table 28. Biotinylated mulitvalent B molecules.

1	$BioB_{tri(30)}$	0.94μmole B_{tri}/mg conjugate and 11.6 nmole Biot/mg conjugate
2	$BioB_{tri(15)}$	0.94μmole B_{tri}/mg conjugate and 23.2 nmole Biot/mg conjugate
3	$BioB_{tri(10)}$	0.94μmole B_{tri}/mg conjugate and 34.8 nmole Biot/mg conjugate



The three different multivalent $BioB_{tri}$ molecules were conjugated to RBC membranes via inserted BioG and avidin, and assessed for transformation by agglutination. $BioB_{tri(30)}$ (Table 28) was the only molecule to generate positive agglutination (Table 29), and was further investigated in subsequent experiments.

Table 29. Trial of the three different multivalent $BioB_{tri}$ molecules. The cells were transformed using the method outlined in protocol 2.2, with 100 μg/mL BioG and varying concentrations of the three different multivalent $BioB_{tri}$ molecules.

Multi-B (μg/mL)	Anti-B*					
	Multivalent B molecule					
	$BioB_{tri(30)}$		$BioB_{tri(15)}$		$BioB_{tri(10)}$	
	Alba	Bio	Alba	Bio	Alba	Bio
1000	2-3	3	0	0	0	0
10	0		0		0	

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were tested against Albaclone and Bioclone anti-B reagents.

$BioB_{tri(30)}$ was trialled in parallel with the monomeric $BioB_{tri(1)}$-3 at equivalent concentrations to evaluate the relative serological transformation of RBCs (Table 30).

RBCs transformed with 500 μg/mL BioG generated negative agglutination with both $BioB_{tri}$ molecules at 10 μg/mL, and were weak at 50 μg/mL for both molecules. At the three higher $BioB_{tri}$ concentrations, the results were essentially the same (4+ for $BioB_{tri(30)}$ and 3-4+ for $BioB_{tri(1)}$-3). The most significant difference occurred at the lowest concentration of BioG trialled (Table 30). At 100 μg/mL BioG, none of the $BioB_{tri(1)}$-3 coated cells were agglutinated by anti-B, while cells with $BioB_{tri(30)}$ gave positive serology. Maximal agglutination was seen with $BioB_{tri(30)}$ down to the

100 μg/mL concentration, decreasing to w+ at 50 μg/mL and negative at 10 μg/mL. The 3-4+ strength of reaction with 500 μg/mL BioG and $BioB_{tri(1)}$-3 at 500 or 100 μg/mL in Table 30 is significantly higher than previous experiments where both concentrations gave 2+ scores (Table 23). In this experiment, the ratio of biotinylated trisaccharide to BioG-avidin RBCs was 1:3 (protocol 2.2, v4), where previously it had been 1:1 (protocol 2.2). Comparison of the $BioB_{tri(1)}$-3 results (Table 30) with previous results (Table 23) showed no difference that was consistent with adding less $BioB_{tri(1)}$-3 into the reaction – it was still in excess.

Table 30. Comparison of cells transformed with $BioB_{tri(1)}$-3 and $BioB_{tri(30)}$.

Biotinylated Saccharide	(μg/mL)	Anti-B*	
		BioG (μg/mL)	
		500	100
$BioB_{tri(1)}$-3	1000	3-4	0
	500	3-4	0
	100	3-4	0
	50	vw	0
	10	0	0
$BioB_{tri(30)}$	1000	4	4
	500	4	4
	100	4	4
	50	w	w
	10	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed using the method in protocol 2.2, v4, and tested against Bioclone anti-B.

These results suggest that the avidin sites were saturated by the 100 μg/mL concentration of $BioB_{tri(1)}$-3, not allowing further attachment and maintaining the score at 3-4+ even though higher amounts of $BioB_{tri(1)}$-3 were introduced. Maximal agglutination, which can mask further attachment of the molecule, is reached with $BioB_{tri(30)}$ at the same concentration (100 μg/mL), but saturation could be assumed to occur at the same level as $BioB_{tri(1)}$-3 (as this is related to the number of biotin/avidin attachment points). However, differences in the molecular weight of the molecules ($BioB_{tri(1)}$-3 vs $BioB_{tri(30)}$) may mean the

concentrations are not directly comparable. If $BioB_{tri(30)}$ was actually at a lower molar ratio (as it would be if its molecular weight was higher than $BioB_{tri(1)}$-3), it may not have reached saturation (which is only determined by extrapolation of the results obtained from $BioB_{tri(1)}$-3). This then, could accommodate the 4+ occurring at the same $BioB_{tri(30)}$ level when the BioG concentration was lower (which should theoretically occur at a lower $BioB_{tri(30)}$ level because of less total biotin-avidin molecules to link to).

While the biotin-avidin sites appeared to have been saturated by the 100 μg/mL $BioB_{tri(1)}$-3 at the 500 μg/mL concentration of BioG, the lack of agglutination at the lower biotinylated ganglioside concentration possibly indicates that there was too little BioG (and hence too little biotinylated saccharide) to support agglutination. Alternatively, the difference may be related to the BioG itself and its behaviour in the membrane. At the lower concentration, BioG may have adopted an arrangement in the membrane that reduced the ability of antibody to support agglutination of the monovalent molecule. The 4+ results seen with $BioB_{tri(30)}$ indicate that this phenomenon did not affect the ability of the cells coated with the multivalent molecule to be agglutinated by anti-B.

These results suggest that $BioB_{tri(30)}$, with 30 copies of the B_{tri} glycotope, is able to generate agglutination at lower concentrations than the $BioB_{tri(1)}$-3 molecule, which bears only the single B_{tri} glycotope.

The interplay between the concentrations of BioG and biotinylated trisaccharide, and the variables at each of the three stages together detract from the significance of a comparison between this system and the insertion of the Syn B glycolipid. As the BioG sample contains a highly heterogeneous mix of gangliosides, molar equivalent concentrations for this and Syn B cannot be calculated, and there is no way to determine the average number of antigens inserted into cells using these two methods. Also, the probable differences in the insertion efficiencies of the double fatty acid tail of Syn B and the natural sphingolipid of BioG further complicates the matter.

Summary

With due consideration to limited methodological development, the attachment of biotinylated saccharides is able to determine differing activity between reagents. However, serious stability issues would need to be resolved before such a technique would be valid for a QC product.

4 RESULTS - SYNTHETIC GLYCOLIPIDS

Natural glycolipid molecules are difficult and time-consuming to extract, and purity had proven elusive. Synthetic analogues of the natural blood group related glycolipid molecules would solve the problems associated with extraction and purification. Furthermore, the capacity for enhancement of the molecule design to improve insertion performance could not be matched with the natural glycolipid.

Ideal performance characteristics:

- Aqueous solubility – the molecule needs to be easily transferable into biocompatible solvents, the most desirable of which is saline or RBC preservative solutions.
- Efficient insertion into membranes – as many of the molecules as possible needed to be able to insert into the membrane so that there was minimal wastage of uninserted product in post-transformation solutions.
- Antibody recognition – the glycolipid needs to be able to insert into the membrane and behave in its environment in such a way that it is detectable with immunoglobulins and/or lectins.

Synthetic molecules were prepared by Lectinity (Bovin *et al.*, 2005a; Bovin *et al.*, 2005b), a large array of which were investigated in this work (Table 31). Early testing highlighted design deficiencies in the initial prototype molecules, prompting modifications that resulted in the development of several molecules capable of successfully transforming cells (Bovin *et al.*, 2005a; Bovin *et al.*, 2005b).

It is important to note that the A and B glycotope requires four terminal sugars to generate core structure. Table 1 shows the different core structures (note specifically types 1 and 2) and Table 2 shows the difference between the A-type-1 and A-type-2 glycotopes. As the synthetic glycolipids have only the three terminal saccharides, they are effectively generic. This was anticipated as a potential advantage in terms of being reactive with antibody clones that are limited by core type specificities.

Table 31. Antigen and linker structures. Lipid tail structures are given in Table 32.

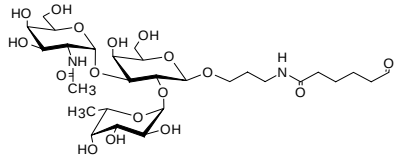
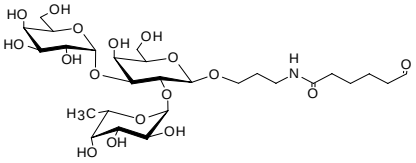
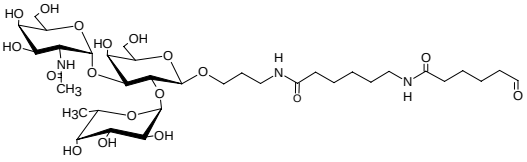
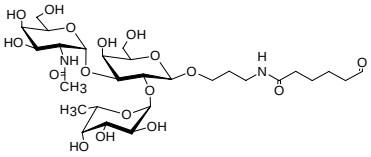
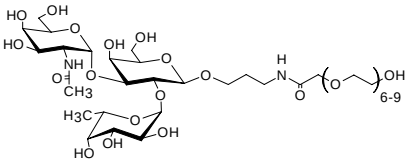
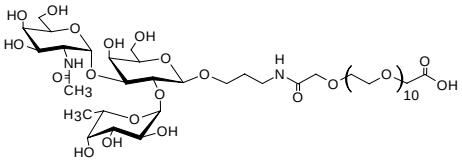
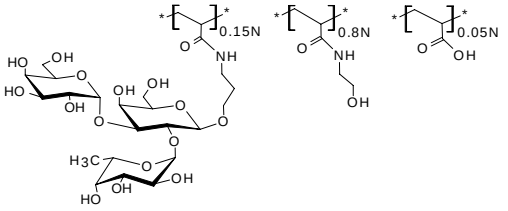
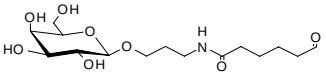
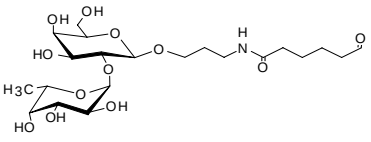
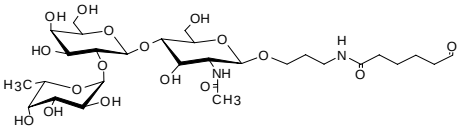
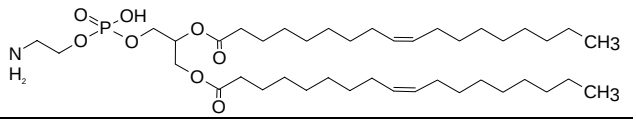
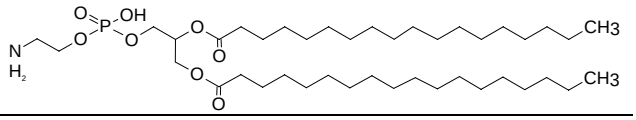
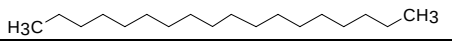
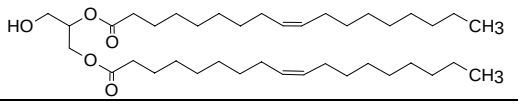
	Name	Antigen and linker	Lipid tail
1	A_{tri}-sp-Ad-DOPE (Syn A)		DOPE
2	B_{tri}-sp-Ad-DOPE (Syn B)		DOPE
3	A_{tri}-sp-Ac-Ad-DOPE (A-sp_2)		DOPE
4	A_{tri}-sp-Ad-DSPE (A-DSPE)		DSPE
5	Lipophilic A_{tri}		Lipo
6	A_{tri}-sp-POE_{10}-DOG (A-DOG)		DOG
7	B_{tri}-PAA-DOPE (B-DOPE)		DOPE
8	Galβ-sp-Ad-DOPE		DOPE
9	H_{di}-sp-Ad-DOPE		DOPE
10	H_{tri}-sp-Ad-DOPE		DOPE

Table 32. Lipid tail structure of the synthetic glycolipid molecules in Table 31.

DOPE	1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine 
DSPE	1,2-O-distearyl-sn-glycero-3-phosphatidylethanolamine 
Lipo	octadecanoic acid 
DOG	rac-1,2-dioleoylglycerol 

4.1 Lipid Anchor Evaluation

The first criterion that synthetic glycolipids should meet is that they are compatible with aqueous solvents e.g. phosphate buffered saline. A number of techniques – heat, sonication – can be employed in order to maximise this solubility (see protocol 1.3). The synthetic glycolipid must also be able to insert into the red cell membrane and be recognisable to the appropriate antibody.

Initial tests on the molecules were designed to establish aqueous solubility and antibody detectability and to identify the most promising contenders (summarised in Table 33).

It is interesting to note that, although soluble in PBS, Lipophilic A_{tri} was not detectable in RBC membranes after incubation (Table 33). Lipophilic A_{tri} (molecule 5, Table 31) had a single rather than a bilipid tail, and possibly did not insert into the membrane bilayer. However, agglutination is dependent on correct antigen presentation, which means it is not a true measure of whether or not insertion occurred. Thus it cannot be ruled out that insertion did take place, but the glycotope was undetectable with antibody. Two molecules, A-DOG and B-DOPE (molecules 6 and 7, Table 31), were found to be insoluble in PBS, and

were not evaluated for insertion in RBC membranes. The remaining A- and B-active synthetic glycolipids were all soluble in PBS and could be detected in RBC membranes following incubation.

Table 33. Solubility of synthetic glycolipids in hot PBS and transformation ability. The results of agglutination testing of selected molecules can be seen in Table 34.

Molecule	Synthetic name	Abbr	Water solubility	Detectable transformation
1	A_{tri}-sp-Ad-DOPE	Syn A	Yes	Yes
2	B_{tri}-sp-Ad-DOPE	Syn B	Yes	Yes
3	A_{tri}-sp1sp2-Ad-DOPE	A-sp_2	Yes	Yes
4	A_{tri}-sp-Ad-DSPE	A-DSPE	Yes	Yes
5	Lipophilic A_{tri}		Yes	No
6	A_{tri}-sp-POE_{10}-DOG	A-DOG	No	No
7	B_{tri}-PAA-DOPE	B-DOPE	No	No
10	Flu-Ad-DOPE	Flu	Yes	Yes

Preparation of the samples was as indicated in protocol 1.3, v1.
Transformation methodology was as outlined in protocol 2.3, v1.

Table 34 shows the serology results for the molecules that gave positive blood group agglutination. Syn A and A-sp_2 generate equivalent serology, with maximal 4+ agglutinations at 0.25 mg/mL against both Albaclone and Bioclone anti-A. A-DSPE produces only 2-3+ and 3+ at this concentration against Albaclone and Bioclone anti-A respectively. The agglutination strength of Syn A decreases at the same rate as A-sp_2 as the concentration of both is reduced. A-DSPE also diminishes, but seems to do so more slowly, so that all three molecules give a similar agglutination score (around 2+) at 0.01 mg/mL.

Note that the DBA lectin, which detects terminal α-GalNAc of the Forsmann disaccharide (GalNAcα1-3GalNAcβ1-R) or the blood group A trisaccharide (GalNAcα1-3[Fucα1-2]Galβ1-R) (Wu *et al.*, 1997), was unable to agglutinate cells transformed with any of the A-active synthetic glycolipids that could be detected by anti-A IgM. This unexpected result was not due to the lectin being inactive because it generated a 4+ agglutination reaction against A_1 cells (results not shown). These results may indicate that there was insufficient antigen present to support DBA agglutination, or that DBA requires more than three sugars or that the A trisaccharide glycotope of the synthetic glycolipid has an orientation that does not allow the lectin to bind or support agglutination.

Table 34. Evaluation of insertion ability of different lipid tails by agglutination.

Molecule	Antisera	Serology*							
		Transformation solution (mg/mL)							
		1	0.5	0.25	0.12	0.1	0.06	0.05	0.01
Syn A	Alba			4		3		2-3	2
	Bio			4†		4†		3†	3
	DBA	0							
Syn B	Alba	2	2	1	0		0		
	Bio	3	2	1	0		0		
A-sp_2	Alba			4		3		2	2
	Bio			4†		3-4†		3†	2
	DBA	0							
A-DSPE	Alba			2-3		2-3		2	2
	Bio			3		2-3		2	2
	DBA	0							

* Serology in tube (protocol 3.1), scoring (appendix 7),
† splatter (appendix 7).
All cells were transformed using the methodology outlined in protocol 2.3, v1.
Cells were tested against Albaclone and Bioclone anti-A and anti-B IgM reagents, and *Dolichos biflorus* anti-A_1 lectin (DBA).

The Syn B molecule produces lower agglutination scores than its A-active counterpart, Syn A. At 0.25 mg/mL, Syn B generates 1+ serology, while Syn A gives maximal agglutination. This is probably due to the known lesser performance of anti-B reagents in general (for further discussion, see section 3.1).

4.2 A-active Molecule Comparison

Three A-active molecules, Syn A, A-sp_2 and A-DSPE (molecules 1, 3 and 4, Table 31), were tested against a panel of monoclonal and polyclonal anti-AB reagents (appendix 10), most of which were expired. Table 35 shows the agglutination results. A-sp_2 gave predominantly maximal 4+ serology scores against these antibodies, with the only exceptions being a 3-4+ against AB-IV, a 2+ against AB-X and a 3+ against AB-XI.

Syn A did not perform as well as A-sp_2 against the anti-AB reagents. At the top of the scale, the only 4+ reactions were against the AB-IX reagent and the human AB-II. Interestingly, the other human reagent, AB-I,

performed quite well against the Syn A and A-sp$_2$ transformed cells, generating a 3+ and a 4+ respectively. At the bottom of the scale, no agglutination was supported by the AB-III or AB-XI reagents. The majority of the agglutination results in between were 2+, with some 3+ reactions and a w+.

Table 35. Comparison of RBC transformation with different 0.25 mg/mL A-active synthetic glycolipids against an anti-AB reagent panel. Anti-ABs are ranked by the strength of their reaction with Syn A transformed cells first, then with A-sp$_2$ transformed cells secondarily and finally with A-DSPE transformed cells.

Anti-AB Reagent	Serology*		
	A-active synthetic glycolipids		
	Syn A	A-sp$_2$	A-DSPE
II	4	4	2
IX	4	4	0
V	3	4	0
I	3	4	0
VII	2	4	0
VIII	2	4	0
VI	2	4	0
IV	2	3-4	0
X	w	2	0
III	0	4	0
XI	0	3	0

* Serology in tube (protocol 3.1), scoring (appendix 7), antisera panel (appendix 10).
Cells were transformed using the method outlined in protocol 2.3, v1, at a concentration of 0.25 mg/mL.

The polyclonal AB-II was the only reagent to agglutinate the A-DSPE transformed cells, and then only giving a 2+ reaction.

From the analyses in this section, two candidate molecules emerged, Syn A (and by association Syn B) and A-sp$_2$. A-sp$_2$ appeared to have superior serology against anti-AB monoclonal reagents (Table 35). However, the difference was not seen against the anti-A or anti-B reagents, and so was not significant enough to warrant the repetition of all the experiments carried out with the Syn A and Syn B molecules, which were completed well before the A-sp$_2$ variant was received. Future work is planned to evaluate B-sp$_2$. Further experiments in this section use Syn A and Syn B to produce cells called KODE™ A and KODE™ B

respectively, and KODE™ AB cells using both Syn A and Syn B (see section 4.4).

4.3 Temperature

The recommended storage temperature for RBCs is 2-8°C, as this slows down cellular metabolism and retards bacterial growth. If cells are healthier for longer when stored at 2-8°C, performing the transformation of RBCs within this temperature range is also possibly better for their long-term health and stability.

Trials were undertaken to determine the characteristics of transformation at refrigeration temperatures (Tables 36 and 37).

After in-house evaluation involving CSL, the concentrations of 0.08 mg/mL Syn A and 0.6 mg/mL Syn B had been chosen for the 37°C method (CSL evaluation of KODE™ - appendix 8a and 8b). These concentrations were used as a starting point.

Detectable transformation was seen after 4 hours with 0.08 mg/mL Syn A (Table 36). Maximal agglutination was observed at 48 hours. The 0.03 mg/mL Syn A transformed cells did not generate any detectable agglutination over the 96 hours testing period.

Table 36. Transformation of RBCs with Syn A at 2°C.

Time[¥] (hours)	Anti-A*		
	Syn A (mg/mL)		
	0.08	0.05	0.03
2	0	0	0
4	1	0	0
6	2	0	0
8	2	0	0
12	2-3	0	0
24	3-4	1	0
30	3-4	1	0
48	4	2	0
72	4	2	0
96	4	2-3	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C (protocol 2.3, v2), and tested against Bioclone anti-A.
[¥]All testing intervals were taken from the commencement of transformation.

Table 37 shows detectable transformation after 6 hours with the cells transformed with 0.6 mg/mL Syn B (w+). At 0.6 mg/mL Syn B, maximal agglutination is reached at 24 hours. At 0.3 mg/mL Syn B, agglutination occurs at 12 hours (w+), while at 0.15 mg/mL Syn B, 2+ insertion is detectable at 24 hours. As there was no testing between 12 and 24 hours, and the score jumped from 0 to 2+ in this time, it is probable that agglutination would have been observed prior to the 24 hour testing point.

It is interesting to note that transformation with Syn A and Syn B appears to occur at different rates. However, the concentration differences and the known performance disparity between A and B reagents preclude a direct comparison to determine the relative transformation rates.

Table 37. Transformation of RBCs with Syn B at 2°C.

Time[¥] (hours)	Anti-B*		
	Syn B (mg/mL)		
	0.6	0.3	0.15
2	0	0	0
4	0	0	0
6	w	0	0
8	2	0	0
12	2	w	0
24	4	3	2
30	4	2-3	0
48	4	3	1
72	4	4	2
96	4	3-4	2-3

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C (protocol 2.3, v2), and tested against Bioclone anti-B.
[¥]All testing intervals were taken from the commencement of transformation.

Testing was discontinued after 10 days (240 hours) because the cells turned brown (scores not shown beyond 96 hours). The reduced survival of these cells was attributed to a number of deleterious factors including: the 48 hour transformation was performed in PBS not Celpresol™, which would provide less than ideal nutrition and support; cell age – cells were already 21 days old when transformed; and also the fact that the cells were transported between Australia and New Zealand in suboptimal

conditions. Subsequent transformations under more desirable conditions resolved this survival issue (see appendix 8c).

The desired strength of agglutination for a weak positive control cell is in the region of 2+ to 3+. The concentrations identified as giving the desired score using the 37°C method were 0.08 mg/mL for Syn A and 0.6 mg/mL for Syn B, which were generating scores from 3+ to as low as 1+ (see appendix 8a).

The results obtained in this temperature insertion study show that agglutination results after insertion of Syn A and Syn B were comparable with those obtained from the 37°C transformation methodology (compare Table 34 with Tables 36 and 37). However, the scores obtained in this trial (Tables 36 and 37) were significantly higher than those obtained at 37°C (appendix 8a). CSL subsequently generated better scores with the 2-8°C method when contrasted against the 37°C method (see appendix 8b).

The results of this trial suggest that 2+ to 3+ scores are achievable at lower concentrations than those chosen by CSL (0.08 mg/mL Syn A and 0.6 mg/mL Syn B). The desired strength of score appears to be achievable with a Syn A concentration between 0.08 mg/mL and 0.05 mg/mL (which stabilise at 4+ and 2+/3+ respectively) and a Syn B concentration of somewhere between 0.3 mg/mL and 0.15 mg/mL (which stabilise at around 3+/4+ and 2+/3+ respectively).

It appears that the 2°C transformation is optimised for insertion of Syn A and Syn B as detected by agglutination. Whether this improvement is due to a greater amount of molecules being inserted or due to improved antigen presentation (possibly related to 2°C being less harmful to the RBC membrane) cannot be ascertained.

In all cases, the rate of insertion at 2°C was significantly slower than occurs at 37°C, and the incubation therefore needed to be longer to achieve scores that were comparable with those obtained after 37°C treatment. Negligible transformation as visualised by agglutination was seen after 8 hours at 2°C for most of the concentrations of glycolipid used, which corresponded with the results obtained using flow cytometry

(see appendix 1). However, it must be remembered that the concentration differences and the known performance disparity between A and B reagents preclude a direct comparison to determine the relative transformation rates.

The direct comparison between results from serology and flowcytometry must take into account the fact that flow cytometry has a wider range of detection of insertion than does serology. Flow cytometry shows that insertion continues beyond 10^2 molecules (see appendix 1), at which point the maximal serological score is achieved. This highlights the fact that the use of serology to measure insertion has its limitations. However, its use in this study was linked to the objective, which was the development of a sensitivity control cell for serological platforms.

This improvement in the dynamics of insertion for the synthetic glycolipids at 2°C was not seen for the natural glycolipids (see section 2.3).

4.4 Simultaneous Insertion

Given that the synthetic glycolipids are used at such low concentrations in relation to their natural counterparts (see section 2.1), it seemed feasible to make cells that simultaneously expressed Syn A and Syn B. A block titre was carried out in order to establish whether the insertion of the two different molecules would occur independently or would affect each other i.e. if the insertion of Syn B at its given high concentration would affect in any way the concurrent insertion of the lower concentration of Syn A. The concentrations for both molecules were chosen based on previous results (see Tables 36 and 37).

The insertion of Syn A appeared to be unaffected by the concomitant insertion of Syn B. For example, cells transformed with Syn A 0.07 mg/mL gave 2+ agglutination with anti-A when the Syn B concentration was 0.3 mg/mL and 0.2 mg/mL (Table 38). This score was obtained on both day 1 and day 5. This trend was also seen across the other two Syn A concentrations.

Against anti-B, the cells showed a similar trend to that seen against anti-A. For example, cells transformed with Syn B 0.3 mg/mL gave

3+ agglutination with anti-B when the Syn A concentrations were 0.07 mg/mL and 0.06 mg/mL (Table 39). When the Syn A concentration was 0.05 mg/mL, the anti-B score was 2+. The fact that these scores were so constant indicates that the insertion of Syn A was not affecting the insertion of Syn B. If anything, the anti-B score (as an indication of Syn B insertion) weakened slightly as the concentration of Syn A decreased, which is contrary to the imagined mechanism of interference.

Table 38. Simulaneous insertion of Syn A and Syn B at 2°C against anti-A.

Day	Syn B (mg/mL)	Anti-A*		
		Syn A (mg/mL)		
		0.07	0.06	0.05
1	0.3	2	1-2	w
	0.2	2	1-2	0
5	0.3	2	1-2	1
	0.2	2	1-2	w

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C for 48 hours (protocol 2.3, v3) and tested against Bioclone anti-A.

Table 39. Simulaneous insertion of Syn A and Syn B at 2°C against anti-B.

Day	Syn B (mg/mL)	Anti-B*		
		Syn A (mg/mL)		
		0.07	0.06	0.05
1	0.3	3	3	2
	0.2	1	1-2	0
5	0.3	2	2	1
	0.2	0	w	vw

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C for 48 hours (protocol 2.3, v3) and tested against Bioclone anti-B.

These results indicate that Syn A and Syn B insert into RBC membranes independently of each other over these concentration ranges. The anti-A agglutination reactions are very stable regardless of the concentration of the Syn B transformation solution. There is more variation in the B agglutinations than the A agglutinations, but variation of one score level is considered to be within the margin of error of this technique. Equivalence in the scores is demonstrated when comparing corresponding

concentrations of the weak AB cells (Tables 38 and 39) with those of the weak A cells (Table 36) and weak B cells (Table 37), indicating that insertion of Syn A is independent and unaffected by simultaneous insertion of Syn B – and vice versa.

4.5 Ratio of Cells to Synthetic Glycolipid

The optimal ratio of cells and synthetic glycolipids for transformation is one that maximises efficiency by balancing the strength of transformation (amount of molecules inserted as assessed by agglutination) with economy of glycolipid consumption. Previous work had established an optimal ratio of packed RBCs to natural glycolipids of 3 parts to 1 part respectively (see section 2.4 and appendix 2). A corresponding assay was carried out for synthetic glycolipids to see if the same principle held true.

Table 40 shows cells transformed with Syn A at varying ratios of RBCs to glycolipids. Maximal (4+), or very near to maximal (3-4+) agglutination results from transformation at the 1:5, 1:2 and 1:1 packed cells to transformation solution ratios. This score drops suddenly to 2-3+ at the 2:1 ratio. The 3:1 ratio was actually suboptimal with the 1 hour 37°C incubation methodology (see section 4.3 for discussion on optimisation of synthetic insertion with respect to other variables), generating an agglutination score of only 1+. Thus, the optimal ratio for Syn A and cells appears to be 1:1, because it produces the highest score using the least amount of glycolipid.

Table 40. The effect on transformation of varying the amount of RBCs with respect to a finite amount of Syn A glycolipid solution. Syn A was at a concentration of 0.08 mg/mL and 10 μL was added to each transformation tube – the RBC amount was varied in relation to this.

	Anti-A*						
	RBC:Syn A ratio						
	1:5	1:2	1:1	2:1	3:1	4:1	5:1
Score	4	4	3-4	2	1	w	1

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour (protocol 2.3, v1), and tested against Bioclone anti-A.

Table 41 shows that the results with Syn B are similar to those obtained with Syn A. Maximal agglutination was produced by transformation at the

1:5, 1:2 and 1:1 ratios. Dropping to a ratio of 2:1 (cells to synthetic glycolipids) showed an immediate and significant drop in agglutination to 2-3+. Thus, the 1:1 ratio of cells to Syn B is optimal, because like Syn A, it generates the highest score using the least amount of glycolipid.

Table 41. The effect on transformation of varying the amount of RBCs with respect to a finite amount of Syn B glycolipid solution. Syn B was at a concentration of 0.6 mg/mL and 10 μL was added to each transformation tube – the RBC amount was varied in relation to this.

	Anti-B*						
	RBC:Syn B ratio						
	1:5	1:2	1:1	2:1	3:1	4:1	5:1
Score	4	4	4	2-3	2	2	1-2

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were transformed at 37°C for 1 hour (protocol 2.3, v1), and tested against Bioclone anti-B.

It is clear to see in yet another respect that the synthetic glycolipids behave quite differently to the natural glycolipids. However, it is important to remember that previous insertion work had all been carried out with glycolipids dissolved in plasma. In plasma, natural glycolipid micelles are incorporated into LDL and HDL particles, and may therefore behave differently to those in saline, which may make it less appropriate to directly compare these two sets of data. Ratio experiments using the natural glycolipids were subsequently carried out to provide a closer comparison (see section 2.3), and showed that the use of PBS rather than plasma produced slightly different results. For this reason, it is inappropriate to directly compare the results presented here with those from the literature.

4.6 Volume Correction

The effect of increasing the volume of non-reactants on the effectiveness of transformation with synthetic glycolipids was investigated. The ratios of cells to synthetic glycolipid were kept constant, allowing the comparison of each ratio with and without added diluent.

Table 42 shows the greatest dilution effect occuring in the 1:5 tube – this tube contained 2 μL RBCs, 48 μL working strength PBS and 10 μL A-6/14 or B-6/14 (in contrast to the 4:1 tube for example, which contained 40 μL

RBCs, 10 μL working strength PBS and 10 μL A-6/14 or B-6/14). As the ratio of cells increased (through 1:2, 1:1 and so on), the factor of dilution decreased until it equated with packed cells in the 5:1 tube, to which no working strength PBS was added. In each set of tubes at a given ratio, the amount of glycolipid present in the reaction in relation to the amount of cells does not change.

Table 42. The effect of increasing the volume of non-reactants on transformation with Syn A glycolipids. Syn A was at a concentration of 0.08 mg/mL and 10 μL was added to each transformation tube. The amount of cells added was varied as shown to provide the stated ratio. In the volume corrected tubes, working strength PBS was the diluent.

Cells:Syn A	RBC (μL)	Syn A (μL)	Diluent (μL)	Total (μL)	Anti-A*
1:5	2	10	-	12	4
	2	10	48	60	3
1:2	5	10	-	15	4
	5	10	45	60	3
1:1	10	10	-	20	3-4
	10	10	40	60	3
2:1	20	10	-	30	2
	20	10	30	60	2
3:1	30	10	-	40	1
	30	10	20	60	1
4:1	40	10	-	50	w
	40	10	10	60	w
5:1	50	10	-	60	1
	50	10	-	60	w

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 37°C for 1 hour (protocol 2.3, v1), and tested against Bioclone anti-A.

The significance of this experiment is in the comparison of the agglutination score obtained from the volume corrected tube with that obtained with packed cells at the corresponding ratio. Comparing thus, at the 1:5 and 1:2 ratios in Table 42, there is a difference of one score level between the volume corrected tubes (3+) and the packed cell tubes (4+). This level of difference is considered to be within the error margins of agglutination scoring. The score differences for the other ratios are less than one level (4+ to 3-4+ or 1+ to w+) or nil.

These results indicate that the addition of non-reactants to the volume corrected tubes did not appear to make a significant difference to Syn A transformation strength as visualised by agglutination.

As with Syn A transformed cells, the difference between the volume corrected tubes and the packed cell tubes is one score level at ratios of 1:5, 1:2, 1:1 and 3:1 with respect to Syn B transformed cells (Table 43). The ratios of 4:1 and 5:1 show score differences of less than one level (2+ to 1-2+ and 1-2+ to 1+ respectively), as would be expected because there is less difference between the packed cell tube and the volume corrected tube at these ratios. Of note is the increase in score from the packed cell tube to the volume corrected tube at the 2:1 ratio, in contrast to the expected and demonstrated pattern of the volume corrected tube producing a slightly lower score than the packed cell tube. However, all of these score differences are within the margin of error for agglutination score assignment.

Most interesting in both tables (Tables 42 and 43) is that the 1:5, 1:2 and 1:1 tubes containing the increased volume of non-reactants were all 3+, i.e. maximal agglutination was not obtained. As the ratios of 1:5 and 1:2 contained two and five times more glycolipid than the 1:1 ratio relative to the volume of cells, they would be expected to have greater insertion as detected by strengthening agglutination scores, but this was not borne out by the results. It appears that increasing the volume of reactants may have reduced the efficiency of synthetic glycolipid insertion, and in this case, held the agglutination detectability to 3+. In other words, even though the 1:5 tube contained more glycolipid relative to cells than the 1:1 tube, the insertion efficiency was slightly reduced so that its agglutination score was the same as for the 1:1 tube.

In the corresponding packed cell reactions, Syn A and Syn B insertion caused maximal agglutination at the ratios of 1:5, 1:2 and 1:1. The ratios of 1:5 and 1:2 contained more glycolipid than the 1:1 ratio relative to the volume of cells contain as discussed above, and would thus be expected to result in more insertion, but this cannot be verified for the packed cell experiments because agglutination cannot detect increases in antigen numbers above the 4+ threshold.

Table 43. The effect of increasing the volume of non-reactants on transformation with Syn B glycolipids. Syn B was at a concentration of 0.6 mg/mL and 10 μL was added to each transformation tube. The amount of cells added was varied as shown to provide the stated ratio. In the volume corrected tubes, working strength PBS was the diluent.

Cells:Syn B	RBC (μL)	Syn B (μL)	Diluent (μL)	Total (μL)	Anti-B*
1:5	2	10	-	12	4
	2	10	48	60	3
1:2	5	10	-	15	4
	5	10	45	60	3
1:1	10	10	-	20	4
	10	10	40	60	3
2:1	20	10	-	30	2-3
	20	10	30	60	3
3:1	30	10	-	40	2
	30	10	20	60	1
4:1	40	10	-	50	2
	40	10	10	60	1-2
5:1	50	10	-	60	1-2
	50	10	-	60	1

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 37°C for 1 hour (protocol 2.3, v1), and tested against Bioclone anti-B.

None of the score differentials (from Table 42 and Table 43) discussed above are highly significant. This means that increasing the volume of the transformation reaction by the addition of non-reactants up to four times the volume of the reactants (volume of reactants – 2 μL RBCs, 10 μL glycolipid, non-reactant volume in the 1:5 volume corrected tube – 48 μL PBS) has no major effect on transformation.

4.7 Insertion Efficiency

In order to determine the quantity of synthetic glycolipids that insert into RBCs, glycolipid solutions that had been used in a large scale RBC transformation experiment (300 mL packed RBCs) were recovered after incubation as supernatants and tested for the presence of residual synthetic glycolipid molecules.

According to Table 44, all the transformations with reused Syn A and Syn B generated negative serology. To further investigate this phenomenon, the 0.08 mg/mL Syn A and 0.6 mg/mL Syn B post-transformation supernatants were concentrated 20x (protocol 5.1) and compared in parallel with transformation solutions of known concentration. Again, the transformation was negative for agglutination (Table 44).

The fact that the agglutination result for the transformation supernatant from the original Syn A 0.08 mg/mL solution was negative means that its glycolipid concentration must be less than 0.03 mg/mL, for which a 1+ result was obtained in the first round (Table 44). This indirect comparison implies that >65% of the molecules are inserted into the RBC membrane on the first pass.

Furthermore, the negative result obtained with the 20x concentrate of the 0.08 mg/mL Syn A post-transformation supernatant indicates that there are not enough molecules in this solution to cause detectable serology. This means that the 20x concentrate of the 0.08 mg/mL Syn A post-transformation supernatant was still lower than 0.03 mg/mL, which equates to >98% of the molecules being used up in the first transformation.

Table 44. Synthetic glycolipid insertion efficiency through transformation with supernatants from a previous insertion experiment. The three Syn A and one Syn B post-transformation supernatants were used at their existing concentrations, and the 0.08 mg/mL Syn A and 0.6 mg/mL Syn B post-transformation supernatants were concentrated 20x (protocol 5.1) for comparative purposes.

Synthetic glycolipid	Original (mg/mL)	Serology*		
		Original	Reuse 1x	Reuse 20x
Syn A	0.08	3	0	0
	0.05	2	0	
	0.03	1	0	
Syn B	0.60	4	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
All cells were transformed at 37°C for 1 hour (protocol 2.3, v1) and tested against Bioclone anti-A and anti-B reagents.

With due consideration of the methodological limitations, these results indicate that > 95% of synthetic glycolipid molecules from a 0.08 mg/mL solution of Syn A were able to be inserted into RBC membranes.

4.7.1 Calculation of Antigen Density

The insertion efficiency result above allows the calculation of the number of synthetic glycolipid antigens that inserted into each RBC in these experiments.

Using the molar masses of the synthetic glycolipids (the molecular weight of Syn A is 1542, and that of Syn B is 1501) it is possible to calculate the number of molecules in each solution (Table 45).

Table 45. Calculation of the number of Syn A and Syn B molecules in 1 μL of transformation solution at the concentrations required to generate serological agglutination.

Syn A		
Agglutination (anti-A)	Syn A concentration (mg/mL)	Syn A molecules in 1 μL
1+	0.02	7.81×10^{12}
Syn B		
Agglutination (anti-B)	Syn B concentration (mg/mL)	Syn B molecules in 1 μL
1+	0.1	40.1×10^{12}

The number of cells in the suspension used for transformation experiments was calculated, using the assumed value of 70% PCV. In order to calculate the number of RBCs in 1 μL of 70% PCV, the cell count of a normal, healthy individual was used as a starting point. The number of cells in 1 μL of whole blood with a hematocrit of 38% was 4.13 million. Calculating from this, there are 7.61 million RBCs in 1 mL of 70% suspension. Using the insertion efficiency figure of 95%, it is then possible to calculate the amount of molecules that inserted into each cell in a reaction containing 1 part transformation solution and three parts packed RBCs (Table 46).

Table 46. Calculation of the theoretical number of Syn A and Syn B molecules that inserted into each RBC to generate weak serological agglutination.

Syn A		
Concentration (mg/mL)	Agglutination (anti-A)	Syn A molecules per cell
		95% insertion
0.02	1+	325,000
Syn B		
Concentration (mg/mL)	Agglutination (anti-B)	Syn B molecules per cell
		95% insertion
0.1	1+	1,670,000

This means that, assuming that 95% of the molecules in the solution insert into the RBC membranes, a 1+ agglutination reaction against the relevant antisera is generated when each RBC contains 325,000 Syn A molecules and 1,670,000 Syn B molecules.

4.8 Antibody Neutralisation

Antibody can recognise and bind free antigen. In an agglutination assay, soluble antigen can combine with antibody and prevent reactivity with cell-bound antigen. This phenomenon is known as antibody neutralisation.

Uninserted synthetic glycolipid could potentially neutralise antibody if not washed away from the cells after transformation, although this effect would be dependent on the amount of antigen that did not insert into RBC membranes, as discussed in section 4.7, and its presentation in solution.

To ascertain how important it was to wash cells after transformation, different concentrations of Syn A solution were incubated with dilutions of monoclonal anti-A (prior to the test, the antibody was tested in serial dilution to establish its endpoint and the range that should be used – results not shown). The reactivity of the neutralised antibody was then assessed by its ability to agglutinate natural group A cells.

Table 47 shows manual tube serology results of this experiment. In this experiment, negative agglutination results show where antibody neutralisation has occurred, and weak scores show partial neutralisation.

The results show that the 5 mg/mL and 2.5 mg/mL Syn A solutions were able to neutralise anti-A and prevent its reaction with group A cells. As the concentration of Syn A was reduced, it was able to cause less neutralisation, as can be seen by the increase in agglutination score down the columns of the table. Looking across the rows of the table, the antibody dilution increases, and was more easily neutralised.

Syn A concentrations above 0.31 mg/mL produced significant neutralisation of the 1:16 dilution of anti-A. As the dilution of anti-A increased, the Syn A concentration at which neutralisation of anti-A occurred decreased. For example, at the 1:256 anti-A dilution, a score (2+) significantly lower than the control score (4+) was obtained for the lowest concentration of Syn A, 0.02 mg/mL. Compare this to the 1:32 anti-A dilution, where a 2+ occurred at 0.16 mg/mL Syn A, an 8-fold higher concentration.

Table 47. Inhibition of diluted antibody with soluble Syn A and subsequent reactivity with group A RBCs.

Syn A (mg/mL)	Anti-A*						
	Anti-A dilutions						
	1:16	1:32	1:64	1:128	1:256	1:512	0
5.0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0
1.25	1	0	0	0	0	0	0
0.63	1	0	0	0	0	0	0
0.31	3	2	0	0	0	0	0
0.16	4	2	1	0	0	0	0
0.08	4	4	4	3	1	0	0
0.04	4	4	3	3	2	0	0
0.02	4	4	4	3	2	0	0
0	4	4	4	4	4	3	0

* Serology in tube (protocol 3.1) against Bioclone anti-A, scoring (appendix 7).

To put these results into context, previous experiments have indicated that > 95% of the Syn A molecules in a 0.08 mg/mL transformation solution are probably being inserted into cell membranes (section 4.7). This corresponds to a post-transformation solution having a concentration of < 0.004 mg/mL. As can be seen from Table 47, at anti-A dilutions of 1:16 to 1:128, a solution of Syn A at 0.02 mg/mL does not significantly

alter agglutination of A cells in comparison with the controls. At the lower dilutions, 1:256 and 1:512, the antibody is neutralised by the 0.02 mg/mL concentration of Syn A, but the 0.02 mg/mL concentration is still 4.5 times higher than the post-transformation solution (0.004 mg/mL). These results indicate that normal agglutination reactions using undiluted anti-A are unlikely to be affected if cells are not washed after transformation.

Direct evaluation of the consequences of not washing away the insertion fluid was undertaken. Two parallel sets of aliquots of transforming cells were removed at timed intervals, one for testing in the unwashed state, and one to be washed prior to testing.

Table 48 shows detectable transformation after 4 hours with 0.08 mg/mL Syn A in both the washed and unwashed states. Maximal agglutination was observed at 48 hours. At all time intervals, the difference in scores for the washed and unwashed Syn A transformed cells was one score level or less.

Table 48. Comparison of anti-A agglutination results of washed and unwashed Syn A transformed cells.

Time (hours)	Anti-A*			
	Syn A 0.08 mg/mL		Syn A 0.05 mg/mL	
	washed	unwashed	washed	unwashed
2	0	0	0	0
4	1	2	0	0
6	2	2	0	0
8	2	2-3	0	0
12	2-3	3	1	0
24	3-4	3-4	2	1
30	3-4	3-4	2	1
48	4	4	2	2
72	4	4	2-3	2
96	4	4	2-3	2-3

*Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C (protocol 2.3, v2) and tested against Bioclone anti-A.

Table 49 shows detectable transformation after 4 hours with the unwashed cells transformed with 0.6 mg/mL Syn B (1+), and after 6 hours with the corresponding washed cells (w+). The difference at

4 hours is only one score level, which is insignificant. At 0.6 mg/mL Syn B, maximal agglutination score is reached at 24 hours. At 0.3 mg/mL Syn B, agglutination occurs at 8 hours for unwashed (w+) and 12 hours for washed (w+). At 0.15 mg/mL Syn B, insertion is detectable at 24 hours, with a 2+ generated by both the washed and unwashed cells. As there was no testing between 12 and 24 hours, and the score jumped from 0 to 2+ in this time, it is probable that agglutination could have been observed prior to the 24 hour testing point.

Like Syn A, the difference in scores for the washed vs unwashed Syn B transformed cells was insignificant at all time intervals.

Table 49. Comparison of anti-B agglutination results of washed and unwashed Syn B transformed cells.

Time (hours)	Anti-B*					
	Syn B 0.6 mg/mL		Syn B 0.3 mg/mL		Syn B 0.15 mg/mL	
	washed	unwashed	washed	unwashed	washed	unwashed
2	0	0	0	0	0	0
4	0	1	0	0	0	0
6	w	1	0	0	0	0
8	2	2	0	w	0	0
12	2	2-3	w	2	0	0
24	4	4	3	3	2	2
30	4	4	2-3	2-3	0	w
48	4	4	3	3	1	2
72	4	4	4	4	2	2
96	4	4	3-4	3-4	2-3	2-3

*Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed at 2°C (protocol 2.3, v2) and tested against Bioclone anti-B.

Cells transformed simultaneously with Syn A and Syn B were also tested for antibody neutralisation in the unwashed versus the washed state.

The results for the washed and unwashed transformed A cells were not significantly different from each other against neat and diluted anti-A (Table 50). On day 1 against neat anti-A, the cells scored 2-3+. By day 3, neat anti-A gave 4+, 1:2 diluted anti-A gave 1-2+ and 1+ (washed being slightly higher than unwashed) and diluted 1:4 anti-A gave w+ reactions.

The washed and unwashed transformed B cells gave 2-3+ against neat anti-B on day 1, which by day 3 became 3+, 1+ against 1:2 anti-B and vw reactions against 1:4 anti-B. As with the Syn A transformed cells, there was no difference between washed and unwashed samples.

The transformed AB cells were tested in the same way and showed the same trend against anti-A – that there was no significant difference in the agglutination scores of washed versus unwashed cells. The cells gave 2+ on day 1, and then 3+ on day 3 against anti-A (neat reagent). The dilutions of anti-A produced 1+ and w+ (1:2 and 1:4 dilution respectively). Against anti-B, the washed cells gave 1+ on day 1, while the unwashed cells were 2+. By day 3, both stes of cells were generating 2-3+ reactions against anti-B.

Table 50. Comparison of agglutination results of washed and unwashed transformed A, B and AB cells. Transformed A RBCs were made with 0.07 mg/mL Syn A, transformed B RBCs with 0.3 mg/mL Syn B and transformed AB RBCs with 0.07 mg/mL Syn A plus 0.3 mg/mL Syn B. Samples were stored unwashed, and aliquots removed for testing. Washed cells were washed immediately prior to testing.

			Dilutions					
			Anti-A*			Anti-B*		
Cells	Status	Day±	1	1:2	1:4	1	1:2	1:4
KODE™ A	Washed	1	2-3					
		3	4	1-2	w			
	Unwashed	1	2-3					
		3	4	1	w			
KODE™ AB	Washed	1	2			1		
		3	3	1	w	2-3	w	vw
	Unwashed	1	2			2		
		3	3	1	w	2-3	w	vw
KODE™ B	Washed	1				2-3		
		3				3	1	vw
	Unwashed	1				2-3		
		3				3	1	vw

* Serology in tube (protocol 3.1), scoring (appendix 7).
A and B cells were transformed using the method outlined in protocol 2.3, while AB cells were transformed as explained in protocol 2.3, v5.
± Testing intervals as shown were determined from the finish of the 48 hour transformation period. Cells were tested against dilutions of Bioclone anti-A.

Taken together, the results of this section indicate that any neutralisation of antisera by uninserted Syn A or Syn B molecules in solution is not significant.

4.9 Storage Temperature

In addition to inserting into RBC membranes, natural glycolipids relocate from the RBC membrane to the plasma *in vivo*, maintaining an equilibrium of one third to two thirds respectively (Schwarzmann, 2001; Wilchek & Bayer, 1987). There has been no work to determine whether this occurs with synthetic glycolipids *in vivo*.

Increasing the temperature at which cells are stored speeds up cellular activity. Membrane events, such as endocytosis or glycolipid exchange etc, may occur more rapidly. Stability trials on cells stored at 2°C have been performed (section 4.12), but the effect of increasing the storage temperature has not been investigated. In order to do this, an extreme storage temperature of 37°C was used to investigate the effect of temperature after transformation on the agglutination results of Syn A and A-6/14 natural glycolipid transformed group O RBCs in comparison with storage at 2°C.

Immediately after transformation (time 0), the Syn A transformed cells produced a 3+ agglutination score (Table 51). The score strengthened to 4+ after 24 hours at both storage temperatures. The A-6/14 glycolipid transformed cells also strengthened up after 24 hours, and then maintained that score (3+) over the next 24 hours, showing no significant difference between the storage temperatures. After 48 hours, the 37°C stored cells were brown and testing was discontinued.

The cells transformed with natural or synthetic glycolipid showed no detectable loss of antigen at 37°C compared to 2°C. This would seem to indicate that both the Syn A and A-6/14 antigens are stable in their expression on the membrane in a range of temperatures between 2°C and 37°C for up to 48 hours.

Table 51. Comparison of 37°C and 2°C as storage temperatures for cells transformed with Syn A and A-6/14 glycolipid. Transformed cells were divided in two and suspended to 5% in CellStab. One set was stored at 2°C and the other was stored in a 37°C waterbath.

A glycolipid (mg/mL)		Time± (hours)	Anti-A*			
			Temperature			
			37°C		2°C	
			Tube	Column	Tube	Column
Syn A	0.1	0	3		3	
		24		4		4
		48		4		4
A-6/14	10	0	1-2		1-2	
		24		3		3
		48		3		3

* Serology in tube (protocol 3.1) and Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The cells were tested against Bioclone anti-A.
A-6/14 cells were prepared using the method outlined in protocol 2.1, and Syn A cells were prepared using the method described in protocol 2.3, v1.
± Time post-transformation.

4.10 Sterile-Filtered Synthetics

Synthetic glycolipids must be sterile to be used for manufacture of a diagnostic reagent red cell product. Sterilisation of the solution will be carried out by filtration through a sterile 0.22 μm membrane. A trial was carried out to investigate the effects of filtration on synthetic glycolipid solutions.

At 0.2 mg/mL Syn A, the agglutination result for the cells transformed with the filtered sample is 4+, which is the same as that for the cells transformed with the unfiltered sample (Table 52). At the three other concentrations of Syn A, there is no significant difference between the serology of the cells transformed with the filtered sample and the cells transformed with the unfiltered sample.

These results indicate that sterile filtration through a 0.2 μM filter did not remove Syn A molecules or change the composition or properties of the transformation solution to the point that insertion as detected by agglutination was affected.

Table 52. RBCs transformed with varying concentrations of sterile-filtered vs unfiltered Syn A.

Syn A (mg/mL)	Anti-A*	
	Sterile-filtered Syn A	Unfiltered Syn A
0.2	4	4
0.1	4	3-4
0.05	2-3	2-3
0.01	0	0
Control	0	

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed using the method outlined in protocol 2.3, v1 and tested against Bioclone anti-A.

4.11 Antisera Comparison

A panel of several monoclonal and polyclonal antisera (appendix 10) were used to assay cells transformed with the synthetic glycolipids (Syn A and Syn B). Transformed cells should perform well against a range of antisera, but importantly, could also highlight unsatisfactory performance of certain reagents. As previously discussed (see section 3.3), antisera formulation can vary across manufacturers and time periods, which can manifest as variation in reactivity. A wide range of reagents were used in order to test the reactivity of the cells with various antibody clones. Historical reagents were used to demonstrate probable reagent deterioration (some were up to 20 years out of date).

Cells expressing controlled low amounts of A antigen only (KODE™ A), B antigen only (KODE™ B), and both the A and B antigen (KODE™ AB) were prepared for testing against the antisera panel. Natural A, B and AB RBCs were tested in parallel for comparative purposes.

Table 53 shows the results of KODE™ and natural A and AB cells against the anti-A panel. The reference reagent is A-XI (it is the only one that is within its expiry date), and reacts maximally with all cells except for a 3+ against KODE™ AB RBCs. Despite the expectation that this should provide the strongest score, and set the benchmark, it was surpassed by reagent A-X, which is made by the same manufacturer, but may be a different clone or concentration.

With the exception of reagent A-XIX, which gives a w+, all reagents maximally agglutinated natural A cells. A-XIX also reacted at 1+ level against natural AB cells and failed to detect KODE™ cells. Three other reagents (A-XII, A-XVII and A-XVIII) failed to detect KODE™ cells, and while giving 4+ reactions against natural A cells, produced 2+, 3+ and 1+ respectively against natural AB cells. The remaining anti-A reagents reagents (other than A-XII, A-XVII, A-XVIII and A-XIX) all gave 4+ reactions against natural AB cells. These results mean that reagents A-XII, A-XVII, A-XVIII and A-XIX had deteriorated, but probably only A-XIX (and maybe A-XVIII) would be judged so from the reactions against natural cells. The KODE™ cells show that reagent A-XV was probably also reduced in its performance.

A surprising result was the trend of weaker agglutination by anti-A of KODE™ AB cells than KODE™ A cells (Table 53). Previous experiments had indicated that insertion of Syn A was independent and unaffected by simultaneous insertion of Syn B – and vice versa (see section 4.4).

Table 53. KODE™ A and AB RBCs against a panel of expired A antisera. The reference reagent (which is within its expiry date) is XI.

Anti-A[†]	Serology*			
	KODE™ cells[±]		Natural cells	
	A	AB	A	AB
III	3	w	4	4
V	3	w	4	4
VIII	4	w	4	4
IX	4	w	4	4
X	4	4	4	4
XI	4	3	4	4
XII	0	0	4	2
XV	2	0	4	4
XVII	0	0	4	3
XVIII	0	0	4	1
XIX	0	0	w	1

* Serology in tube (protocol 3.1), scoring (appendix 7).
[†] Reagent descriptions (appendix 10).
[±] Transformation solution for A cells contained 0.2 mg/mL Syn A, and 0.2 mg/mL Syn A plus 0.75 mg/mL Syn B for AB cells, and cells were prepared using the method described in protocol 2.3, v1.

Table 54 shows the results of KODE™ and natural B and AB cells against the anti-B panel. The reference reagent was B-X and this showed

maximal agglutination against all cells. Most of the expired anti-B reagents reacted strongly with the natural cells, with only two giving less than a 4+ score; B-XI gave a 2+ against B cells and a 1+ against AB cells, while B-XV generated a 2+ against both natural cell samples. As expected, both these lower performing anti-B reagents were negative against the KODE™ cells. The lower results against both natural and KODE™ RBCs shows clearly that these two antisera had deteriorated in their performance. B-II, B-VI and B-VII all showed lower than maximal reactions against KODE™ cells, indicating that they too had deteriorated, but not to an extent that was detectable with natural cells. The other anti-B reagents (B-V, B-IX, B-XIII and B-XVI) all reacted maximally with KODE™ A and AB RBCs. As a side issue, these results indicate that anti-B reacts equally with the B antigen on KODE™ B and AB cells.

Table 54. KODE™ B and AB RBCs against a panel of expired B antisera. The reference reagent (which is within its expiry date) is X.

Anti-B[†]	Serology			
	KODE™ cells[±]		Natural cells	
	B	AB	B	AB
II	2	3	4	4
V	4	4	4	4
VI	w	w	4	4
VII	2	2	4	4
IX	4	4	4	4
X	4	4	4	4
XI	0	0	2	1
XIII	4	4	4	4
XV	0	0	2	2
XVI	4	4	4	4

* Serology in tube (protocol 3.1), scoring (appendix 7).
† Reagent descriptions (appendix 10).
± Transformation solution for B cells contained 0.75 mg/mL Syn B, and 0.2 mg/mL Syn A plus 0.75 mg/mL Syn B for AB cells, and cells were prepared using the method described in protocol 2.3, v1.

Table 55 shows the results of KODE™ and natural A, B and AB cells against the anti-AB panel. The reference reagent, B-IX, showed maximal agglutination against all natural cells and the KODE™ B and AB cells, but gave a 3+ against the KODE™ A cells. Most of the expired anti-AB reagents also reacted strongly with the natural cells and less well with the KODE™ cells. After the reference reagent, the next best performers

against KODE™ cells were the two polyclonal reagents, AB-II and AB-IV. AB-I was the poorest performer against natural cells, with a 3+ against A and AB cells, but a w+ against B cells. AB-VII and AB-XIV were normal against natural A and AB RBCs, but reacted less strongly with natural B RBCs. All three of these reagents (AB-I, AB-VII and AB-XIV) reacted very poorly with KODE™ cells, the only positive reaction amongst them being a 1+ with B-VII against KODE™ AB cells. These results demonstrate that the antisera had deteriorated.

Notably, reagent AB-XVI was unable to agglutinate any of the KODE™ cells despite returning 4+ scores against the natural cells, which is indicative of degradation that could not be detected by cells expressing normal amounts of A and/or B antigen. Reagents that showed 4+ reactivity with natural cells, but decreased reactivity with KODE™ (mainly A) cells (with respect to the reference reagent – AB-IX) were AB-VI, AB-X, AB-XI, AB-XII and AB-XIII.

Table 55. KODE™ A, B and AB RBCs against a panel of expired AB antisera. The reference reagent (which is within its expiry date) is IX.

Anti-AB[†]	Serology*					
	KODE™ cells[±]			Natural cells		
	A	B	AB	A	B	AB
I	0	0	0	3	w	3
II	2	4	4	4	4	4
IV	2	4	4	4	4	4
VI	0	1	3	4	4	4
VII	0	0	1	4	3	4
VIII	w	3	3	4	4	4
IX	3	4	4	4	4	4
X	0	2	3	4	4	4
XI	0	4	3	4	4	4
XII	0	2	4	4	4	4
XIII	0	4	4	4	4	4
XIV	0	0	0	4	2	4
XVI	0	0	0	4	4	4

* Serology in tube (protocol 3.1), scoring (appendix 7).
† Reagent descriptions (appendix 10).
± Transformation solution for A cells contained 0.2 mg/mL Syn A, 0.75 mg/mL Syn B for B cells, and 0.2 mg/mL Syn A plus 0.75 mg/mL Syn B for AB cells, and cells were prepared using the method described in protocol 2.3, v1.

Overall, agglutination results against anti-AB were stronger with the A antigen than with the B antigen on natural cells, but the trend was reversed with the KODE™ cells. This is possibly because the KODE™ A cells were transformed at a much lower concentration than the KODE™ B cells, and because some anti-AB reagents are blends of monoclonal anti-A and anti-B clones.

These results show that KODE™ cells can react with a range of antisera, and that they can also detect deterioration of antisera that would be overlooked with normal RBCs.

4.12 Stability Trials

As discussed in section 2.6, useful blood grouping sensitivity controls should be stable for a minimum shelf life of 8 weeks. The stability of cells transformed with natural glycolipids has been evaluated and show a gradual loss of reactivity over time (see section 2.6).

In order to evaluate the stability of cells transformed with synthetic glycolipids, RBCs were transformed with various concentrations of Syn A and Syn B, and stored in two different cell preservative solutions – CellStab and Alsevers. Results for the stability of the synthetic glycolipid-transformed RBCs are provided in Tables 56 and 57.

The results for Syn A (Table 56) show stability data over 86 days (12 weeks). The first thing that is apparent from these results is that the Alsevers stored samples stopped giving scores at day 57 (for the lowest concentrations of Syn A). This was because the samples had become bacterially contaminated and testing was discontinued due to haemolysis and blackening of the cells. CellStab did not suffer with this problem, probably because it contains antibacterial agents.

At the highest Syn A concentration, 0.1 mg/mL, maximal agglutination was obtained and sustained for virtually the entire time, with only the last three weeks' scores weakening slightly to 3-4+ against Albaclone anti-A.

At 0.05 mg/mL Syn A, the Alsevers stored cells started at 2+ against Albaclone anti-A and strengthened to 3+, staying at or between 2+ to 3+ for the 57 day duration (Table 56). These same cells against Bioclone

anti-A started at 3+ and strengthened to 4+, with a single irregularity of 2-3+ at day 15. The CellStab stored cells against Albaclone anti-A started at 2+ and strengthened to 3+, with two weeks at 4+ (days 15 and 22), one at 3-4+ (day 43), and dropping back to 2+ in the final three weeks. The CellStab stored cells against Bioclone anti-A started at 3+, strengthening to 4+, with one weeks drop to 2-3+ at day 15, and finally settling back to 3+ after day 57.

The 0.025 mg/mL Syn A transformed cells were essentially negative against Albaclone anti-A (Table 56). Against Bioclone anti-A, the Alsevers stored cells started at 1+ and reached 2+ by day 43, just after two weeks at w+. The CellStab stored cells also started at 1+, achieving 2+ at day 29 before dropping back to 1+ and then negative at day 63.

There were some limited periods of significant difference between the two cell preservative solutions, with CellStab producing the stronger serology, e.g. the 0.05 mg/mL transformed cells on day 15 against Albaclone anti-A, Alsevers 2+ and CellStab 4+. Overall, there was no significant difference in the effect of the two cell preservative solutions on the serology of Syn A transformed cells. Where they did differ significantly was their respective abilities to restrict bacterial growth.

The results of the stability trial of Syn B transformed cells are given in Table 57. At 1 mg/mL Syn B, maximal agglutination was seen for cells stored in Alsevers and CellStab against both Albaclone and Bioclone anti-B reagents. The CellStab stored cells were tested for 116 days, but testing of the Alsevers stored cells was discontinued at day 72 due to bacterial contamination causing haemolysis and browning.

At 0.5 mg/mL Syn B, 4+ scores were obtained for all Alsevers stored cells against both Albaclone and Bioclone anti-B until testing was discontinued at day 51 due to bacterial contamination. The CellStab stored cells also gave 4+ agglutination but weakened to 3-4+ at day 87 against both anti-Bs and day 116 against Bioclone anti-B. By day 116, the CellStab stored cells were 3+ against Albaclone anti-B.

Table 56. Stability of RBCs transformed with Syn A concentrations of 0.1 mg/mL, 0.05 mg/mL and 0.025 mg/mL.

Day	Cell storage solution	Anti-A*					
		Albaclone			Bioclone		
		Syn A (mg/mL)					
		0.1	0.05	0.025	0.1	0.05	0.025
2	AL	4	2	0	4	3	1
	CS	4	2	0	4	3	1
8	AL	4	3	0	4	4	1
	CS	4	3	0	4	4	1
15	AL	4	2	0	4	2-3	1
	CS	4	4	0	4	4	1
22	AL	4	2-3	0	4	3	w
	CS	4	4	0	4	4	1
29	AL	4	2	0	4	3	w
	CS	4	3	0	4	4	2
43	AL	4	3	w	4	4	2
	CS	4	3-4	0	4	4	1
50	AL	4	3	w	4	4	2
	CS	4	3	0	4	4	1
57	AL	4	2-3		4	4	
	CS	4	3	0	4	3	w
63	AL						
	CS	3-4	2	0	4	3	0
71	AL						
	CS	3-4	2	0	4	3	0
86	AL						
	CS	3-4	2	0	4	3	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Abbreviations: Al – Alsevers, CS – CellStab.
Cells were transformed using the methodology outlined in protocol 2.3, v4. Blank spaces indicate that testing was not undertaken due to insufficient cells.

The 0.25 mg/mL Syn B transformed cells stored in Alsevers started at 2+ and strengthened to 3+ before dropping again to 2+ against Albaclone anti-B before testing was discontinued at day 72 (Table 57). The same Syn B transformed cells stored in CellStab also started at 2+, and strengthened to 3+ after the first week, beginning to drop at day 58 to be negative at day 116 against Albaclone anti-B.

Against Bioclone anti-B, the Alsevers stored 0.25 mg/mL Syn B cells started at 2+, dropped to 1+ after the second week, before strengthening

to 3+ for some five weeks followed by a brief drop to 2+ and finishing on 3+ when testing was discontinued at day 72 due to bacterial contamination (Table 57). The CellStab stored 0.25 mg/mL Syn B cells started at 2+, strengthening to 3+ after the first week and then to 3-4+ at day 44, and finally dropping to 1+ at day 116 after some fluctuation.

Table 57. Stability of RBCs transformed with Syn B concentrations of 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL.

Day	Cell storage solution	Anti-B*					
		Albaclone			Bioclone		
		Syn B (mg/mL)					
		1	0.5	0.25	1	0.5	0.25
2	AL	4	4	2	4	4	2
	CS	4	4	2	4	4	2
9	AL	4	4	2	4	4	2
	CS	4	4	3	4	4	3
16	AL	4	4	2	4	4	1
	CS	4	4	3	4	4	3
23	AL	4	4	3	4	4	3
	CS	4	4	3	4	4	3
30	AL	4	4	3	4	4	3
	CS	4	4	3	4	4	3
37	AL	4	4	3	4	4	3
	CS	4	4	3	4	4	3
44	AL	4	4	2	4	4	3
	CS	4	4	3	4	4	3-4
51	AL	4	4	2	4	4	3
	CS	4	4	3	4	4	3
58	AL	4		1	4		2
	CS	4	4	2	4	4	2
72	AL	4		2	4		3
	CS	4	4	2-3	4	4	3
87	AL						
	CS	4	3-4	1	4	3-4	1-2
116	AL						
	CS	4	3	0	4	3-4	1

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Abbreviations: Al – Alsevers, CS – CellStab.
Cells were transformed using the methodology outlined in protocol 2.3, v4. Blank spaces indicate that testing was not undertaken due to insufficient cells.

The stability of Syn B seems to be dependent on the concentration of the transformation solution: it is not quite so good at the lowest concentration (0.25 mg/mL) especially at the end. Note, however, that 116 days is double the required eight weeks (56 days). Within the 56 day time frame, the serology of the 0.25 mg/mL Syn B transformed cells was within one score level of the opening score.

The cells that generate a 4+ reaction may mask the loss of antigen detectability (through loss of the glycolipids from the cell membrane or a change in the presentation of the glycotope) i.e. cells that have double the number of antigens required to generate 4+ agglutination can therefore lose over 50% of those antigens before the loss becomes detectable with antibody. Furthermore, it is reasonable to expect that the cells that start with the lowest amount of antigen, a result of the lowest concentration transformation solution, would become negative first. This means that the cells transformed with Syn B concentrations of 1 and 0.5 mg/mL appear to have better stability, but in reality, it cannot be ruled out that they are losing antigen at a similar rate to the cells transformed with 0.25 mg/mL Syn B (Table 57).

The results in Tables 53 and 54 indicate that CellStab is an effective preservative for cells that are going to experience continual exposure to non-sterile conditions over long periods, such as being opened often. Under conditions where the cells were opened weekly, Alsevers could not prevent contamination that lead to the discontinuation of testing as early as 57 days.

It can be seen from these results that Bioclone anti-A gives stronger agglutination reactions than Albaclone (Table 56), but the two anti-B reagents perform equivalently against cells transformed with Syn B glycolipids (Table 57).

The results of the stability trials indicate that the 0.05 mg/mL concentration Syn A, which gives scores in the range of 2+ to 3+ (the desired range for a sensitivity control agglutination reaction), generates serologically stable reactions for periods of up to 80 days. The cells transformed with 0.25 mg/mL Syn B, which gives scores in the range of

2+ to 3+, are serologically stable for periods of around 70 days. After this time the scores of the Syn B transformed cells start to reduce.

Both these time frames are longer than the required 56 day (8 week) shelf life for a commercial reagent RBC product.

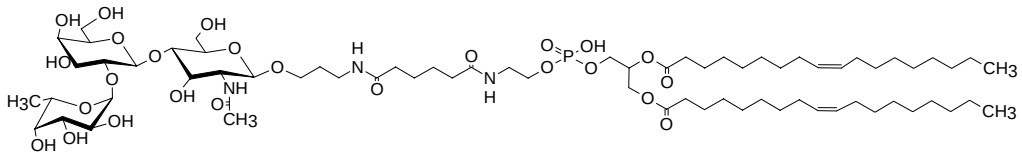
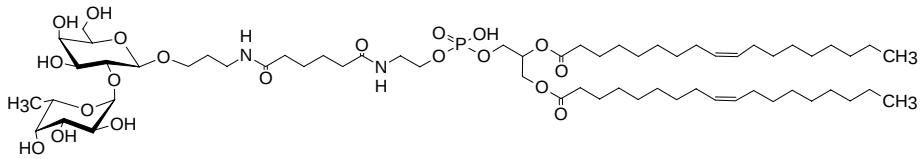
4.13 H Synthetics

H is the name given to the structure that is the precursor for the construction of A or B antigens. Individuals of blood group O express this antigen on their tissues. The H terminal trisaccharide structure can be seen in Table 3.

Two synthetic glycolipids with different glycotopes (Table 58) were trialled to assess reactivity against a monoclonal and anti-H reagent and the lectin *Ulex europaeus*. Both molecules had the DOPE tail (see Table 32 for the lipid tail structures). The synthetic H_{tri}-sp-Ad-DOPE was a type 2 structure, while the disaccharide was generic – i.e. having no core structure (similar to Syn A and Syn B in this respect).

Murine RBCs, which do not express the H antigen, were transformed with each of these Syn H synthetic glycolipids. The anti-H reagents were a monoclonal IgM and the *Ulex europaeus* lectin (UAE), which strongly recognises H type 2. A biotinylated version of UAE was trialled to ascertain whether biotinylation of the lectin affected its agglutination ability.

Table 58. Synthetic glycolipids trialled against anti-H reagents.

H_{tri}-sp-Ad-DOPE 
H_{di}-sp-Ad-DOPE 

The anti-H IgM did not agglutinate cells transformed with 0.25 mg/mL (or lower) H_{di}-sp-Ad-DOPE in tube or Diamed cards (results not shown).

These results indicate that the anti-H IgM is unable to recognise the H disaccharide glycotope of H_{di}-sp-Ad-DOPE. It is possible that this molecule was not able to insert into the cells, being different from all other synthetic glycolipids used in that it had reduced numbers of sugars. This may have altered the amphipathic nature of the molecules and prevented insertion. However, these molecules theoretically should be more non-polar than the ones with three sugars, but they were soluble in aqueous solvents, and thus the difference cannot have been too great.

The anti-H IgM gave a strong (4+) reaction in tube serology with murine RBCs transformed with 0.25 mg/mL H_{tri}-sp-Ad-DOPE (Table 59). Against cells transformed with 0.1 mg/mL H_{tri}-sp-Ad-DOPE, the serology was 3+, and 1+ at 0.05 mg/mL. IgM anti-H did not agglutinate cells transformed with 0.01 mg/mL H_{tri}-sp-Ad-DOPE. The results using the Diamed system were slightly weaker, with only a 3+ seen at 0.25 mg/mL H_{tri}-sp-Ad-DOPE transformed cells, and a 2+ with 0.1 mg/mL H_{tri}-sp-Ad-DOPE transformed cells. Both the lower concentrations were negative in Diamed cards.

Table 59. Comparison of synthetic H glycolipids with trisaccharide and disaccharide glycotopes to transform murine RBCs.

Synthetic	Conc mg/mL	Anti-H*				
		IgM		UEA		BioUEA
		Tube	Diamed	Tube IS	Tube T20	Tube
Syn H_{tri}	1			2		2
	0.25	4	3			1
	0.1	3	2			
	0.05	1	0			
	0.01	0	0			
	0	0		0	0	
Human O RBCs		4		1	2-3	4

* Serology in tube (protocol 3.1) and Diamed gel-cards (protocol 3.2), scoring (appendix 7). Abbreviations: B-UEA – biotinylated *Ulex europaeus*, IS – immediate spin, T20 – antisera and cells incubated for 20 minutes before centrifugation.
The cells were transformed as outlined in protocol 2.3, v1 and tested against the anti-H reagents IgM, *Ulex europaeus* and biotinylated *Ulex europaeus*.

At 0.25 mg/mL H_{tri}-sp-Ad-DOPE, the agglutination reaction with anti-H IgM was equivalent to that seen with natural human O cells, but this does not mean that there were the same number of H determinants on both cell types. A 4+ agglutination reaction reflects a range of antigen

quantites, for example, a 4+ may occur when the cells express 500,000 H antigens, but cells with double that will also produce a 4+ reaction. It is quite likely that the transformed cells may have just reached the threshold, whereas the natural cells were well over it.

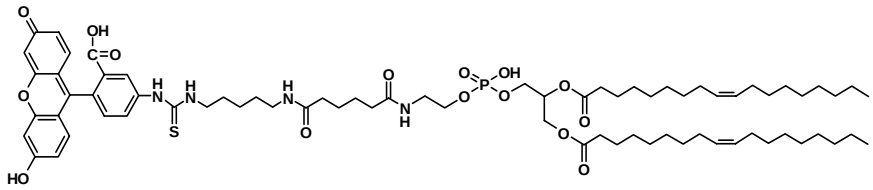
UEA and biotinylated UEA were only weakly reactive with H_{tri}-sp-Ad-DOPE compared with the IgM anti-H, giving only a 2+ reaction for cells transformed with 1 mg/mL H_{tri}-sp-Ad-DOPE (Table 59). This concentration was four times higher than the 0.25 mg/mL H_{tri}-sp-Ad-DOPE transformed cells that generated a 4+ against the IgM. However, UEA showed less reactivity with human O cells than did the IgM, giving a 1+ (which strengthened to a 2-3+ after 20 minutes incubation) compared with 4+ for the IgM anti-H. Taking into account this lower reactivity against cells naturally expressing the H antigen, the difference in the reactivity of UEA with cells bearing the synthetic H compared to the IgM is of less significance.

These results indicate that the anti-H IgM is unable to recognise the H disaccharide glycotope of H_{di}-sp-Ad-DOPE. The anti-H IgM was able to agglutinate murine cells transformed with H_{tri}-sp-Ad-DOPE. Taken together, these results indicate that the IgM anti-H required a minimum of three sugars in the glycotope.

4.14 Fluorescent-labelled DOPE Molecules

The predominant technique for the detection of insertion of glycolipids (natural and synthetic) in this research was agglutination of transformed cells with antibodies directed to the epitope of the inserted molecule. In order to detect insertion in RBCs using a different technique, a molecule in which the glycotope was replaced with a fluorescein label (Table 60) was trialled. This molecule could be visualised under fluorescence microscopy.

Table 60. Fluorescent-labelled synthetic molecule.

Fluo-Ad-DOPE


The Ad-DOPE tail was conjugated to a FITC molecule and used to try to visualise insertion at different concentrations under a fluorescence microscope. Positive insertion was seen for 1 mg/mL and 0.5 mg/mL transformed cells (see Table 61 and Figure 10). Positive insertion was also seen for 0.1 mg/mL transformed cells, but was too weak for an exposure to be taken (Table 61).

Table 61. Fluorescence of RBCs transformed with Fluo-Ad-DOPE at varying concentrations.

	Fluo-Ad-DOPE (mg/mL)			
	1	0.5	0.1	0.05
Fluorescence score	medium	weak	very weak	negative

Cells were transformed using the methodology outlined in protocol 2.3.
Cells had been transformed 44 days earlier and stored at 2°C in the dark.

These results provide alternative evidence that the synthetic molecule becomes associated with the membranes of RBCs in a relatively enduring fashion. The fact that the fluorescein label is still visible in the RBC membrane after 44 days complements the stability trial agglutination data (see section 4.12).

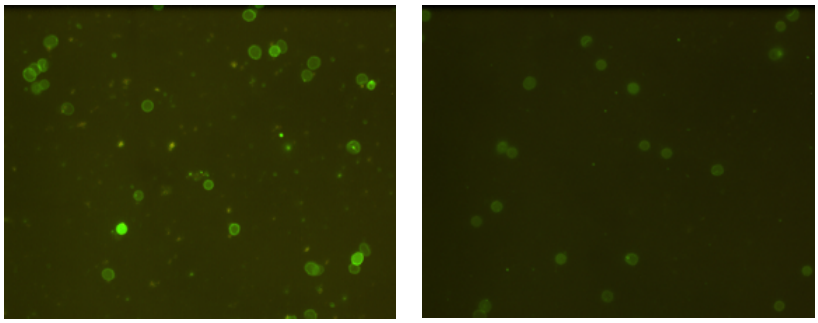


Figure 10. Fluorescent microscope photos of RBCs inserted with Fluo-Ad-DOPE at concentrations of 1 mg/mL (left) and 0.5 mg/mL (right). Exposures were taken through a 40x objective at exposure times of 7 seconds with excitation at 460-490 nm.

Cells were transformed using the methodology outlined in protocol 2.3.
Cells had been transformed 44 days earlier and stored at 2°C in the dark.

5 RESULTS - GLYCOSIDASES

The α-galactosidase enzyme is able to remove the terminal sugar of blood group B antigens – the α1-3galactose saccharide. Removal of this monosaccharide from the B antigen generates the H antigen, which is the blood group structure of natural group O RBCs. Total removal of the B-active sugars of blood group B cells will thus generate cells that appear as blood group O. Stopping the reaction prior to this total removal should result in cells that express low amounts of the B antigen, and this could be an alternative method for the preparation of weak B cells for use as sensitivity controls. Only blood group B antigen digestion was studied (as proof of principle) because the galactosidase enzyme is easily obtained. Stripping of the A-active sugars from blood group A cells could foreseeably be accomplished with an *N*-acetylgalactosaminidase enzyme. It is anticipated that digestion of the A-active sugars from blood group A cells would occur in a similar manner to that demonstrated with the galactosidase.

Two different green coffee bean derived enzyme preparations were used in this study. One was a commercially available preparation (Glyko – GKX-5007, Glyko, USA) while the other (Lab) was prepared as described in protocol 6.1. According to the Glyko package insert, green coffee bean α-galactosidase can cleave galactose linked through α1-4 and α1-6 bonds in addition to the α1-3 bonds that are found on blood group B structures. A series of enzyme digested cells were prepared and tested for the presence of B antigen. Both enzyme preparations were able to remove α-galactose from blood group B RBCs.

The results in Table 62 show that undiluted anti-B was able to maximally agglutinate all cells treated with α-galactosidase for up to 48 hours (and control), which indicates that the stripping is incomplete. Because the undiluted antibody has such a high affinity for the B antigen, it cannot show loss of antigen when the final numbers of antigen are still above the 4+ threshold, which can be far below the the number of antigens initially present on the cell. For example, if 100,000 antigens is the 4+ threshold, a reduction from 900,000 to 100,000 (a 9-fold reduction in antigen

numbers) will not be detectable. This means that a significant proportion of the B-active sugars need to be removed before the loss is detectable with neat anti-B.

To generate more information about the loss of B reactivity, dilutions of anti-B were also used. The threshold for maximal agglutination with antibody dilutions is higher than for the neat reagent, and increases as the antibody is further diluted. The ability of the antibody to agglutinate unmodified B cells is reduced as it is diluted. This means that the loss of B reactivity is seen earlier with diluted anti-B. Comparisons between the antibody dilutions cannot be made, but within each dilution, an untreated (saline) blood group B cell provides a reference score.

Table 62. Removal of the B-active sugar (α-galactose) by two α-galactosidase enzymes.

Anti-B	Enzyme	Serology*			
		Time (hr)			
		2	4	24	48
Neat	Glyko	4	4	4	4
	Lab	4	4	4	4
	Saline	4	4	4	4
1:64	Glyko		3-4	2-3	1-2
	Lab		3-4	2-3	2-3
	Saline		3-4	3-4	3-4
1:128	Glyko		3	2	1
	Lab		3	2	2
	Saline		3	3	3
1:256	Glyko		2	w	w
	Lab		2	w	1
	Saline		2	2-3	2-3

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were prepared with 0.8 Units of the Glyko enzyme by the method in protocol 6.2, v2, and with an enzyme prepared from green coffee beans according to the method in protocol 6.2, v1. The cells were tested against Bioclone anti-B dilutions at the intervals shown.

At four hours, there was no difference between the untreated control cells and either of the enzyme treated cells (Table 62). For example, at the 1:256 dilution of anti-B, the agglutination scores for all cells were 2+. This indicates that any α-galactose stripping that had occurred was insufficient to be detected even by the most diluted antibody.

After 24 hours, there was significant variation between the enzyme treated cells and the untreated saline control detected by the 1:256 dilution – with scores of 2-3+ for the untreated cells and w+ for both enzyme treated cells.

After 48 hours, the difference was significant for the commercial enzyme (Glyko) against the 1:128 and 1:64 anti-B dilutions also. The Glyko enzyme treated cells vs the control cells were 1+ vs 3+ respectively against the 1:128 dilution and 1-2+ vs 3-4+ against the 1:64 dilution.

After 48 hours at 37°C, all samples became brown (results not shown). This was independent of the enzyme because it occurred in the untreated cells also – it was a result of the extended 37°C incubation in saline.

These results indicate that both enzyme preparations were able to remove α-galactose from blood group B RBCs. As expected, the commercial (Glyko) enzyme appeared to have a slightly higher activity than the enzyme (Lab) prepared in the laboratory. This suggests that the enzyme prepared in the laboratory was at a lower concentration than 5 U/mL.

The appropriate strength for a weak sensitivity control cell is in the range of 2+ to 3+ against undiluted antibody. This was not achieved after 48 hours with an enzyme concentration of 5 U/mL. Higher concentrations of the Glyko enzyme were used to establish a working range for the effective stripping of α-galactose from natural group B RBCs and the results are shown in Table 63.

The 2 hour results show fairly conclusively that at high enzyme concentrations (> 50 U/mL) antigen stripping has begun – all diluted anti-B reagents show a reduction in score when compared with the control cells (Table 63). This trend is most conclusive against the 1:256 diluted antibody. The first negative score (reduction in antigen levels below the threshold of reagent detection) shows up against the 1:128 and 1:256 anti-B dilutions after 4 hours of treatment with 100 U/mL of enzyme.

After 24 hours, the cells treated with 25 U/mL of enzyme or greater had been stripped of their α-galactose sugars to the degree that undiluted anti-B could no longer detect B antigen.

Table 63. Glyko α-galactosidase digestion of group B RBCs. Each reaction contained 25 μL RBCs and 40 μL enzyme at the concentrations shown.

Enzyme (U/mL)	Anti-B	Serology*			
		Time (hr)			
		2	4	8	24
100	Neat	4	4	3	0
50		4	4	4	0
37.5		4	4	4	0
25		4	4	4	0
12.5		4	4	4	4
0		4	4	4	4
100	1:64	2-3	1-2	0	0
50		3	2-3	1	0
37.5		3-4	3	1-2	0
25		3-4	3-4	2	0
12.5		3-4	3-4	3	0
0		3-4	3-4	3-4	4
100	1:128	2	0	0	0
50		2-3	2	0	0
37.5		2-3	2	w	0
25		2-3	2-3	1-2	0
12.5		2-3	3	2-3	0
0		3	3	3-4	3-4
100	1:256	1	0	0	0
50		2	1-2	0	0
37.5		2	2	0	0
25		2-3	2	w	0
12.5		3	2	2-3	0
0		2-3	2-3	2-3	2-3

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were prepared according to the method in protocol 6.2, v3, and tested against Bioclone anti-B dilutions at the intervals shown.

These results provide an indication of how much enzyme is needed to make weak B RBCs. Against neat anti-B, a 3+ score is achieved after 8 hours with the enzyme at 100 U/mL. The 2+ reaction would have occurred at some stage between 8 and 24 hours, but no data was recorded in this time. Given that RBCs should spend as short a time as possible at 37°C if they are to remain healthy for 8 weeks (the required

shelf life of reagent RBCs), it would not be prudent to produce these cells at any enzyme concentration lower than 100 U/mL. Incubation of RBCs at 37°C for more than 1-2 hours is less than ideal, and to continue using this incubation time would necessitate an enzyme concentration significantly greater than 100 U/mL.

Although this method was proven to work, extensive analysis was not undertaken because sourcing the enzymes is problematic and the end result is believed to be less controllable than other treatments because of its dependence on starting antigen density, which can be highly variable.

6 RESULTS - GLYCOSYLTRANSFERASES

Another possible method for the creation of RBCs expressing low amounts of blood group active antigens is to use glycosyltransferase enzymes to assemble antigens on RBCs. Two functional transferases account for the existence of the human blood group A and B antigens: they are α3-N-acetylgalactosaminyltransferase (GTA) and α3-galactosyltransferase (GTB). These enzymes catalyse the transfer of activated α-N-acetylgalactosamine and α-galactose (the A and B sugars – referred to as donors or substrates) respectively to the acceptor molecule, the H antigen. *In vivo*, this catalysis occurs in the Golgi apparatus of the cell, where the natural membrane bound enzymes reside. However, soluble (and in many cases, recombinant) forms of these enzymes have been shown to be active towards synthetic acceptor molecules in many *in vitro* kinetics studies. Other glycosyltransferase enzymes had also been used to construct antigens on intact RBCs (Galili & Anaraki, 1995).

Recombinant analogues of human A and B blood group glycosyltransferase enzymes, GTA and GTB, derived from a synthetic gene construct were kindly supplied by Dr Monica Palcic of the University of Alberta, Canada (Seto *et al.*, 1995).

6.1 Enzyme and Donor Interaction

Incubation of the enzymes with the non-complementary substrate (eg GTA with UDP-Gal) as well as their complementary substrate (eg GTA with UDP-GalNAc) was undertaken to assess the absolute specificity of each of the glycosyltransferases.

Table 64 shows that the enzymes could effectively modify group O RBCs to A or B, producing 4+ serology with the complementary substrate against the relevant antisera as expected. Neither of the enzymes appeared to be able to utilise the non-complementary monosaccharide, as shown by the negative reactions of cells created with GTA and UDP-Gal against anti-B and cells transformed with GTB and UDP-GalNAc against anti-A. As expected, the cells created with GTB and UDP-Gal were

negative against anti-A, and the cells created with GTA and UDP-GalNAc were negative against anti-B.

Table 64. Glycosyltransferase enzyme synthesis of A and/or B antigens on group O RBCs.

Donor	Serology*			
	GTA		GTB	
	Anti-A	Anti-B	Anti-A	Anti-B
UDP-GalNAc	4		0	
UDP-Gal		0		4

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
The enzyme and substrate were in excess.
Cells were transformed as described in protocol 7.1 and tested against the relevant Bioclone antisera.

To further understand the specificity of the glycosyltransferases, experiments were conducted in which multiple enzyme and substrate combinations were used to transform cells. Accordingly, a reaction containing both enzymes and both substrates was carried out to determine if each enzyme could transfer independently in the presence of the other. To further elucidate any interaction, each enzyme was incubated with both substrates in combination to determine if the presence of the non-complementary substrate affected the transfer of the complementary substrate. Other experiments revealed that GTA needs to be used neat, while GTB could be used at a 1:200 dilution (results not shown, but see Table 67). The substrates were added in excess (60 mM).

Table 65 shows the agglutination results of this trial. As expected, GTB in combination with UDP-Gal and UDP-GalNAc produced cells that were maximally agglutinated by anti-B, but not agglutinated at all with anti-A. This shows that the presence of UDP-GalNAc does not interfere with the transfer of UDP-Gal by GTB. This is also seen for the cells transformed with both enzymes and both substrates, which are maximally agglutinated with anti-B. Surprisingly though, these results show that GTA is unable to transfer UDP-GalNAc in the presence of UDP-Gal: no agglutination can be observed against anti-A for the cells transformed with both substrates and either GTA alone or GTA plus GTB (Table 65). Because this effect is seen in the cells that were not created with GTB, it appears that the inhibition can be put down to the presence of the UDP-Gal.

Table 65. Transformation of O RBCs with combinations of GTA, GTB, UDP-GalNAc and UDP-Gal.

	Serology*		
Enzyme	GTA	GTA + GTB	GTB
UDP sugars	GalNAc + Gal	GalNAc + Gal	GalNAc + Gal
Anti-A	0	0	0
Anti-B	0	4	4

* Diamed serology (protocol 3.2), scoring (appendix 7).
Cells were transformed as described in protocol 7.1, but GTB was used at a 1:200 dilution.
Cells were tested against the relevant Bioclone antisera.

A possible mechanism for this inhibition of GTA by UDP-Gal could be found in the structure of the molecular components. GTA has relatively large binding site designed to accommodate the *N*-acetyl group of the GalNAc residue, into which the smaller UDP-Gal – having only a small hydroxyl group where GalNAc has the larger *N*-acetyl group – could easily fit. It has previously been reported that wild-type GTA can transfer the B donor (UDP-Gal) with three times greater efficiency than wild-type GTB can transfer the A donor (Seto *et al.*, 1999). This is in accordance with the mechanism discussed above, but in the current work, the UDP-Gal could not actually be transferred to the H acceptor. This may be due to some difference between the recombinant and wild-type enzymes.

6.2 Enzyme and Donor Dilutions

Cells were transformed with dilutions of the nucleotide donor monosaccharides (UDP-GalNAc and UDP-Gal) and excess quantities of the respective enzyme, and vice versa, with dilutions of the enzyme and excess quantites of the respective substrate. This was done to better understand the dynamics of the reaction, which would lead to a better ability to control the outcome.

The results of RBC transformations with GTA and different concentrations of UDP-GalNAc are shown in Table 66. The range of substrate concentrations tested was 60 μM to 0.06 μM. Cells created with GTA and 60 μM (neat) UDP-GalNAc gave 4+ agglutination against anti-A, and the scores weakened from this value as the substrate was further diluted (Table 66). No agglutination could be observed for the 1.5 μM UDP-GalNAc transformed cells.

GTB and UDP-Gal created cells generated positive agglutination with anti-B to much lhigher dilutions. The first negative reaction occurred with RBCs transformed at 0.06 μM UDP-Gal (Table 66). UDP-GalNAc 1.5 μM generated cells that could no longer be agglutinated with anti-A, while 4+ agglutination was seen with RBCs transformed with the 0.6 μM UDP-Gal.

Table 66. Enzymatic transformation of O RBCs with dilutions of the donor monosaccharides. The enzyme was in excess.

	Serology*											
	UDP-donor concentrations (μM)											
	60	12	6	3	1.5	0.6	0.3	0.15	0.1	0.08	0.06	0
GTA + UDP-GalNac	4	2-3	1	vw	0	0	0	0	0	0	0	0
GTB + UDP-Gal	4	4	4	4	4	4	3	2-3	2	1	0	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed as described in protocol 7.1, v1 and tested against Bioclone anti-A and anti-B.
Controls contained enzyme, but no substrate.

Table 67 shows the results of RBCs transformed with dilutions of the GTA and GTB enzymes from 30 mU/mL to 0.02 mU/mL and excess donor monosaccharides (60 μM). Both enzymes, in their undiluted form, were able to catalyse the transfer of enough substrate to the cells to generate 4+ agglutination against the relevant antisera. The cells transformed with 7.5 mU/mL GTA were 3+, and no agglutination was seen for the cells transformed with 3.75 mU/mL GTA. However, for GTB transformed cells, 4+ agglutinations were maintained right through to the 0.12 mU/mL concentration. The negative reaction was observed with cells transformed with 0.02 mU/mL GTB. These results indicate that GTB was a much more active enzyme than was GTA, being able to transfer enough substrate to RBCs to generate maximal agglutination at the 0.12 mU/mL concentration, a transfer capacity which ended at 15 mU/mL GTA.

Table 67. Enzymatic transformation of O RBCs with dilutions of enzyme. The donor saccharides were in excess.

	Serology*											
	Enzyme concentrations (mU/mL)											
	30	15	7.5	3.75	1.88	0.94	0.47	0.24	0.12	0.06	0.03	0.02
GTA/UDP-GalNAc	4	4	3	0	0	0						
GTB/UDP-Gal	4	4	4	4	4	4	4	4	4	2-3	2	0

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
Cells were transformed as described in protocol 7.1, v2 and tested against Bioclone anti-A and anti-B.
Controls contained substrate, but no enzyme.

Overall, these results show that GTB is a more active and efficient enzyme than GTA. It was able to continue to catalyse enough monosaccharide transfer to be detectable with antibody at 25x lower concentrations of substrate and 250x lower enzyme concentrations than could GTA. However, this research does not seek to determine or enter into significant discussion of the relative kinetics of these two recombinant glycosyltransferases. These experiments were conducted to determine the experimental conditions required to generate 2+ to 3+ RBC agglutination reactions with blood group A and B glycosyltransferases.

6.3 GTA / GTB Cells

A significant advantage for blood grouping sensitivity control cells would be the ability to make cells that simultaneously control for both group A and group B, in other words, by the creation of a cell that simultaneously expresses controlled low amounts of the A and B antigens. Experiments with the synthetic glycolipids have shown that this is possible with KODE™ technology (section 4.4). It also seems feasible with glycosyltransferase enzymes to construct both A and B antigens on the H precursor structures present on group O RBCs. Accordingly, experiments were conducted to investigate how a cell weakly expressing both the A and B antigen could be made using glycosyltransferase enzymes.

Given the existence of a possible inhibition of GTA activity by UDP-Gal detected in section 6.1, it seemed prudent to carry out the enzyme incubations separately. Thus, a two-step block titre of GTA and GTB dilutions with excess substrate was performed. The GTA/UDP-GalNAc incubation was carried out first to minimise the amount of UDP-Gal present, after which the cells were washed and the GTB/UDP-Gal incubation was done.

Table 68 shows the anti-A agglutination results of cells transformed with GTA then GTB (and their respective substrates) in block titre. The results show that transformation with GTA and UDP-GalNAc appears to be affected by transformation with GTB and UDP-Gal. This is most noticeable at the 7.5 mU/mL GTA concentration combined with the 0.075 mU/mL GTB concentration. The scores range between negative (immediately

post-modification) and 2+ (three days later), but appear to settle out at 1+. This is in contrast to the 4+ scores seen when the cells were transformed with 7.5 mU/mL GTA and 0.038 mU/mL GTB or no GTB. It thus appears that the agglutination score against anti-A increases as the GTB concentration decreases.

Table 68. Two-step block titre transformation of group O RBCs with different concentrations of GTA then GTB in the presence of excess substrate.

GTA (mU/mL)	Day	Anti-A*			
		GTB (mU/mL)			
		0.075	0.05	0.038	0
15	1	3	4	4	4
	4-21¥	4	4	4	4
	28	3	4	4	4
10	1	2-3	4	4	4
	4-21¥	3	3-4	4	4
	28	2	3-4	3-4	3-4
7.5	1	0	3	3	3
	4-21¥	2	3	3	3
	28	1	3	3	3

* Diamed gel-cards (protocol 3.2), scoring (appendix 7).
¥ Days tested were 4, 7, 14 and 28 – results were essentially similar, only day 4 results are shown.
Cells were transformed as described in protocol 7.1, v3, and stored in Celpresol.
Agglutinations perfomed against Bioclone anti-A.

At the higher concentrations of GTA (15 and 10 mU/mL), the same trend is also seen. For example, the cells transformed with 15 mU/mL GTA and then 0.075 mU/mL GTB give predominantly 3+ scores, while cells transformed with the lower concentrations of GTB (0.05 and 0.038 mU/mL, and also no GTB) give slightly stronger scores (3-4+ and 4+).

Thus, these results indicate that some kind of interaction may occur between the two antigens that affects agglutination with anti-A. The effect is the most significant at the lowest GTA concentration (7.5 mU/mL).

The results against anti-B do not show the same trend. Even at the lowest concentration of GTB, 0.038 mU/mL, maximal 4+ scores were seen for all cells (results not shown). It is possible that the trend seen against

anti-A also occurred with the B antigen against anti-B, but could not be seen because of the strength of the anti-B reactions. When a maximal agglutination result is obtained, it is not clear whether antigen expression is only just or significantly above the threshold required to give a 4+ reaction, and the GTB enzyme at the dilutions used in this experiment may have constructed significantly more B antigens than required to generate a 4+ score. Any loss of antigen reactivity with anti-B could then go undetected. In this study, the loss of antigen reactivity with anti-B was not of sufficient magnitude to be reflected in agglutination scores (results not shown).

Because the cells were transformed with GTA before GTB, the explanation for the reduction in anti-A agglutination scores as the GTB enzyme concentration increased cannot be that the GTB enzyme was so efficient that it consumed most of the H antigen acceptor structures. It would seem most likely that the opposite would be true – that the anti-B agglutinations would be affected when the cells were transformed with GTA first i.e. that the anti-B agglutinations would be weaker with the high concentrations of GTA. Clearly, there are enough H antigens on these cells to allow the construction of adequate quantities of both the A and B antigens to generate agglutinations ranging up to 4+ against anti-A and consistently at 4+ against anti-B. No explanation for this phenomenon was obvious, and it was not investigated further.

The correct agglutination strength against anti-A was achieved using GTA 15 mU/mL, but the 0.038 mU/mL GTB concentration was still too high to generate 2+ to 3+ scores against anti-B.

6.4 Antisera Comparison

Comparison of the performance of a panel of historical antisera (appendix 10) was conducted using natural AB RBCs as controls and group O RBCs that were enzymatically transformed to express both A and B antigens by the two-step method (section 6.3). Only antisera that gave a positive reaction with natural AB cells was taken into the trial against enzymatically created cells. As most of the antisera samples were

expired, this check was carried out to exclude antisera whose performance had deteriorated.

The cells were first transformed with GTA at a dilution of 1:2 (15 mU/mL), and then with GTB at a dilution of 1:800 (0.0375 mU/mL). These cells had been enzymatically transformed 12 days prior to undertaking this antisera comparison, and had been stored in Celpresol at 4°C.

Table 69 shows the results of testing against the historical A antisera. Four of the antisera, A-II, A-III, A-XII and A-XIII, were able to detect the enzymatically transformed cells with the same ability as they were able to detect the natural AB cells (and this was the 4+ for both). In general, with the exception of the four antisera mentioned above, all the reagents gave a lower score with the enzymatically transformed cells than the natural cells (and these always scored 4+ against natural cells, so were probably more severely deteriorated).

Table 69. Enzymatically transformed AB RBCs against a panel of A antisera.

A antisera	Serology*	
	RBCs	
	Natural AB	Enzyme created AB
II	4	4
III	4	4
IV	3	1-2
VI	3	1-2
VII	2-3	0
VIII	2-3	0
IX	3-4	3
X	3	1-2
XI	2-3	1
XII	4	4
XIII	4	4

* Diamed gel-cards (protocol 3.2), scoring (appendix 7), antisera (appendix 10).
Cells were transformed as described in protocol 7.1.

In the case of the reagents which gave 4+ for both cell groups, it must be remembered that a maximal agglutination result does not indicate whether antigen expression is only just or significantly above the threshold required to give a 4+ reaction. Thus, the enzymatically

transformed cells possibly expressed lower levels of antigen than the natural AB cells, which could explain the reaction pattern generated by the other anti-A reagents.

Of note, the A-VII and A-VIII reagents were unable to detect the enzymatically transformed cells, but along with the A-XI reagent, were positive (2-3+) against the natural cells.

All the anti-B reagents gave 4+ reactions against the natural AB cells except for BVI, which gave a 3-4+ score (Table 70). This same reagent was unable to detect the enzymatically transformed cells. The B-V reagent gave the next lowest agglutination reaction at 2+, but was strongly reactive with natural AB cells. Two 3+ agglutinations were given by the B-VII and B-VIII reagents, indicating some reagent deterioration. The remaining anti-B reagents gave 4+ reactions against the enzymatically transformed cells.

Table 70. Enzymatically transformed AB RBCs against a panel of B antisera.

B antisera	Serology*	
	RBCs	
	Natural AB	Enzyme created AB
I	4	4
II	4	4
III	4	4
IV	4	4
V	4	2
VI	3-4	0
VII	4	3
VIII	4	3
IX	4	4
X	4	4
XI	4	4

* Diamed gel-cards (protocol 3.2), scoring (appendix 7), antisera (appendix 10).
Cells were transformed as described in protocol 7.1.

Of all the antisera that could detect the A and B antigens on the natural AB cells, most showed a reduced ability, with some being completely unable, to detect the antigens on the enzymatically transformed AB cells. The difference in antisera performance between natural and enzymatically

transformed AB cells is probably predominantly due to quantitative differences, although qualitative issues may also be involved as the whole range of potential structures may not be present. Internal core structures may mean that some H antigens are not accessible to one or other of the enzymes, although differential specificity is unlikely due to the considerable homology between the two enzymes. The enzymes may also have differential preferences in terms of sugar chain length, but again, the structural homology between the two enzymes makes it more probable that their preferences are closely aligned. Some of the A and B antigen variants may be present in lesser frequencies than on natural cells, and therefore may affect the performance of some specific antibody clones.

In summary, an expected reduction in the potency of some of the antisera may have rendered them unable to agglutinate enzyme created cells expressing a reduced level of a specific antigenic structure (see B-VI in Table 70). In the case of some of the antisera, a reduced potency is also noticeable against cells expressing antigenic structures at normal levels (see A-VII and A-VIII in Table 69).

7 DISCUSSION

The purpose of this research was the development of RBCs that express controlled amounts of ABO blood group antigens, to thus create an ABO sensitivity control with clinical relevance, in unlimited quantity, that could ensure reproducibility of results in blood grouping laboratories. A critical factor in the success of this research was achievement of control over the amount of antigen present on cells, to create a cell that performed at the clinical threshold, which would be of most value as a weak positive control for grouping procedures. Failure to detect this control cell would indicate that system performance was not of adequate sensitivity.

Several novel methods were evaluated in their ability to create RBC's expressing specific amounts of bioactive A and B molecules.

In clinical practice, transfusion of blood occurs in life-threatening situations and the decision to transfuse is not taken lightly. Clinicians and the public expect that blood will be correctly typed to thus avoid the occurrence of transfusion reactions. Issitt and Anstee cite several articles reporting the incidence of ABO-incompatible transfusion (as reviewed by Issitt & Anstee, 1998). From these reports and taking into account supposed under-reporting or under-detection of the less serious events, they conclude that about 1:10,000 units of blood transfused is of incompatible group. Death has been reported to ensue in 10% of incompatible cases, which is an incidence of 1:100,000. This figure is corroborated by the NIH (USA), and they further estimate that 1:4,000 units transfused causes a non-fatal transfusion reaction (NIH, 1988). Some 33% of the errors leading to incompatible transfusion have been attributed to breakdowns in blood banking procedure (Issitt & Anstee, 1998), with the remainder occurring at the bedside.

One source of error from blood banks is the incorrect typing of blood. This can occur with subgroups, the detection of which depends largely on antisera quality and reactivity. An important factor in the performance of ABO reagents is the concentration of the antibody (mg/mL), with minor dilution often resulting in loss of activity against cells expressing low levels of antigen. Antisera are critical to the effective performance of blood

grouping laboratories, but can deteriorate for a variety of reasons, such as sub-optimal conditions during transport and storage. They may be able to lose up to 95% of their analytical sensitivity and still give normal reactions with the common blood groups (appendix 11.1), but not effectively detecting weak (subgroup) cells. Other causes of error are poorly selected clones for reagent manufacture, inappropriate use, bacterial contamination or cross-contamination with other reagents. The performance of blood group determination laboratories could be improved through the implementation of routine, standardised quality control programmes. This was clearly shown when KODE™ cells were used in the RCPA trial (appendix 6).

In many blood grouping laboratories, routine quality control of the blood determination procedure is not carried out. In other labs, some form of quality control is implemented on some of the steps involved, but all instances of possible error are not covered. Furthermore, most of the methods used are indirect – for example, the most common method of testing antibody potency involves normal cells against diluted antibody to find the point at which the antibody fails to detect antigen. Titre testing of performance in this way is based on extrapolation, because the true test is diluted antigen against undiluted antibody. It also fails to take account of the fact that modern monoclonal reagents are often blends of multiple antibody clones at different concentrations, formulated to specific performance characteristics that may be fundamentally altered by dilution.

Some laboratories avoid antibody titration through the use of natural weak subgroup cells as controls. However, there are two problems with this method; the cells are hard to reliably come by, and there is a range of antigen count within a single defined subgroup. Group A_2B is often used because it is the easiest to come by, but the levels of both A and especially the B antigen expression are too high for it to adequately function as a weak positive control for either anti-A or anti-B. A_x RBCs are the generally accepted benchmark for anti-A analytical sensitivity (appendix 11.2), but are scarce. The variation in antigen density within a subgroup may affect the reproducibility of quality control testing, and thus contraindicate the use of natural weak subgroup cells for this purpose.

Further to the problem of antisera performance is the impact of human error. Automation has reduced this to a significant degree in all aspects of testing, specifically the transcription of scores and tracking or labelling of samples and determination of reaction. However, full automation is available only in some laboratories – most have partial automation and some still have no automation, even in first world countries (primarily because the laboratories are only small and automation is not cost-effective).

Routine quality assurance should encompass the entire testing process to ensure that test results are reproducible, accurate and sensitive. As part of the quality assurance program, quality control of the systems used in blood group determination should be standardised from laboratory to laboratory, and the standard components or reagents involved should come from an external accredited source. Internally manufactured standards will fail in their objective if characterised by and used to control for the same potentially flawed system.

Quality control of reagents should involve testing of avidity, specificity and sensitivity. There should always be controls for provision of negative and positive reactions. The negative control in blood banking should have antigens against the most likely contaminating antibody, while the positive should poorly express the antigens required to be detected. Specificity can be assured by the inclusion of all four common ABO blood groups, and the use of a weak positive control can detect a loss of sensitivity against weak subgroup cells that would not show up against a strong positive control.

In order to test the performance of the entire process, the controls should be tested alongside, and no differently than, patient samples. These controls should therefore simulate patient samples, and be designed to uncover any deviation from established methodology throughout the entire testing procedure. This is especially pertinent with automated systems, which can often be set up to run process controls differently from the normal process, entirely defeating the purpose. However, manual techniques should also apply this same strategy of testing controls

in the same manner as routine samples. Routine use may also assist in the detection of transcription errors.

A reliable supply of a standardised and reproducible weak positive control RBCs for routine use in blood banks would, when treated as patient samples, be useful for controlling the entire testing procedure. A weak blood group A and/or B cell is ideal for this purpose, but the antigen strength of natural weak subgroup cells is too variable and the most appropriate subgroups are too rare to be useful.

The alternative then is an unnatural cell expressing specific controlled levels of antigen. The origin of the current research is creation of such cells artificially, based on the natural phenomenon in which glycolipids are able to be taken up from plasma by the RBC membrane. Glycolipids are amphipathic molecules composed of a polar hydrophilic carbohydrate chain attached to a non-polar hydrophobic bilipid tail, which naturally form micelles in solution (e.g. plasma or isotonic saline). The unique properties of glycolipids and membranes, whereby the lipids of the bilayer are capable of rearrangement, allow the spontaneous incorporation of the glycolipids from the surrounding milieu into the membranes of RBCs.

The phenomenon of insertion, as it is called, was first identified in animals (Rendel *et al.*, 1954; Stormont, 1949). The phenomenon was then investigated and established for Lewis structures in humans (Sneath & Sneath, 1955). That insertion occurred only with lipid-borne antigens was determined later (Marcus & Cass, 1969), after unsuccessful experiments with glycoproteins (Lewis *et al.*, 1960; Makela & Makela, 1956). Confirmation that ABO antigens could also be acquired by RBCs followed (Renton & Hancock, 1962; Tilley *et al.*, 1975).

It is now clear that there is free exchange of glycolipids between plasma and RBC membranes, and the glycolipids are thought to be divided between the membrane and plasma (in the lipoprotein fraction) compartments at an equilibrium of one third to two thirds respectively (Schwarzmann, 2001; Wilchek & Bayer, 1987).

More recent work with gangliosides has revealed that assemblies of these molecules (usually micelles or lipoprotein particles) are able to interact

with the cell membrane in three ways; either loosely associated with the outside of the cell membrane such that they are easily washed away, and in a trypsin-sensitive or trypsin-stable manner (as reviewed by Mobius *et al.*, 1999). It is this last trypsin-stable association that is true insertion. Because gangliosides are positively charged glycolipids, their non-insertion interaction with the membrane may be quite different than for neutral glycolipids, and indeed, no equivalent phenomenon has been reported for the neutral blood group glycolipids.

Previous unpublished experiments conducted by members of this laboratory have investigated various features of glycolipid insertion. One study examined insertion at different temperatures using flow cytometry (appendix 1). Significant insertion of natural glycolipids occurred within 8 hours at 37°C. The rate of insertion reduced as the temperature was decreased, as could be seen when the level of insertion that corresponded with serological detection was achieved after the first hour at 37°C, but did not occur until after the fifth hour at 22°C. Insertion at refrigeration temperature (2-8°C) was almost non-existent (appendix 1). This is in contrast to the work of Callies (Callies *et al.*, 1977), which found that gangliosides could be taken up by different cell types at temperatures between 0°C and 4°C. Furthermore, the amount of associated (uninserted) ganglioside was seven times lower after low temperature incubation than when incubated at 37°C. However, the interaction of negatively charged gangliosides with the membrane may be different than that for neutral glycolipids.

In this research, natural glycolipids were successfully inserted into RBCs to create weak ABO antigen expressing cells. Natural glycolipids produced transformed cells with good stability when tested using column technology, but not when the testing method was manual tube serology, which showed significant variability of up to three score levels from week to week. The 25°C transformation method produced lower agglutination scores by manual serology than the 37°C method, indicating that insertion at 25°C was suboptimal for natural glycolipids in this instance. Transformation at 2°C may have resolved these issues, but was not investigated because of the indication that reducing the temperature

below 37°C became suboptimal and also the advent by that time of the synthetic glycolipids.

The major downside of natural glycolipids is the difficult and time consuming method required to extract and purify them from tissues. A batch of 100 units of blood can take up to 3 months to process and generate less than 2 g of usable blood group glycolipid (unpublished data). In addition, the final product has a significant degree of lipid tail heterogeneity. These factors make them less than ideal for the purpose of manufacturing a commercial product in large scale with the absolute demand for reproducibility.

To address the problems of using natural glycolipids for manufacture, the synthetic glycolipid was created. Synthesis offers the twin advantages of product homogeneity – a sample that is completely homogeneous in terms of the lipid and carbohydrate moieties can be produced, and guarantee of supply – almost any quantity of product can be generated.

The synthetic glycolipid was designed to capture the desired features of the natural molecule, comprising a carbohydrate antigenic determinant and an 'insertable' bi-lipid tail, while simultaneously incorporating several 'improvements' (Bovin *et al.*, 2005a; Bovin *et al.*, 2005b).

The carbohydrate portion of the synthetic A and B glycolipids is a trisaccharide antigenic determinant (see Figure 1 for the trisaccharide determinants of A and B). This confers a generic specificity because the trisaccharide is the basis of commonality for all appropriate antibodies – i.e. all anti-A clones are able to recognise the basic A trisaccharide, regardless of their chain type specificity. Had a fourth sugar been included, its glycosidic linkage would have conferred a chain type to the antigen, as exists in natural glycolipids (see Table 1 for chain types), and thus decreased the range of antibody clones that would have been reactive. The generic glycotope is an improvement over the natural glycolipid as it ensures that the transformed cell will be able to react with as many antibodies as possible.

The lipid tail and sugar antigen are joined together by a specially designed linker that confers aqueous solubility to the molecule. This unique feature

makes the synthetic glycolipid easier to work with than its natural counterpart, which is difficult to dissolve in aqueous media.

In practice, synthetic glycolipids appear to have a very high insertion efficiency (between 65% and 98% - see section 4.7), compared to the partitioning of natural glycolipids to 1/3 in cell membranes and 2/3 in plasma (Schwarzmann, 2001). The superior insertion exhibited by synthetic glycolipids may be the result of their improved aqueous solubility, but equally could be related to the lipid tail design. In structure, the lipid portion of the synthetic glycolipid is more similar to the glycerophospholipids than it is to the lipid moiety of natural glycolipids – this is because it lacks the long chain base of the sphingolipid, having instead two fatty acid chains, and therefore has quite different physicochemical properties (see sections 1.2.1, 1.2.3 and 1.2.4). It is thus possible to speculate that the different physicochemical properties of this lipid tail may cause it to have different insertion characteristics compared to natural glycolipids.

RBCs transformed with synthetic glycolipids under optimal (but not sterile) conditions were stable for at least 70 days, which is longer than the required 56 day (8 week) shelf life for a commercial reagent RBC product. A gradual decrease in score over time was indicative of loss of antigen detectability – either through a change in antigen configuration or their loss from the cell. The transfer of natural glycolipids from RBC membranes back to the plasma *in vivo* and *in vitro* has been well documented (Schwarzmann, 2001; Wilchek & Bayer, 1987), but may differ between the natural and synthetic glycolipids because of their different physicochemical properties, and the rate of loss of the glycolipid antigens in saline or cell preservative may be different from plasma.

It is important to note that in this research, a ubiquitous finding was that the B antigen in the transformation solution (synthetic or natural) had to be at higher concentration (up to 10 x) than the A to be detected serologically. It is well known that anti-B's are generally less sensitive than anti-A. This is because of the similarity between the B antigen and the Galili antigen (Galili is B minus the fucose) that all animals possess.

Thus, the B antigen is less immunogenic in the animal cell lines the monoclonals are derived from.

A critical factor in the success of this research was to achieve control over the amount of antigen on cells. To be a useful sensitivity control product, the transformed cell must operate at the clinical threshold, performing at the level of an A_x cell, which has been implicated in a haemolytic transfusion reaction in a recipient who had high titre anti-A (as reviewed by Mollison *et al.*, 1997). Failure to obtain a positive reaction with the control cell could indicate that the detection system was mis-typing A_x cells (and possibly even some of the more immunogenic subgroups), or more importantly, the system may not detect normal A expression of a low level, thus risking potential incompatible transfusions.

Transformation with synthetic glycolipids has been demonstrated to be controllable in the current research. The strength of agglutination reaction can be varied according to requirements by varying the amount of antigen expressed. This can be achieved simply by changing the concentration of the synthetic transformation solution that is contacted with the cells under optimal incubation conditions.

The favourable performance characteristics of the synthetic glycolipid molecule in the transformation of RBCs has made it possible to manufacture a cell with a precisely defined reactivity. Securacell® contains the first synthetically modified commercial ABO blood grouping RBC sensitivity control cell – termed KODE™. The product, manufactured and marketed by CSL Biosciences Ltd in Australasia, consists of four samples (appendix 11.1 and 11.2). Three of these are natural cells with added alloantibodies for testing in an antibody panel. The fourth sample is the KODE™ A_wB_w cell, which is the synthetically transformed sensitivity control.

One of the strengths of the product is that it is able to be used to control the entire blood group testing procedure. As a sensitivity control, its technical function is to detect antisera failure, but due to its capacity to be processed like a patient sample, it can detect errors at all stages of testing. The samples are manufactured and validated by an external

source (not the testing laboratory itself) and so can prevent the repetition of internal errors, and are also useful for staff education and practice at scoring weak reactions. An additional benefit of the KODE™ cell is that manufacture can be easily regulated up or down to accommodate demand.

The need for a sensitivity control product such as this was verified by a trial conducted by the Royal College of Pathologists of Australasia (RCPA) as a quality assurance educational exercise for 310 member laboratories. KODE™ A cells at three different strengths (agglutination score strengths) and one KODE™ B cell were produced by CSL and sent out by the RCPA as a clinical problem. Testing was carried out by the laboratories using a wide variety of reagents in tube, tile and column agglutination and in a range of automated systems. The results (presented in appendix 6) show some serious mistyping of these weak cells. The strongest KODE™ A cell was typed as blood group O in 3.3% of tests across all methods. A cell with this level of antigen could potentially cause a fatal transfusion reaction. The middle strength KODE™ A cell was typed as group O in 8.6% of tests and the weakest KODE™ A sample in 32.7% of tests. These mistypings could results in the occurrence of a serious to mild transfusion reaction. The KODE™ B cell was typed as O in 7.9% of tests and as an A cell in one case, both of which could cause a serious transfusion reaction.

Across the results of the RCPA educational exercise, the poorest performing method on average at this instance was column agglutination, while the best was microplate, which did not record any O results except for the weakest KODE™ A cell (although this method had the smallest sample size at ≤ 20, and the result thus has less statistical relevance). It is of note that, one year later, column technology has substantially improved its performance (S Henry, personal communication), which is believed by this author to be a direct consequence of the introduction of KODE™ technology.

The RCPA trial thus unquestionably demonstrated the exigency and utility of a standardised sensitivity control cell. There is no natural cell with characteristics appropriate for such a control due to limitations of supply and variability in antigen strength, leaving the modification of natural cells

as the only alternative. Insertion of blood group active molecules into RBCs as a method to alter their antigenic profiles originated from observations of a natural phenomenon whereby RBCs acquire blood group glycolipids from plasma. Natural blood group glycolipids were the primary candidates, but are difficult to extract from tissues, are not homogeneous, and can differ in species profile and purity from one batch to the next. The synthetic analogue of the natural blood group glycolipid does not exhibit these disadvantageous characteristics. Furthermore, it has the added benefits of structural improvements that confer aqueous solubility, superior insertion performance and stability in the membrane as well as imparting a generic antigenic determinant capable of reacting with a wide range of antisera. Samples of the molecule have a high degree of homogeneity with excellent batch to batch reproducibility. These features make the synthetic glycolipid ideal for the creation of a standardised sensitivity control cell.

As an intermediate proof-of-principle step in the creation of a synthetic glycolipid, biotinylated synthetic trisaccharides were attached through avidin to membrane anchors composed of natural charged glycolipids (gangliosides) modified with biotin. The charged nature of gangliosides comes from sialic acid, which has a highly reactive carboxyl group that can be used for the attachment of biotin (Wilchek & Bayer, 1987).

Cells expressing low levels of antigen using the BioG-avidin-biotinylated trisaccharide system did not have the long-term stability required for a commercial reagent RBC. Antigen recognition dropped significantly over a relatively short period of time – from as little as one week.

Cells not possessing the $BioA_{tri}$-3 molecule, in an experiment in which it was conjugated to pre-made, stored BioG-avidin cells on each testing day, were no more stable than fully transformed cells (Table 25). This indicates that the mechanism for the reduction of antigen recognition is independent of the $BioA_{tri}$-3 molecule. Therefore, it seems most likely that the phenomenon is related to the BioG molecule or the BioG-Avidin complex. The BioG itself may be unable to remain in the membrane, or in an appropriate domain or it may be unable to retain a configuration that adequately presents the glycotope for antibody recognition. This cannot

be explained by the structure of the ganglioside ceramide, as it is similar to that of the natural glycolipids, and for this reason would be expected to behave in a very similar manner (natural glycolipids were stable for 10 weeks). This effect could be related to the fact that Avidin is quite a large molecule, at around 55 kDa in size, which could change the insertion characteristics of the BioG that it complexes with. Alternatively, the carbohydrate moiety has some influence on the location of the entire structure in the membrane and its association with its neighbours (Hakomori, 2003; Hakomori & Handa, 2002). It may be that the carbohydrate chains of biotinylated gangliosides, which have quite different properties to those of neutral glycolipids, account for the different performance of the ganglioside as a membrane anchor.

The biotinylated saccharide with 30 copies of the B_{tri} glycotope was able to generate agglutination at lower concentrations than the $BioB_{tri(1)}$-3 molecule, which bears only a single B_{tri} glycotope. As would be expected, lower concentrations of BioG than could be used with $BioB_{tri(1)}$-3 were able to generate agglutination with $BioB_{tri(30)}$. This was not surprising, as the molecule provides more B glycotopes for antibody recognition, and there is some evidence that the spacing of multiple glycotopes along PAA coils improves multivalent IgM binding (Bovin, 2002).

These experiments showed that the generic trisaccharide antigen could be detected by antibody, which provided support to the more ambitious project of engineering an all-in-one synthetic glycolipid.

Alternative methods for the creation of weak antigen expressing RBCs that were unrelated to insertion were also evaluated. Glycosidase and glycosyltransferase enzymes were investigated for their ability to modify the expression of ABO blood group molecules on RBCs.

The α-galactosidase from *Coffea canephora* (green coffee beans) has been extensively used in the quest for universal O RBCs, by stripping the immunodominant B sugar (α-Gal) from group B RBCs (Lenny *et al.*, 1994) (Phillips & Smith, 1996; Zhu *et al.*, 1996).

Seroconversion of blood group A RBCs to group O has been more difficult because an appropriately active enzyme has been hard to obtain

(Levy & Aminoff, 1980; Phillips *et al.*, 1995). However, isolation of an α-3-*N*-acetylgalactosaminidase from a bacterial strain found in the human intestinal tract that can hydrolyse the α1-3 bond between GalNAc and Gal of A glycotopes has been reported (Falk *et al.*, 1991; Hoskins *et al.*, 1997).

Two different enzyme preparations were used in the current work to explore the possibilities of enzyme stripping as a method to create RBCs with controlled amounts of blood group antigen.

Both enzymes appeared to be equally efficient in the removal of α-galactose from B RBCs. However, in order to generate the desired 2-3+ agglutination result, enzyme concentrations as high as 100 U/mL were required, combined with an incubation period of 8 to 12 hours. The use of lower concentrations, which are easier to obtain, would probably necessitate the use of longer incubation times, but the less time spent at 37°C the better for the health of the RBCs (the cells turned brown after 48 hours incubation, and there is the minimum 8 week stability requirement to consider). Studies using an α-galactosidase from *Glycine max* (soybean) found that its activity by weight was 50-100 times that of the *Coffea canephora* enzyme (Hobbs *et al.*, 1995; Hobbs *et al.*, 1996), and it may therefore be a more appropriate alternative.

This method was able to create cells with weak expression of B antigens (Gilliver *et al.*, 2004). However, an important disadvantage of this method for the production of cells weakly expressing the B antigen is that it is dependent on the initial antigen expression quantity of the cells, which is in turn dependent on the efficiency of the blood group glycosyltransferase that constructed them when the cell was formed. This factor could compromise batch equivalence if the method were to get to manufacturing stage. Total control of antigen expression is imperative, and this could not be guaranteed to be the same for each different batch of blood.

Enzymatic synthesis of A and B epitopes on the H structures of blood group O cells using the ABO blood group glycosyltransferases α3-*N*-acetylgalactosaminyltransferase (GTA – the A glycosyltransferase

enzyme) and α3-galactosyltransferase (GTB – the B glycosyltransferase enzyme) has not previously been investigated for the creation of weak control cells. However, there are several examples of the use of various glycosyltransferases to modify carbohydrate expression on cells through constructive addition of saccharides. Srivastava (Srivastava *et al.*, 1992) used a Lewis α3/4-fucosyltransferase to transfer a preassembled B trisaccharide that was covalently bound to the fucose donor, to type 1 or type 2 precursor structures on O Le(a-b-) RBCs. In a further set of experiments, Galili and co-workers (Galili & Anaraki, 1995; La Temple *et al.*, 1996) used an α3-galactosyltransferase to construct the Galili antigen on RBCs by adding α-Gal to de-sialylated LacNAcs to make tumor cells more immunogenic during autologous immunisation.

The GTA and GTB enzymes were shown to effectively modify group O RBCs to weak A or B. However, when the enzymes were used one after the other to make weak AB cells, there appeared to be some kind of interaction between the two. When the activity of GTB was high (caused by decreasing the dilution factor), the strength of the anti-A agglutination decreased. As the GTA reaction was performed first, the explanation did not lie in the GTB enzyme having consumed most of the H antigen acceptor structures. Exactly why this occurred cannot be explained, but it is enough to indicate that the manufacture of weak AB cells using glycosyltransferases needs some refinement to be entirely controllable.

Another drawback to the glycosyltransferase enzyme synthesis system is the 37°C incubation requirement of both enzyme methods, because reagent RBCs have a longer shelf-life if stored and handled at 2-8°C.

On the positive side, modification of the target structures on RBCs by enzymes, for example, from H to A or B by glycosyltransferases, or from A or B to O by glycosidases, should theoretically take place on glycoproteins as well as glycolipids, and also potentially on all different core types, thus most closely approximating the situation on natural cells. Moreover, cells modified by either glycosidases or glycosyltransferases have the advantage that no new/different lipids have been introduced, therefore the membrane itself is not altered. However, the addition of new lipids into the membranes of RBCs by insertion of either natural or synthetic

glycolipids has not appeared to have any deleterious effect that was not also seen with enzymatically modified cells.

Antigen density paradox

Quantitative differences between the subgroups are well established (as reviewed by Issitt & Anstee, 1998). In the current research, 325,000 Syn A antigens and 1,670,000 Syn B antigens were required to produce a 1+ agglutination result. This differs significantly with the numbers of blood group determinants on subgroup erythrocytes proposed by others (Economidou *et al.*, 1967) – Table 4 shows the results of serum adsorption of radiolabelled antibodies with respect to A and B antigens. These figures put the above Syn A cells into the A_2 group and the Syn B cells into the B groupings outlined by Economidou. As both of these subgroup cells normally generate 4+ agglutination reactions with the appropriate antisera, this raises the question of whether previous studies have underestimated the amount of antigen present on RBCs.

One possible explanation for the discrepancy is that there is a need for higher antigen density with synthetics or that the antigens behave differently in the membrane. For example, the membrane anchors of all the antigens inserted to make KODE™ cells are lipid based, and because of this, the molecules may relatively freely migrate and cluster, which is not so easy for the glycoprotein antigens, present in addition to glycolipids on natural A and B cells. This difference in the profile of the anchor for the antigenic determinants, and the fact that clustering affects antibody binding in various ways (see below), may account for the inconsistency.

On the other hand, the use of antibody interaction as a measure of the number of antigens on RBC surfaces, as Economidou did, has several limitations. Firstly, it requires some assumption to be made about the antigen-antibody relationship. Economidou and co-workers assumed that one antibody would combine with one antigenic site. There are several reasons why this hypothesis may not be valid. Firstly, IgG antibodies (such as those used by Economidou) have two possible binding domains, and cross-linking may occur, which means one antibody may bind two antigens. Secondly, glycolipid antigens are mobile in the RBC membrane

(Namork, 1994) and tend to cluster, which is an important facet of antibody binding (as reviewed by Bovin, 2002; Feizi, 2001; as reviewed by Hakomori & Kannagi, 1983; Kannagi *et al.*, 1983; Nores *et al.*, 1987). Binding of one antibody to an antigen in a cluster of antigens that are in close proximity to each other may prevent a second antibody from approaching, which means that clustering of antigen may result in incomplete saturation of some of the antigenic sites. Both these factors make it possible that the antibody interaction method may underestimate the number of antigens on the RBC surface.

Furthermore, as the antigen-antibody complex is reversible, i.e. the antibody frequently lets go and binds again to the same or another antigen, the maximum adsorption was obtained by extrapolation (Economidou *et al.*, 1967). It was estimated by the authors that this introduced no more than a 10% error into their measurements. The authors themselves highlighted other potential errors in this methodology, such as during the cell count and the assumption that the specific activity of the isolated IgG fraction was the same as the immunoglobulin sample that it derived from. Nevertheless, these antigen density figures remain uncontested to date.

7.1 Conclusion

This research has resulted in five different methods to create ABO sensitivity control cells; cells transformed by inserted natural glycolipids, cells transformed by inserted modified natural glycolipids and the biotin avidin system, cells transformed by inserted synthetic glycolipids, cells transformed by the removal of the immunodominant sugar using glycosidases, and cells transformed by the addition of the immunodominant sugar using glycosyltransferases. These methods have produced multiple patent applications (Blake *et al.*, 2002; Blake *et al.*, 2005; Gilliver & Henry, 2004; Gilliver *et al.*, 2004).

All the methods trialled were able to produce cells that showed weak expression of antigen against normal ABO antisera. However, each had its own set of advantages and/or disadvantages. The natural glycolipids are notoriously difficult to extract and purify to the required degree. Cells

transformed using the modified natural glycolipids (gangliosides), avidin and biotinylated trisaccharide were poorly stable, but proved that the trisaccharide was visible to antibody. The synthetic glycolipids showed improved insertion over natural glycolipids (both in efficiency and in low temperature insertion), improved stability of the resultant cells, and could be prepared with a pure homogeneous reproducible profile in limitless quantities. The glycosidase method is highly dependent on the starting antigen strength, which can vary between batches of RBCs (depending on the donors) and thus compromise batch reproducibility. The glycosyltransferases appeared to have interaction problems when used sequentially to modify cells to A_wB_w. In addition, the enzymes (glycosidase and glycosyltransferases) all require incubation at 37°C, which is not ideal for RBCs.

The conclusion reached in this research was that synthetic glycolipids offered the best combination of characteristics for the production of cells expressing controlled weak amounts of antigen. Accordingly, the synthetic glycolipid has been used to engineer a sensitivity control cell (present in the Securacell® product) for use by the blood banking industry.

Future perspectives

During the course of this project, a variety of non-ABO molecules were evaluated. Some of these molecules were promising and are now major aspects of new research (results not shown). Of most interest is the peptide antigen concept, for which the prototype is one of the Miltenberger peptide antigens, Mi^a. KODE™ constructs incorporating this antigen are currently being synthesised. Successful insertion of the constructs into RBCs and detection with anti-Mi^a will provide the base technology for other peptide antigens to follow. Various peptides with diagnostic antibody test applications using KODE™ technology have already been identified.

KODE™ technology has an extensive range of applications; its power resides in its potential to modify the membrane of any cell type with any molecule of interest.

EXPERIMENTAL PROTOCOLS

Reagents and materials

Manufacturer	Catalogue ref	Lot	Expiry
Column agglutination technology (CAT) cards.			
Diamed	NaCl, enzyme and cold agglutinins		
Anti-A reagents.			
Albaclone, SNBTS	Anti-A	Z0010770	12.12.04
Seraclone, Biotest	801320100	1310401	12.04.03
Bioclone, OCD	Anti-A, experimental reagent	DEV01102	-
Biolab	Dolichos biflorus lectin (DBA)	8203	02.83
Anti-B reagents.			
Albaclone, SNBTS	Anti-B	Z0110670	01.07.05
Bioclone, OCD	Anti-B, experimental reagent	DEV01103	-
Anti-Le^b reagent.			
CSL	Anti-Le^b IgM	12801	-
Anti-human globulin reagent.			
CSL	Anti-human globulin, anti-IgG – C3d	03701	Jan 00
Anti-D IgG reagent.			
CSL	Human anti-D IgG (MCAD6)	3111015	13.12.08
NIH (WHO)	Anti-D quantitation standard (human)	4a-1	-
Anti-Mi^a reagent.			
Gamma Biologicals	Anti-Mi^a (GAMA-210)	Mi^a(210)112701	-
Secondary anti-mouse Ig.			
Chemicon	Anti-mouse Ig (FITC)	AP326F	Nov 2004
Anti-H reagents.			
Lorne Labs	*Ulex europaeus*	11549E	6.2004
Japanese Red Cross	IgM clone HIRO-75	-	-
EY Labs	Bio-UEA BA2201-2	201105-2	-

EXPERIMENTAL PROTOCOL

Protocol 1.1

Preparation of transformation solution: Natural glycolipids and biotinylated gangliosides

OBJECTIVE

To prepare natural glycolipids or biotinylated gangliosides in an isotonic solution.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- Dried HPLC purified natural glycolipid or biotinylated gangliosides (from protocol 4.1)

Reagents

- Chloroform (02405E21, SDS)
- 10 x PBS
- Methanol (15250, BDH)
- Nitrogen gas (Air Liquide)
- Deionised water
- Working strength PBS

Equipment

- Micropipettes (10 – 100 μL, 100 – 1000 μL)
- Glass Kimble tubes (KIM45048-18150, Biolab)
- Heating block (Wealtec HB-2)
- Glass freeze dry vials (BO2168, Bonnet Equipment)
- -85°C freezer
- Freeze-dryer (Heto LyoLab 3000)
- Parafilm (PM-996, Pechiney Plastc Packaging)
- Ultrasonic water bath (Grant XB14)

FINAL METHOD

- Dissolve the natural glycolipid sample or biotinylated ganglioside sample in chloroform-methanol 2:1 (v/v) to a concentration of 50 mg/mL.
- Transfer 500 μL of the 50 mg/mL natural glycolipid sample or biotinylated ganglioside sample to a glass Kimble tube.
- Add 50 μL of 10 x PBS along with enough methanol to make the chloroform and water miscible.
- Dry the solution in a 70°C heating block under a gentle stream of nitrogen gas. If the solution separates out into two phases at any point, add a small amount of methanol. Dry the glycolipid sample fully.
- Heat deionised water in the 70°C heating block, and add 500 μL to the dried glycolipid sample or biotinylated ganglioside sample to produce a 50 mg/mL solution.
- Seal the tube with Parafilm and sonicate it for 2 min, then place the sample in the 70°C heating block for up to two hours to ensure it is completely dissolved.
- Dilute as required in working strength PBS.

Method Variations

Variation 1.

- As for final method with the exception that drying of the sample was freeze-dried – it was first frozen at −85°C for about 30 min (dependent on the size of the sample) and then freeze-dried.

EXPERIMENTAL PROTOCOL

Protocol 1.2

Preparation of transformation solution: Biotinylated saccharides

OBJECTIVE

To prepare biotinylated saccharides in an isotonic solution.

SAMPLES, REAGENTS AND EQUIPMENT

Samples
- Dried biotinylated saccharide (Syntesome, Russia)

Reagents
- Deionised water
- Working strength PBS

Equipment
- Vortex (Thermolyne Maximix Plus M63210-26)

METHOD

- Dissolve the biotinylated saccharide sample in deionised water to a concentration of 1 mg/mL.
- Vortex to ensure the sample is fully dissolved.
- Dilute as required in working strength PBS.

EXPERIMENTAL PROTOCOL

Protocol 1.3

Preparation of transformation solution: Synthetic glycolipids

OBJECTIVE

To prepare synthetic glycolipids in an isotonic solution.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- Dried synthetic glycolipid (Lectinity, Russia - (Bovin et al., 2005a; Bovin et al., 2005b))

Reagents

- Cell preservative solution

Equipment

- Parafilm (PM-996, Pechiney Plastc Packaging)
- Waterbath (Julabo F18)
- Heating block (Wealtec HB-2)
- Micropipettes (100 – 1000 μL)
- Vortex (Thermolyne Maximix Plus M63210-26)
- Glass Kimble tubes (KIM45048-18150, Biolab)
- Ultrasonic water bath (Grant XB14)

FINAL METHOD

- Dissolve the synthetic glycolipid sample in RT cell preservative solution to the desired concentration.
- Seal the tube with Parafilm and sonicate it for 2 min, and then place the sample in a 37°C waterbath for up to two hours to ensure it is completely dissolved.
- Dilute as required in cell preservative solution.

Method Variations

Variation 1.

- Dissolve the synthetic glycolipid sample in 70°C working strength PBS to the desired concentration.
- Seal the tube with Parafilm and sonicate it for 2 min, and then place the sample in the 70°C heating block for up to two hours to ensure it is completely dissolved.
- Dilute as required in 1 x PBS.

EXPERIMENTAL PROTOCOL

Protocol 2.1

Insertion: Natural glycolipids

OBJECTIVE

To transform RBCs with natural blood group glycolipids by insertion.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- HPLC purified glycolipid in working strength PBS (from protocol 1.1)

Cells
- Group O blood

Reagents
- Working strength PBS
- Cell preservative solution

Equipment
- Micropipettes (1 – 10 μL, 10 – 100 μL)
- Centrifuge (Immufuge II, American Dade, USA)
- Eppendorf microcentrifuge tubes, 1.5 mL (3445, Raylab)
- Waterbath (Julabo F18)
- Centrifuge (Sorvall MC-12V, Du Pont, USA)
- Flat bottomed dropper bottles

FINAL METHOD

37°C/60 min.
- Dilute the glycolipid sample to the desired concentration with working strength PBS.
- Wash the group O blood three times in working strength PBS by centrifugation in an immufuge.
- Add 20 μL of the A glycolipid sample to 60 μL of the washed packed group O RBCs in an eppendorf tube.
- Incubate the tube in a 37°C waterbath for one hour, with mixing being carried out every 15 min.
- Wash the RBCs three times by centrifugation in working strength PBS, and then suspend in saline/cell preservative for serology testing in tube or the Diamed gel-card platform.

Method Variations

Variation 1. 37°C/90 min.
- As for final method with the exception that the incubation period was 90 min in a 37°C waterbath.

Variation 2. Comparison of 1 hr and 2 hr at 37°C.
- As for final method with the exception that one set of cells was incubated for one hour in a 37°C waterbath, and another was incubated for two hours in a 37°C waterbath.

Variation 3. 25°C/4 hr.
- As for final method with the exception that the incubation was carried out for four hours in a 25°C waterbath.

Variation 4. Comparison of 37°C and 25°C.
- As for final method with the exception that one set of cells was incubated for 90 min in a 37°C waterbath, and another was incubated for 4 hours in a 25°C waterbath.

Variation 5. Comparison of 37°C and 2-8°C.
- As for final method with the exception that one set of cells was incubated for 60 min in a 37°C waterbath, and another was incubated in a 2-8°C fridge for time periods of 24, 48 and 72 hours.

Variation 6. Stability trial.

- As for final method except that the cells were transformed at blood group glycolipids concentrations of 10 mg/mL, 5 mg/mL, 2 mg/mL [two identical tubes were made] and 1 mg/mL).
- The tubes containing cells transformed with the 10 mg/mL, 5 mg/mL and 1 mg/mL glycolipid solutions, and one of the 2 mg/mL transformed tubes were washed three times with PBS and then suspended in cell preservative solution. The tube that remained unwashed was suspended directly in the cell preservative solutions. For all samples, a 5% suspension was made for standard tube serology testing and a 0.9% suspension was made for Diamed gel-card testing. All suspensions were made in flat bottomed dropper bottles for storage.
- The samples were stored at 2-8°C. After the cells were allowed to settle overnight, the supernatant was removed from all tubes and replaced with fresh cell preservative solution.

EXPERIMENTAL PROTOCOL

Protocol 2.2

Insertion and conjugation: Biotinylated gangliosides, avidin and biotinylated saccharides

OBJECTIVE

To transform RBCs via the attachment biotinylated synthetic trisaccharides through avidin to inserted natural ganglioside membrane anchors modified with biotin.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- Biotinylated ganglioside in working strength PBS (from protocol 1.2, v1)
- Biotinylated saccharide in working strength PBS (from protocol 1.2, v2)

Cells
- Group O blood

Reagents
- Working strength PBS
- Avidin (A-9275, Sigma)
- Cell preservative solution

Equipment
- Micropipettes (1 – 10 μL, 10 – 100 μL)
- Centrifuge (Immufuge II, American Dade, USA)
- Eppendorf microcentrifuge tubes, 1.5 mL (3445, Raylab)
- Waterbath (Julabo F18)
- Centrifuge (Sorvall MC-12V, Du Pont, USA)
- Diamed gel-card platform (Diamed, Australia)

FINAL METHOD

Insertion 37°C/60 min, saccharides:RBCs 1:1.

Step 1. Biotinylated ganglioside insertion.
- Dilute the biotinylated ganglioside sample to the desired concentration with working strength PBS.
- Wash the group O blood three times in working strength PBS by centrifugation in an immufuge.
- Add one part biotinylated ganglioside sample to three parts of the group O RBCs in an eppendorf tube.
- Incubate the tube in a 37°C waterbath for one hour, with mixing being carried out every 15 min.
- Wash the RBCs three times by centrifugation in working strength PBS.

Step 2. Avidin conjugation.
- Dissolve the avidin in working strength PBS to a concentration of 1 mg/mL.
- Add two parts avidin sample to three parts washed packed biotinylated ganglioside-RBCs in an eppendorf tube.
- Incubate the tube in a 25°C waterbath for 30 min, with mixing being carried out every 10 min.
- Wash the biotinylated ganglioside/avidin RBCs three times by centrifugation in working strength PBS.

Step 3. Biotinylated saccharide conjugation.
- Dilute the biotinylated saccharide sample to the desired concentration with working strength PBS.
- Add one part biotinylated saccharide sample to one part washed packed biotinylated ganglioside/avidin RBCs.
- Incubate the tube in a 25°C waterbath for 30 min, with mixing being carried out every 10 min.
- Wash the biotinylated ganglioside/avidin/biotinylated saccharide RBCs three times by centrifugation in working strength PBS, and then suspend in saline/cell preservative for serology testing.

Method Variations

Variation 1. Insertion 37°C/90 min, saccharides:RBCs 1:1.

Step 1. Biotinylated ganglioside insertion.
- As for final method with the exception that the incubation period was 90 min in a 37°C waterbath.

Step 2. Avidin conjugation.
- As for final method.

Step 3. Biotinylated saccharide conjugation.
- As for final method.

Variation 2. Insertion 25°C/4 hr, saccharides:RBCs 1:1.

Step 1. Biotinylated ganglioside insertion.
- As for final method with the exception that the incubation period was four hours in a 25°C waterbath.

Step 2. Avidin conjugation.
- As for final method.

Step 3. Biotinylated saccharide conjugation.
- As for final method.

Variation 3. Biotinylated saccharides added before testing.

Step 1. Biotinylated ganglioside insertion.
- As for final method with the exception that the incubation period was four hours in a 25°C waterbath.

Step 2. Avidin conjugation.
- As for final method.

Step 3. Biotinylated saccharide conjugation.
- As for final method with the exception that only half of the cells had the biotinylated saccharide conjugated the same day as the other steps were carried out.
- The remainder of the cells were stored as Av-BioG cells, and the biotinylated saccharides were conjugated as for the final method on each day of testing.

Variation 4. Insertion 37°C/ 60 min, saccharides:RBCs 1:3.

Step 1. Biotinylated ganglioside insertion.
- As for final method.

Step 2. Avidin conjugation.
- As for final method.

Step 3. Biotinylated saccharide conjugation.
- As for final method with the exception that the only one part biotinylated saccharide sample was added to three parts washed packed Av-BioG RBCs.

EXPERIMENTAL PROTOCOL

Protocol 2.3

Insertion: Synthetic glycolipids

OBJECTIVE

To transform RBCs through the insertion of synthetic blood group active glycolipids.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- Synthetic glycolipid in PBS or cell preservative solution (from protocol 1.3 or protocol 1.3, v1)

Cells
- Group O blood

Reagents
- Working strength PBS
- Cell preservative solution

Equipment
- Micropipettes (1 – 10 μL, 10 – 100 μL)
- Centrifuge (Immufuge II, American Dade, USA)
- Eppendorf microcentrifuge tubes, 1.5 mL (3445, Raylab)
- Fridge or cool room (2-8°C)
- Waterbath (Julabo F18)
- Centrifuge (Sorvall MC-12V, Du Pont, USA)
- Flat bottomed dropper bottles

FINAL METHOD

2-8°C/48 hr.
- Dilute the synthetic glycolipid sample to the desired concentration with cell preservative solution.
- Wash the group O blood three times in working strength PBS by centrifugation in an immufuge.
- Add 1 part synthetic glycolipid sample to three parts washed packed group O RBCs in an eppendorf tube.
- Incubate the tube in a 2-8°C fridge for 48 hours, with mixing being carried out hourly during the day.
- Wash the RBCs three times by centrifugation in working strength PBS, and then suspend in saline/cell preservative for serology testing.

Method Variations

Variation 1. 37°C/60 min.
- As for final method with the exception that the incubation period was 60 min in a 37°C waterbath.

Variation 2. 2-8°C time trial.
- As for final method with the exception that the cells were incubated in a 2-8°C fridge for time periods of 2, 4, 8, 12, 24, 48, 72 and 96 hours.
- Cells were removed for testing at intervals against the relevant antisera, and were tested in both washed and unwashed states (i.e. washed samples had the transformation solution removed).
- After 48 hours Celpresol was added to the cells so that the final cells:suspension liquid ratio was 3:5 (v/v). The cells continued to be tested in the Diamed gel-card platform at intervals.

Variation 3. Simultaneous transformation at 2-8°C.
- As for final method with the exception that the cells were incubated with Syn A and Syn B transformation solutions combined in block titre and incubated in a 2°C fridge.
- Cells were removed for testing at intervals against the relevant antisera, and were tested in both washed and unwashed states (i.e. washed samples had the transformation solution removed).
- After 48 hours Celpresol was added to the cells so that the final cells:suspension liquid ratio was 3:5 (v/v). The cells continued to be tested in the Diamed gel-card platform at intervals.

Variation 4. Stability trial.

- As for final method with the exception that the cells were incubated for 60 min in a 37°C waterbath, and then suspended in saline/cell preservative and stored at 2-8°C for testing at weekly intervals over 10 weeks.

Variation 5. AB cells for antibody neutralisation.

- As for final method.
- After 48 hours Celpresol was added to the cells so that the final cells:suspension liquid ratio was 3:5 (v/v).
- Cells were removed for testing at days 1 and 3 (post-48 hour transformation period) against the relevant antisera, and were tested in both washed and unwashed states (i.e. washed samples had the transformation solution removed).

EXPERIMENTAL PROTOCOL

Protocol 3.1

Agglutination: Tube Method

OBJECTIVE

To test for RBC antigens by agglutination with the relevant antisera in tube.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Cells
- RBCs to be tested

Reagents
- Working strength PBS
- Relevant antisera

Equipment
- Micropipettes (10 – 100 μL)
- Centrifuge (Immufuge II, American Dade, USA)
- Kimble glass serology tubes (735001075, Biolab)

METHOD

- Wash the RBCs three times in working strength PBS by centrifugation in an immufuge.
- Suspend to about 3% in PBS.
- Add 1 drop (about 50 μL) of cell suspension to a glass serology tube.
- Add 1 drop (about 50 μL) of the relevant antisera to the tube.
- Shake the tube to ensure the cells and antisera are well mixed.
- Spin the tube in a balanced immufuge for 15 seconds on low (about 2200 rpm).
- Read the agglutinate(s) or lack of in the tube using an eyepiece and score using the 4 point system.
- Record the agglutination score (scoring see appendix 7).

EXPERIMENTAL PROTOCOL

Protocol 3.2

Agglutination: Diamed column method

OBJECTIVE

To test for RBC antigens by agglutination with the relevant antisera in the Diamed gel-card platform.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Cells
- RBCs to be tested

Reagents
- Working strength PBS
- Relevant antisera
- Red cell diluent (Diluent 2 or CellStab, Diamed, Australia)

Equipment
- Micropipettes (1 – 10 μL, 10 – 100 μL)
- Immufuge (Immufuge II, American Dade, USA)
- Centrifuge (Diamed, Australia)
- Blank gel-cards (NaCl, Enzyme and cold agglutinins, Diamed, Australia)

METHOD

- Wash the RBCs twice in working strength PBS and a final time in Diluent 2 or CellStab by centrifugation in an immufuge.
- Suspend to 0.8% in Diluent 2 or CellStab.
- Add 50 μL RBC suspension to a well in the card.
- Add 50 μL relevant antisera to the well.
- Place the card in the balanced card centrifuge and spin.
- Record the agglutination score (scoring see appendix 7).

EXPERIMENTAL PROTOCOL

Protocol 4.1

Biotinylation of gangliosides

OBJECTIVE

To attach a biotin residue to the carboxyl group of the sialic acid residue of gangliosides.

SAMPLES, REAGENTS AND EQUIPMENT

Samples

- Dried purified gangliosides

Reagents

- Working strength PBS
- $NaIO_4$ (S-1878, Sigma)
- BACH reagent (B3770, Sigma)
- DMSO (D8779, Sigma)
- KBH_4 (29587, BDH)
- MilliQ water
- Methanol (15250, BDH)
- Wet ice
- Nitrogen gas (Air Liquide)

Equipment

- Rotary evaporation flasks (50 mL, 100 mL and 500 mL)
- Rotary evaporator (Buchi EL101) with waterbath (Buchi 461)
- 14 kDa dialysis tubing (44146, Serva)
- Large sink or waterbath
- Magnetic stirrer (Selby SMS11) and bar
- 500 Da dialysis tubing (131054, Purescience)
- Glass Kimble tubes (KIM45048-18150, Biolab)

METHOD

- Add 10 mL of working strength PBS to 100 mg gangliosides in a 50 mL rotary evaporation flask.
- Rotate on the rotary evaporator in a 60°C waterbath (do not apply vacuum) until dissolved.
- Prepare 0.5 M NaIO4 aqueous solution just prior to use.
- Add another 10 mL of working strength PBS and 400 μL of the 0.5 M NaIO4 aqueous solution to the sample and mix on a magnetic stirrer on ice for 30 min.
- Dialyse over night in a 14 kDa dialysis bag at 4°C in 2 L of working strength PBS with two changes of buffer.
- Transfer the dialysed sample into a 100 mL rotary evaporation flask.
- Dissolve 100 mg BACH reagent in 4 mL DMSO then add this mixture dropwise to the ganglioside solution.
- Incubate at RT with mixing for 1.5 hr followed by 1 hr at 37°C and then another 1.5 hr at RT.
- Dialyse overnight in working strength PBS in a 500 Da dialysis bag.
- Transfer the dialysed sample into a 100 mL rotary evaporation flask.
- Prepare 0.5 M KBH4 solution in water just prior to use, and add 470 μL to the dialysed sample.
- Incubate at RT for 10 min.
- Dialyse for 2 hr in MilliQ water with two changes.
- Place sample in a 500 mL rotary evaporation flask and evaporate to dryness.
- Prepare 50% methanol in water.
- Resuspend the sample in 6.5 mL 50% methanol in water and dry under nitrogen gas in a preweighed kimble tube.
- Store at 4°C.

EXPERIMENTAL PROTOCOL

Protocol 5.1

20 x concentration of post-transformation supernatants

OBJECTIVE

To obtain 20x concentrates of post-transformation supernatants in an isotonic solution.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples

- Syn A 0.08 mg/mL post-transformation supernatant
- Syn B 0.6 mg/mL post-transformation supernatant

Reagents

- Deionised water
- Chloroform (02405E21, SDS)
- Methanol (15250, BDH)
- Nitrogen gas (Air Liquide)
- Working strength PBS

Equipment

- 20 mL pipette
- 500 Da dialysis tubing (131054, Purescience)
- Rotary evaporator (Buchi EL101) with waterbath (Buchi 461)
- Rotary evaporation flasks
- Glass Kimble tubes (KIM45048-18150, Biolab)

METHOD

- Dialyse post-transformation supernatants (20 mL) against deionised water for 2 days in a 500 Da dialysis bag.
- Reduce the samples under ambient air in a fumehood.
- Transfer the samples to rotary evaporation flasks and set on the rotary evaporator to rotate under vacuum with no heat overnight.
- Warm waterbath to 40°C and continue to dry samples while rotating on the rotary evaporator.
- Wash over into smaller vessels with chloroform-methanol 2:1.
- Dry the CM 2:1 samples.
- Washed over into kimble tubes with chloroform-methanol 2:1 and dry under nitrogen gas.
- Redissolve the samples in 1 mL of working strength PBS for transformation experiments.

EXPERIMENTAL PROTOCOL

Protocol 6.1

Preparation of α-galactosidase from green coffee beans

OBJECTIVE

To extract α-galactosidase from green coffee beans (*Coffea canephora*).

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples

- Dried green coffee beans (Coffea Canephora) (Kenyan Robusta variety)

Reagents

- Acetone (2435, Mallinckroft)
- MilliQ water
- $FeCl_3$ (F7134, Sigma)
- Toluene (8608, Mallinckroft)
- Tannic acid (T0125, Sigma)
- Methanol (15250, BDH)
- $NaPO_4$, dibasic (S-5136, Sigma)
- Citric acid (38640, Serva)

Equipment

- Coffee grinder (Breville CG-2)
- Magnetic stirrer (Selby SMS11) and bar
- Buchner funnel
- Whatmans filter paper number 1
- Vacuum pump (Buchi Vac V-500)
- Mortar and pestle
- Cheese cloth
- 14 kDa dialysis tubing (44146, Serva)
- Balance (Mettler Toledo PB1502-S)
- Ph meter (MeterLab PHM201)
- Centrifuge (Hitachi 05PR-22)
- Millipore Amicon Ultra-15 centrifugation filters/concentrators (MILUFC903008, Biolab)

METHOD

- Grind 200 g of green coffee beans to a fine powder in a coffee grinder.
- Place the powder into a ready prepared beaker containing an ice-cold solution of acetone-water 3:1 (1.2 L) as it is being produced. Incubate with stirring on ice for 1.5 hr.
- Vacuum filter the prep with Whatmans filter paper number 1 using a Buchner funnel. Continue to wash the solids with pre-cooled acetone-water 3:1 until tannins are no longer present in the filtrate – test with FeCl3.
- Air dry the sample and store at 4°C.
- Grind the coffee prep in a mortar and extract with 700 mL toluene saturated water for 24 hr at RT on a mixer.
- Continue to extract in the toluene saturated water for a further 24 hr at 4°C.
- Filter the suspension through cheese cloth, and centrifuge the filtrate at 5500 rpm for 40 min at 4°C.
- Add 90 mL of a 2% aqueous tannin solution to the supernatant and stir for 1 hr at RT on a magnetic mixer.
- Centrifuge the sample at 5500 rpm for 40 min at 4°C.
- Suspend the precipitate in 10ml pre-cooled (in -20°C freezer overnight) acetone and pour into the Buchner funnel. Keep the acetone cold in a methanol ice bath, and use to wash the precipitate repeatedly until there is no colour reaction when FeCl3 is added to the filtrate.
- Dry the sample under vacuum to get rid of acetone. Store dessicated at 4°C.
- Resuspend 1 g dry precipitate in 100 mL MilliQ water and extract overnight at 4°C.
- Dialyse the supernatant in a 14 kDa dialysis bag against Milli Q water for 48 hr with 2 changes.
- Prepare 100 mM citrate-phosphate buffer by making 500 mM NaPO4 dibasic solution in water, and titrate to pH 6 with a 500 mM citric acid in water solution. Dilute the 500 mM solution 5x in water to make a 100 mM citrate-phosphate buffer solution.
- After the 48 hr dialysis in water, dialyse the sample for 2 hr or more in 100 mM citrate phosphate buffer.
- Centrifuge the sample at 5000 rpm for 40 min and store the supernatant overnight at 4°C.
- Concentrate the supernatant until too viscous to be further concentrated in Amicon Ultra (Millipore) concentrating devices spun at max 2900 rpm.

EXPERIMENTAL PROTOCOL

Protocol 6.2

Transformation with α-galactosidase

OBJECTIVE

To transform natural group B RBCs to weak group B RBCs with α-galactosidase enzymes.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- α-galactosidase enzyme (from protocol 7.1)
- α-galactosidase lyophilised powder (GKX-5007, 10 Units, Lot 173 002a, Glyko)

Cells
- Group B blood

Reagents
- Buffer (supplied by Glyko with enzyme)
- MilliQ water
- NaPO4, dibasic (S-5136, Sigma)
- Citric acid (38640, Serva)
- Working strength PBS
- Cell preservative solution

Equipment
- Microcentrifuge (Sorvall MC-12V, Du Pont)
- Centrifuge (Immufuge II, American Dade)
- Eppendorf microcentrifuge tubes, 1.5 mL (3445, Raylab)
- Micropipettes (10 – 100 μL)
- Waterbath (Julabo F18)

Method Variations

Variation 1. Coffee bean enzyme.
- Wash the group B blood three times in working strength PBS by centrifugation in an immufuge.
- Prepare 100 mM citrate-phosphate buffer by making 500 mM NaPO4 dibasic solution in water, and titrate to pH 6 with a 500 mM citric acid in water solution.
- To an eppendorf tube, add 40 μL concentrated enzyme, 16.25 μL 500 mM citrate-phosphate buffer and 25 μL washed packed group B RBCs (in that order).
- Incubate the tube in a 37°C waterbath, with mixing being carried out every hour during the day.
- At prescribed intervals, remove and wash 2 μL RBCs twice by centrifugation in working strength PBS, and then suspend to 0.8% in working strength PBS or cell preservative solution for serology.

Variation 2. Glyko enzyme.
- Spin the lyophilised powder enzyme in microcentrifuge for 2 min at highest speed.
- Resuspend powder in 47 μL of MilliQ water – the concentration is 10 U/47 μL or about 212 U/mL.
- Dilute Glyko buffer 5x in MilliQ water.
- Make 168 μL of 5 U/mL solution in working strength buffer by combining 4 μL enzyme and 164 μL working strength buffer.
- Wash the group B blood three times in working strength PBS by centrifugation in an immufuge.
- Prepare 100 mM citrate-phosphate buffer by making 500 mM NaPO4 dibasic solution in water, and titrate to pH 6 with a 500 mM citric acid in water solution.
- To an eppendorf tube, add 160 μL 5U/mL enzyme, 65 μL 500 mM citrate-phosphate buffer and 100 μL washed packed group B RBCs (in that order).
- Incubate the tube in a 37°C waterbath, with mixing being carried out every hour during the day.
- At prescribed intervals, remove and wash 2 μL RBCs twice by centrifugation in working strength PBS, and then suspend to 0.8% in working strength PBS or cell preservative solution for serology.

Variation 3. Glyko enzyme concentration comparison.
- As for variation 2 with the exception that the enzyme is diluted in 100 mM citrate-phosphate buffer in eppendorf tubes to concentrations of:
- 100 U/mL – 18.8 μL enzyme and 21.2 μL 100mM citrate-phosphate buffer.
- 50 U/mL – 9.4 μL enzyme and 30.6 μL 100mM citrate-phosphate buffer.
- 37.5 U/mL – 7.05 μL enzyme and 32.95 μL 100mM citrate-phosphate buffer.
- 25 U/mL – 4.7 μL enzyme and 35.3 μL 100mM citrate-phosphate buffer.
- 12.5 U/mL – 2.35 μL enzyme and 37.65 μL 100mM citrate-phosphate buffer.
- To each of the eppendorf tubes above, add 16.25 μL 500mM citrate-phosphate buffer and 25 μL washed packed group B RBCs (in that order).

EXPERIMENTAL PROTOCOL

Protocol 7.1

Glycosyltransferase Modification

OBJECTIVE

To transform RBCs using glycosyltransferase enzymes and activated sugars.

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- Lyophilised enzyme (from Dr Monica Palcic of the University of Alberta, Canada)
- UDP-GalNAc disodium salt (U5252, Sigma)
- UDP-Gal disodium salt (U4500, Sigma)

Cells
- Group O blood

Reagents
- Normal saline
- Mops buffer (Sigma)
- MnCl2 (10152, BDH)
- BSA (30060-578, GibcoBRL)
- NaCl (102415K, BDH)
- MilliQ water
- Cell preservative solution

Equipment
- Micropipettes (10 – 100 μL)
- Centrifuge (Immufuge II, American Dade)
- Waterbath (Julabo F18)

METHOD

- Group O RBCs were washed three times in saline, and packed after the final wash.
- The treated samples contained enzyme (GTA and/or GTB) plus substrate (UDP-GalNAc and/or UDP-Gal) while controls contained no substrate or enzyme.
- The enzyme was dissolved in 1 x Mops buffer (50 mM Mops buffer [pH 7], 20 mM MnCl2 [pH 7], 1 mg/mL BSA).
- The ingredients were added in the following manner: add water, buffer, NaCl2 and substrate, then mix well, then add the cells followed by the enzyme to start the reaction. The final concentrations in the reaction mixture were: 50 mM Mops buffer (50 mM Mops buffer [pH 7], 20 mM MnCl2 [pH 7], 1 mg/mL BSA), 150 mM NaCl, 60 μM substrate (UDP-GalNAc or UDP-Gal), 0.03 mU/μL enzyme (GTA or GTB) and MilliQ water in a total volume of 39.65 μL. This mixture was used to modify 26.35 μL washed packed RBCs.
- For the controls 1 x Mops buffer was substituted for the enzyme and MilliQ water was substituted for the substrate.
- The reaction mixture was incubated in a 37°C waterbath for 2 hours and 40 min.
- After this time the RBCs were washed three times in normal saline, and then suspended in saline/cell preservative for serology testing.

Method Variations

Variation 1. Different concentrations of activated sugar donors.
- As for METHOD but with the exception that the substrates (UDP-GalNAc or UDP-Gal) were used at concentrations of 60, 12, 6, 3, 1.5, 0.6, 0.3, 0.15, 0.1, 0.08 and 0.06 μM.

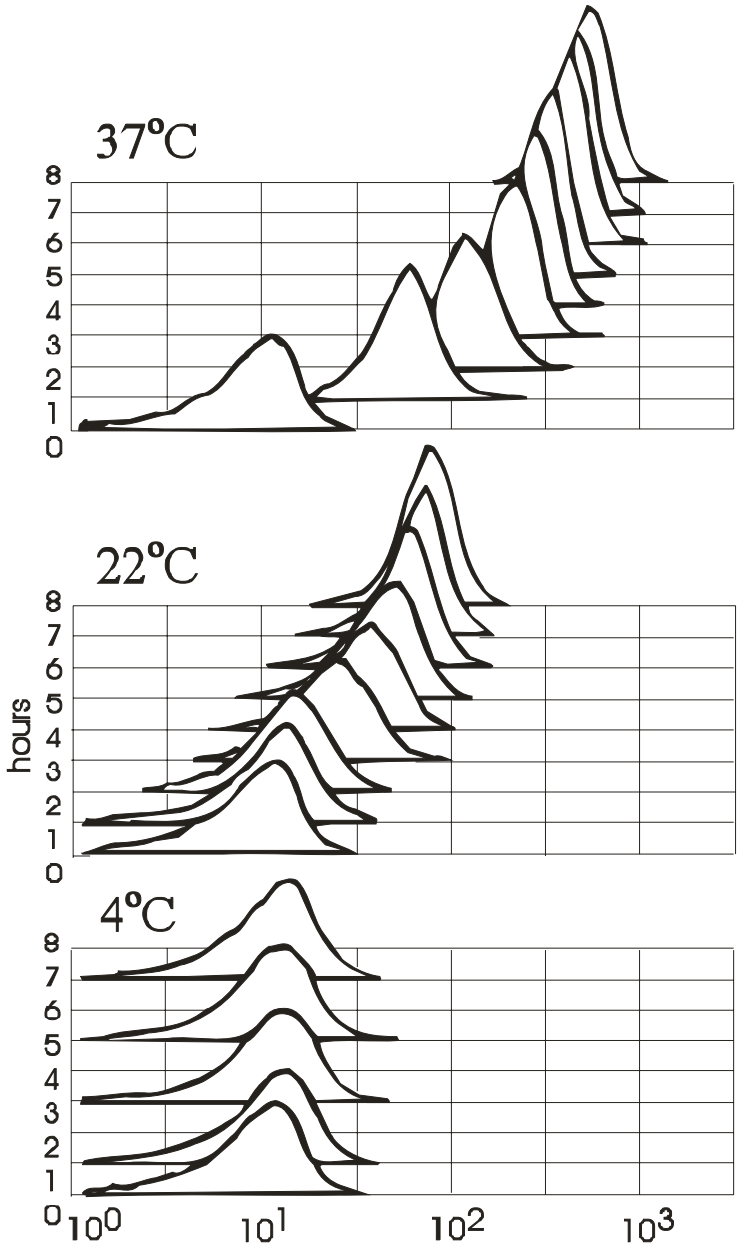
Variation 2. Different concentrations of enzyme.
- As for METHOD but with the exception that the enzymes (GTA and/or GTB) were used at concentrations of 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47, 0.24, 0.12, 0.06, 0.03 and 0.02 mU/mL.

Variation 3. GTA/GTB cells.
- As for METHOD but with the exception that the cells were first incubated with GTA (at 15, 10 or 7.5 mU/mL) and UDP-GalNAc, washed, and then incubated with GTB (at 0.075, 0.05 and 0.038 mU/mL) and UDP-Gal.

APPENDICES

Appendix 1

Flow Cytometry of Glycolipid Insertion



In vitro transformation of human Le(a-b-) red cells with natural Le^b-6 glycolipid over time at three transformation temperatures, 37°C (top), 22°C (middle) and 4°C (bottom). Natural Le^b-6 glycolipid was dissolved in Le(b-) plasma and used to transform RBCs at a final glycolipid concentration of 2 mg/mL and a final cell suspension of 10%. Reactivity was determined by FACS analysis using a Gamma anti-Le^b. Serological detection level occurs around 102 molecules.

Henry S, (1996) Unpublished data.

Appendix 2

Optimal Ratio of RBCs:glycolipids

Tube serology of the effect of changing the ratio of packed RBCs to glycolipid transformation media. Different volumes of group O RBCs were transformed with Nat A or Nat B at a concentration of 9.6 mg/mL and tested against anti-A or anti-B. Cells were tested against Bioclone anti-A and CSL anti-B.

RBC:glycolipid ratio	Anti-A	Anti-B
1:1	4+	4+
2:1	4+	4+
3:1	4+	4+
4:1	2+	3+
5:1	2+	2+
6:1	2+	2+

From: Chen JI, (2001) Creating artificial weak blood group A and B antigen phenotyping erythrocytes using KODE™ technology. MAppSc Thesis, Auckland University of Technology.

Appendix 3

Dilution of Glycolipids in Transformation

Tube serology of rat cells transformed with natural Le^b-6 glycolipid. Cells were used at a 10% suspension made up of 1 part packed cells and 9 parts rat plasma. Cells were transformed at 37°C. Cells were removed at timed intervals for agglutination testing against Gamma anti-Le^b.

Le^b (μg/mL)	Incubation (hours)								
	0.5	1	2	4	8	12	24	36	48
24	2+	3+	4+	4+	4+	4+	4+	4+	2+
12	1+	2+	3+	4+	4+	4+	4+	4+	1+
6	0	1+	2+	2+	3+	3+	3+	3+	0
3	0	0	1+	1+	2+	2+	2+	2+	0
1.5	0	0	0	0	1+	1+	0	0	0
0.8	0	0	0	0	0	0	0	0	0
0.4	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0

Tube serology of cells rat transformed with natural Le^b-6 glycolipid. Cells were used at a 25% suspension made up of 1 part packed cells, 4 parts saline and 5 parts rat plasma. Cells were transformed at 37°C. Cells were removed at timed intervals for agglutination testing against Gamma anti-Le^b.

Le^b (μg/mL)	Incubation (hours)								
	0.5	1	2	4	8	12	24	36	48
24	2+	3+	4+	4+	4+	4+	4+	4+	3+
12	1+	2+	4+	4+	4+	4+	4+	4+	2+
6	0	1+	3+	3+	3+	3+	3+	3+	1+
3	0	0	2+	2+	2+	3+	4+	3+	0
1.5	0	0	1+	2+	2+	2+	2+	2+	0
0.8	0	0	0	0	1+	1+	1+	0	0
0.4	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0

Tube serology of rat cells transformed with natural Le^b-6 glycolipid. Cells were used at a 50% suspension made up of 1 part packed cells and 1 part rat plasma. Cells were transformed at 37°C. Cells were removed at timed intervals for agglutination testing against anti-Le^b.

Le^b (μg/mL)	Incubation (hours)								
	0.5	1	2	4	8	12	24	36	48
24	2+	3+	4+	4+	4+	4+	4+	4+	3+
12	2+	2+	4+	4+	4+	4+	4+	4+	2+
6	1+	2+	3+	3+	4+	4+	4+	3+	2+
3	0	1+	2+	2+	3+	3+	4+	2+	1+
1.5	0	0	1+	1+	2+	2+	2+	1+	0
0.8	0	0	0	0	1+	1+	1+	0	0
0.4	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0

Henry S, (1996) Unpublished data.

Appendix 4

CSL Securacell® Manufacturing Protocol

Preparation of Transformation Solutions

SAMPLES, REAGENTS AND EQUIPMENT

Samples
- Synthetic A glycolipid powder (Lectinity, Russia)
- Synthetic B glycolipid powder (Lectinity, Russia)

Reagents
- Celpresol (CSL, Australia)

Equipment
- Containers (appropriate size for the amount of solution to be made)
- Sterile containers (appropriate size for the amount of solution to be made)
- Peristaltic pump and tubing
- 0.2 μm sterile filter (Millex GV, Invitrogen)

METHOD

Synthetic A solution (1 mg/mL)
- In a container, dissolve the synthetic A glycolipid sample in 60% of the amount of RT cell preservative solution required to make a solution with a concentration of 1 mg/mL.
- Using a peristaltic pump, filter the solution through a 0.2 μm sterile filter into a sterile container.
- Follow the solution through the pump with RT cell preservative solution to make up to 1 mg/mL.

Synthetic B solution (5 mg/mL)
- As for the synthetic A solution, except the final concentration should be 5 mg/mL.

Transformation

SAMPLES, CELLS, REAGENTS AND EQUIPMENT

Samples
- Synthetic A glycolipid in cell preservative solution (1 mg/mL)
- Synthetic B glycolipid in cell preservative solution (5 mg/mL)

Cells
- Group O blood

Reagents
- Working strength PBS
- Cepresol (CSL, Australia)

Equipment
- Micropipettes (1 – 10 μL, 10 – 100 μL)
- Centrifuge (Immufuge II, American Dade, USA)
- Eppendorf microcentrifuge tubes, 1.5 mL (3445, Raylab)
- Waterbath (Julabo F18)
- Centrifuge (Sorvall MC-12V, Du Pont, USA)

METHOD

Weak A Cells.
- Wash the group O blood three times in cell preservative solution by centrifugation in an immufuge.
- Measure the packed cell volume.
- At 1:30 pm, add the required amount of synthetic A glycolipid solution and cell preservative to have the final mix at the desired concentration at 50% PCV. An appropriate concentration range for the final solution is 0.02 mg/mL to 0.012 mg/mL. Use the following equation:

  To work out how much of the 1 mg/mL A solution (x) to add to every 1 mL of cell suspension (Note: initial PCV must be greater than 50%):
  x (mL) = PCV x final desired concentration
  (Note: PCV is expressed as a ratio of 1)
  Then, to work out what volume of cell preservative needs to be added to make up the volume (z):
  z (mL) = PCV – (1 – PCV) – x

Multiply x and z by the number of millilitres of cell suspension you want to transform to get the final volumes to add.

- Incubate at 25°C for 3 hours.
- At 4:30 pm, move the transformation batch to a fridge or cool room (2-8°C) for overnight (18 hour) incubation.
- At 10:30 am the next day, wash the cells three times in cell preservative solution by centrifugation in an immufuge.
- Suspend to 25% in cell preservative solution.

Weak B Cells.

- Wash the group O blood three times in cell preservative solution by centrifugation in an immufuge.
- Measure the packed cell volume.
- At 1:30 pm, add the required amount of synthetic B glycolipid solution and cell preservative to have the final mix at the desired concentration at 50% PCV. An appropriate concentration range for the final solution is 0.2 mg/mL to 0.18 mg/mL. Use the following equation:

  To work out how much of the 5 mg/mL B solution (y) to add to every 1 mL of cell suspension:

  $$y \text{ (mL)} = \frac{\text{PCV x final desired concentration}}{5}$$

  (Note: PCV is expressed as a ratio of 1)

  Then, to work out what volume of cell preservative needs to be added to make up the volume (z):

  z (mL) = PCV – (1 – PCV) – y
- Multiply y and z by the number of millilitres of cell suspension you want to transform to get the final volumes to add.
- Incubate at 25°C for 3 hours.
- At 4:30 pm, move the transformation batch to a fridge or cool room (2-8°C) for overnight (18 hour) incubation.
- At 10:30 am the next day, wash the cells three times in cell preservative solution by centrifugation in an immufuge.
- Suspend to 25% in cell preservative solution.

Weak AB Cells (A_wB_w).

- Wash the group O blood three times in cell preservative solution by centrifugation in an immufuge.
- Measure the packed cell volume.
- At 1:30 pm, add the required amounts of synthetic A glycolipid solution, synthetic B glycolipid solution and cell preservative to have the final mix at the desired concentration at 50% PCV. An appropriate concentration range for the final solution is 0.02 mg/mL to 0.012 mg/mL for synthetic A and 0.2 mg/mL to 0.18 mg/mL for synthetic B. Use the following equation:

  To work out how much of the 1 mg/mL A solution (x) to add to every 1 mL of cell suspension (Note: initial PCV must be greater than 50%):

  x (mL) = PCV x final desired concentration

  (Note: PCV is expressed as a ratio of 1)

  To work out how much of the 5 mg/mL B solution (y) to add to every 1 mL of cell suspension:

  $$y \text{ (mL)} = \frac{\text{PCV x final desired concentration}}{5}$$

  Then, to work out what volume of cell preservative needs to be added to make up the volume (z):

  z (mL) = PCV – (1 – PCV) – x – y

  Multiply x, y and z by the number of millilitres of cell suspension you want to transform to get the final volumes to add.
- Incubate at 25°C for 3 hours.
- At 4:30 pm, move the transformation batch to a fridge or cool room (2-8°C) for overnight (18 hour) incubation.
- At 10:30 am the next day, wash the cells three times in cell preservative solution by centrifugation in an immufuge.
- Suspend to 25% in cell preservative solution.

Appendix 5

CSL Evaluation of KODE™ Technology

Validation testing of KODE™ CAE control cells was performed by CSL comparing Securacell (batch 1 – produced using the method outlined in Appendix 4 with Syn A at 0.02 mg/mL and Syn B at 0.18 mg/mL) and natural weak ABO subgroup cells. The KODE™ cells are AwBw (number 15) in the tables below. Note that these results highlight performance issues with some of the platforms tested.

Validation results across all methods against anti-A.

Cell	Type	Testing platform							
		Tube	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
15	A_wB_w	2+	2+	2+	2+	0	0	0	0
3	A_1B	4+	4+	4+	4+	4+	4+	4+	4+
5	A_2B	3+	4+		3+	3+	1+	2+	3+
11	A_3	4+	4+		3+	3+	1+	1+	3+
12	A_3B	3+	3+		3+	2+	w+	w+	2+
7	A_x	1+	2+		2+	0	0	0	0
2	A_x	w+	2+		2+	0	0	0	0
4	A_x	w+	2+		2+	0	0	0	0
6	A_x	w+	2+		2+	0	0	0	0
8	A_x	w+	2+		2+	0	0	0	0
10	A_x	w+	2+		2+	0	0	0	0
1	A_x	w+	2+		1+	0	0	0	0
9	A_x	0	1+		1+	0	0	0	0
13	B_3	0	0	0	0	0	0	0	0
14	B_3	0	0	0	0	0	0	0	0

Validation results across all methods against anti-B.

Cell	Type	Testing platform							
		Tube	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
15	A_wB_w	3+	1+	1+	1+	0	0	0	0
5	A_2B	4+	4+		4+	4+	3+	3+	4+
3	A_1B	4+	4+	4+	4+	4+	3+	3+	4+
12	A_3B	4+	4+		4+	4+	4+	4+	4+
13	B_3	2+	3+	2+	2+	2+	2+	2+	2+
14	B_3	2+	2+	2+	2+	2+	1+	1+	2+
1	A_x	0	0		0	0	0	0	0
2	A_x	0	0		0	0	0	0	0
4	A_x	0	0		0	0	0	0	0
6	A_x	0	0		0	0	0	0	0
7	A_x	0	0		0	0	0	0	0
8	A_x	0	0		0	0	0	0	0
9	A_x	0	0		0	0	0	0	0
10	A_x	0	0		0	0	0	0	0
11	A_3	0	0		0	0	0	0	0

Validation results across all methods against anti-AB.

Cell	Type	Testing platform							
		Tube	CAT 1	CAT 2	CAT 3	CAT 4	CAT 5	CAT 6	CAT 7
15	A_wB_w	3+							3+
3	A_1B	4+							4+
5	A_2B	4+							4+
11	A_3	4+							4+
12	A_3B	4+							4+
2	A_x	4+							3+
6	A_x	4+							3+
7	A_x	4+							3+
9	A_x	4+							2+
4	A_x	3+							3+
8	A_x	3+							3+
10	A_x	3+							3+
1	A_x	3+							2+
13	B_3	2+							2+
14	B_3	2+							2+

Reagents and cards used in validation testing.

Method	Reagent		
Tube	Epiclone		
Ref	Card manufacturer and type	Batch	Expiry
CAT 1	OCD BioVue ABD/Rev	ABR528A	16.06.05
CAT 2	OCD BioVue ABD/Rev	ABR521A	06.05.06
CAT 3	OCD BioVue ABD/ABD	ACC255A	24.05.05
CAT 4	Diamed ID-MTS	50092.10.02	Apr-05
CAT 5	Diamed ID-MTS Donor typing	51051.05.04	Mar-05
CAT 6	Diamed ID-MTS Recipient typing	50053.07.02	Apr-05
CAT 7	Diamed ID-MTS Cord typing	50961.08.03	Jul-05

Appendix 6

Royal College of Pathologists of Australasia Educational Exercise

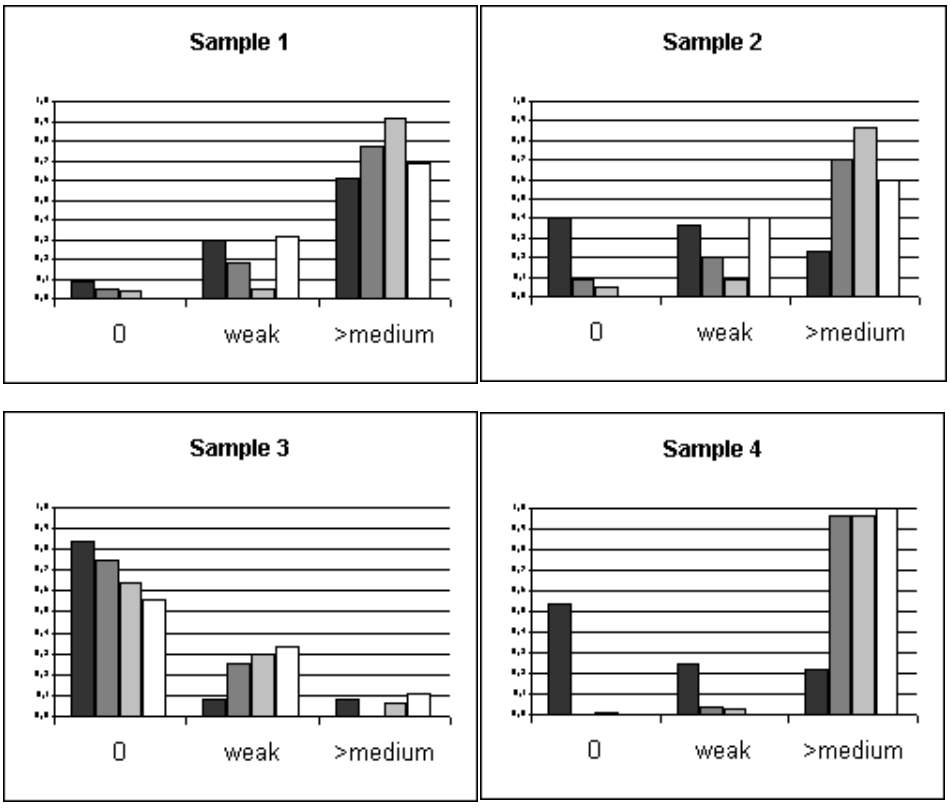
The Royal College of Pathologists of Australasia sent out KODE™ cells as an educational exercise. The following clinical history and exercise were set:

> A family of four donates blood for the first time. Unusual reactions are noted and samples are referred to your laboratory. You are asked to perform an ABO (Forward and Reverse) and Rh(D) Grouping on all 4 samples using all grouping techniques available in your laboratory. If your laboratory uses an ABD checking procedure, please also test these samples.

The kit contained KODE™ A cells transformed to three different strengths (medium - 0.08 mg/mL, weak - 0.05 mg/mL and very weak - 0.03 mg/mL) and a KODE™ B cell (weak - 0.6 mg/mL). The kit was sent out to 310 participating laboratories in the Asia-Pacific region. Results indicated that significant mistyping of these cells had occurred. The medium KODE™ A cells were typed as group O in just under 4% of tests across all methods – but rose to almost 6% for column alone. The weak KODE™ A cell was typed as O in almost 9% of tests across all methods, and 19% in column testing, while the very weak KODE™ A cell typed as O in 23% of tests across all methods and 40% of column tests. Of note, microplate also performed poorly with this very weak KODE™ A cell – 41%. The weak KODE™ B cell was typed as O in almost 8% if tests across all methods and almost 20% in column. Interestingly, this cell was reported as group A in just over 1% of column tests.

This table presents the number of standardised serological reactions by strength (medium - 2+, 3+ or 4+, weak – w or 1+) against the testing platform used. Highlighting indicates the expected serological score.

			Serological Score					
	Technology		nil	weak		>medium		
		n	0	(+)	1+	2+	3+	4+
Sample 1 Anti-A	Column	256	23	32	44	76	56	25
	Tile	92	4	7	10	24	34	13
	Tube	345	13	3	13	51	96	169
	Microplate	19	0	1	5	2	3	8
Sample 2 Anti-A	Column	257	103	49	46	31	22	6
	Tile	92	8	6	13	21	33	11
	Tube	345	15	8	23	67	110	122
	Microplate	20	0	1	7	2	2	8
Sample 3 Anti-A	Column	258	217	9	11	20	1	0
	Tile	91	68	14	9	0	0	0
	Tube	345	220	59	45	18	1	2
	Microplate	18	10	4	2	2	0	0
Sample 4 Anti-B	Column	254	137	26	36	43	8	4
	Tile	91	0	1	2	9	39	40
	Tube	342	4	2	7	16	76	237
	Microplate	20	0	0	0	3	4	13



RCPA Field trial results by testing method. Samples 1, 2 and 4 should have been scored in the medium range (2+, 3+ or 4+), and sample 3 in the weak range (w, 1+). As can be seen for sample 2, a significant proportion of CAT users typed it as group O. This was also seen with sample 4. Also of concern, sample 3 was returned as a group O result by the majority of laboratories, when it should have been scored as a w or 1+ group A.

World's first blood grouping sensitivity control: created with KODE™ technology

Lissa Gilliver and Steve Henry

AUT biotechnology research institute

OBJECTIVE

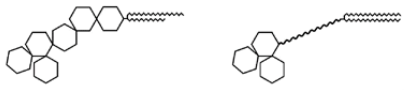
To engineer the surface of red blood cells with synthetic glycolipids to create ABO blood grouping sensitivity controls.

INTRODUCTION

The ABO blood group system is clinically the most important blood group system in man. The amount of blood group antigen on red cells varies markedly between individuals. Failure to correctly determine ABO blood type may have fatal transfusion consequences. A quality control system is an important requirement in all laboratory analytical testing. Despite the importance of accurate blood typing, no natural cell is available or suitable as an analytical sensitivity control. In order to meet this market need we designed a synthetic molecule which could express AB blood group antigens on the outside of group O red cells in a controllable manner.

SCIENCE

For many years it has been known that the natural glycolipid molecule was capable of inserting into cell membranes. Using the basic format of the natural glycolipid, we set out to construct a "smart" synthetic analogue which captured the desired features of the natural molecule. The figure below demonstrates the general relationship between the structures of the natural and synthetic molecules.



Natural ABO glycolipid Synthetic ABO glycolipid

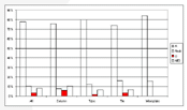
The synthetic glycolipid molecule was developed and refined over the course of extensive research. Variation of the conditions under which insertion occurred allowed expression of specific levels of blood group A and/or B antigen on cells. Subsequent validation experiments established total control of the insertion phenomenon, and in combination with long term stability trials, confirmed that no loss in cell viability or functionality occurred.

FEATURES

The novel synthetic molecule incorporated several new features. Principally, the molecule was directly soluble in water and could be successfully inserted into cell membranes over a very large temperature range - 4°C to 37°C. Being synthetic in nature, an unlimited amount of standardised product could be created, allowing for the future manufacture of control cells in sufficient quantities to meet forecast market demand. Another novel feature was the broad spectrum specifity of the glycotope, which was conferred by attachment to a flexible non-carbohydrate linker. This allows for a generic presentation of antigen, which enables reactivity with all active antibodies.

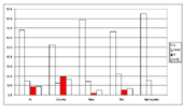
CLINICAL FIELD TRIAL

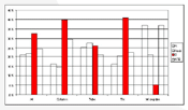
Synthetic A and B KODE™ molecules were used to transform O red cells to produce red cells expressing low levels of blood group A and B antigens. These transformed cells were formulated to three different strengths of A and one of B (figures below). These samples were used in a Royal College of Pathologists of Australasia Quality Assurance Educational Exercise and tested by 310 laboratories in the Asia-Pacific region. The results, obtained from a wide range of reagents in tile, tube, column agglutination and on a range of automated instruments, are shown in the figures below.
Red bars indicate a failure to obtain the correct result.



Medium A - failure to obtain the correct result (red bar) may cause a fatal transfusion reaction

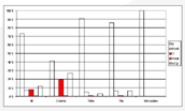
Weak A - failure to obtain the correct result (red bar) may cause a serious transfusion reaction





Very weak A - failure to obtain the correct result (red bar) may cause a mild transfusion reaction

Weak B - failure to obtain the correct result (red bar) may cause a serious transfusion reaction



All of the reactions shown above should have been detected by adequately performing systems. Thus the clinical trial clearly established the suitability of the product and the need for these ABO blood grouping sensitivity controls.

INTELLECTUAL PROPERTY

The intellectual property relating to the KODE™ controls, two competitive technologies and the structures of the synthetic molecules have been protected by patent applications.

COMMERCIALISATION

Following the successful field trial and further validation trials, a product known as Securacell® was prepared and launched on the Australian market by CSL Biosciences. Already the control has detected deficiencies within blood accreditation systems.

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Appendix 7

Agglutination Scoring

Insertion of blood group molecules (natural or synthetic) is assessed visually by agglutination with the relevant IgM antibodies. The methods used include manual tube serology and the column method – specifically Diamed ID Micro Typing System. Both methods can accommodate various antisera – the Diamed system allows this in its 'NaCl, enzyme test and cold agglutinins' card, which is not pre-loaded with antisera. The advantage of this is being able to control which antisera is used, as the antisera of different manufacturers perform differently (based on clone identity and formulation). It is important when comparing the results of different experiments that the antisera used is taken into account.

The relationship between two common agglutination scoring methods is shown below. The system used in this work is based on the 4 point score system (4+, 3+, 2+, 1+, w+, vw, 0).

Manual tube serology involves the placement of the cell suspension (3%, 1 drop) and antisera (1 drop) into a glass serology tube. The tube(s) are placed in a centrifuge and spun at 2200 rpm for 15 seconds. Scores are assigned based on the appearance of the cells in the bottom of the tube.

The column agglutination technique involves the use of a card or cassette containing microtubes pre-filled with buffered sephadex gel. The cell suspension (0.8%, 50 μL) and antisera (50 μL) are placed into a reservoir above the gel. The card is centrifuged at a pre-programmed speed and for 10 minutes. Scores are assigned according to the position and pattern of the cells in the gel.

The 4 point scoring system used in this work is shown in the left hand column, and its relationship with the 12 point scoring system is also shown.

		Tube	CAT
4+ spl		Cells strongly agglutinated as well as adhered to glass tube	
4+	12	Single firm disc	Single sharp band at top of column
3+	10	Single disc that breaks into large clumps	More diffuse band at top of column.
2+	8	Several soft clumps	Cells spread out from top to bottom of column
1+	5	Many small soft clumps	Diffuse band at base of column
w+	3	Most cells negative, but some evidence of minor agglutination	Some cells above band at base of column
vw	1	Most cells negative, but some evidence of stickiness	A few cells above band at base of column
0	0	No clumps	Single sharp band at base of column
‡		Cells unreadable – impossible to score.	

Variation of one score level is considered to be within the margin of error of this technique, and not considered significant. Significant score variances are those of more than one score level.

Haemolysis was also scored using a 4 point scale (see Table 6 in section 2.1).

APPENDIX 8a

CSL KODE™ Experimental Protocol and Results

CSL conducted extensive evaluation experiments on KODE™ technology, beginning in the second half of 2003. Abridged results of those initial experiments are presented here.
From the outset, the desired reaction strength for a commercial sensitivity control product was intended to be around w+ for Tube and Tile, and 1+ in the BioVue and DiaMed-ID MTS CAT systems.

Method

Preparation of transformation solutions
- Dilute reagent solution in working strength PBS to desired concentration(s).

Preparation of red cells
- Sample red cells from donor packs – ensure that samples are labelled with all required identification details.
- Dispense 0.5 mL of cells into 75x12mm glass tubes.
- Wash 4 times in PBS.
- Pool washed cells into 2 tubes.
- Centrifuge and remove as much supernatant as possible.

Transformation
- Label tubes.
- Add 3 parts cells to 1 part transformation solution.
- Incubate for 1 hour at 37°C.
- Wash at least 3 times with PBS.
- Wash once with cell preservative solution.
- Make up to desired concentration for testing with cell preservative solution e.g. 0.8%, 3%, 40%.

The Syn A results are presented first, followed by the Syn B results.

Results - Transformation of O cells to A cells.

Tube blood grouping results using CSL Epiclone anti-A.

Syn A (mg/mL)	Donor			
	1	2	3	4
0.09	3+	2+	2+	w+
0.08	3+	2+	1+	1+
0.07	2+	1+	1+	w+
0.06	3+	3+	1+	w+
0.05	3+	1+	1+	w+
0.04	1+	1+	w+	w+
0.03	w+	w+	0	w+
0.02	w+	w+	0	0
0.01	0	0	0	0
0.00	0	0	0	0

Comparison of Tube and CAT methods. Tube testing was performed against CSL Epiclone anti-A and anti-AB. Diamed testing was performed in DiaMed DiaClon ABD Donor Grouping cards. BioVue testing was carried out in Ortho BioVue Grouping cassettes.

Syn A (mg/mL)	Tube		Diamed	BioVue	
	Anti-A	Anti-AB	Anti-A	Anti-A	Anti-A
0.10	4+	3+	3+	4+	4+
0.09	4+	3+	3+	4+	3+
0.08	3+	2+	3+	3+	3+
0.07	2+	1+	2+	3+	3+
0.06	w+	w+	2+	2+	2+
0.05	w+	vw	2+	2+	2+
0.04	w+	vw	1+	2+	2+
0.03	w+	vw	w+	2+	1+
0.02	w+	0	0	2+	1+
0.01	0	0	0	1+	vw
0.00	0	0	0	0	0

Tile blood grouping results against CSL Epiclone anti-A and anti-AB.

Syn A (mg/mL)	Anti-A			Anti-AB		
	Agglutination time (sec)	Reaction @ 2 minutes	Reaction @ 5 minutes	Agglutination time (sec)	Reaction @ 2 minutes	Reaction @ 5 minutes
0.1	16	1+	2+	35	w+	1+
0.09	11	1+	2+	35	w+	1+
0.08	12	1+	2+	35	w+	1+
0.07	9	1+	2+	45	w+	1+
0.06	16	1+	1+	45	w+	1+
0.05	19	w+	w+	73	w+	w+
0.04	49	w+	w+	75	w+	w+
0.03	45	w+	w+	-	vw	w+
0.02	-	0	0	-	0	0
0.01	-	0	0	-	0	0
0.00	-	0	0	-	0	0

All O blood group red cell samples were successfully transformed to A red cell samples at Syn A concentrations greater than 0.03 mg/mL. The Syn A concentrations producing the desired reaction strengths in each of the serological methodologies are set out in the table below.

Technique	Agglutination	Syn A concentration mg/mL
Tube	w+	0.03-0.05
Tile	w+	0.03-0.05
BioVue	1+	0.03
DiaMed-ID MTS	1+	0.02-0.04

These results showed that a formulation of 0.04 mg/mL was appropriate for transformation with Syn A for weak reactions, and further experiments were conducted around this concentration level. The results are presented in the tables below.

Comparison of Tube and CAT methods. Tube testing was performed against CSL Epiclone anti-A and anti-AB. Diamed testing was performed in DiaMed DiaClon ABD Donor Grouping cards. BioVue testing was carried out in Ortho BioVue Grouping cassettes.

Syn A (mg/mL)	Tube		Diamed	BioVue
	Anti-A	Anti-AB	Anti-A	Anti-A
0.06	4+	1+	1+	3+
0.05	3+	1+	1+	2+
0.04	2+	1+	0	2+

Tile blood grouping results against CSL Epiclone anti-A and anti-AB.

Syn A (mg/mL)	Anti-A		Anti-AB	
	2 minutes	3 minutes	2 minutes	3 minutes
0.04	1+	1+	w+	w+
0.05	1+	2+	w+	w+
0.06	1+	2+	w+	w+

Results - Transformation of O cells to B cells.

Comparison of Tube and CAT methods. Tube testing was performed against CSL Epiclone anti-A and anti-AB. BioVue testing was carried out in Ortho BioVue Grouping cassettes.

Syn B (mg/mL)	Tube		BioVue	
	Anti-B	Anti-AB	Anti-B	Anti-AB
1.0	3+	2+	2+	4+
0.9	2+	2+	1+	4+
0.8	2+	2+	1+	4+
0.7	3+	3+	1+	4+
0.6	4+	2+	w+	4+
0.5	3+	3+	w+	4+
0.4	3+	3+	w+	4+
0.3	4+	3+	w+	4+
0.2	1+	1+	0	4+
0.1	1+	1+	0	4+
0.0	0	0	0	4+

All O blood group red cell samples were successfully transformed to B red cell samples at Syn B concentrations greater than 0.3 mg/mL. The Syn B concentrations producing the desired reaction strengths in each of the serological methodologies are set out in the table below.

Technique	Reaction Grade Strength	Concentration mg/mL
Tube	w+	0.1
BioVue	1+	0.3-0.6

A Syn B concentration of 0.4 mg/mL is recommended for use to achieve weak reactions.

APPENDIX 8b

CSL Evaluation of Different KODE™ Transformation Methods

CSL conducted experiments to evaluate the different temperature transformation methods (2-8°C, 21°C and 37°C) and the effect of simultaneous transformation with both Syn A and Syn B. Abridged results of those experiments are presented here.

Method

The transformation method was essentially the same as that set out in Appendix 8a with the exception of the incubation times and temperatures:

- 37°C for 1 hour
- 21°C for 5 hours
- 2-8°C for 40 hours

and concentrations:

- Aw – Syn A 0.08 mg/mL
- Bw - Syn B 0.6 mg/mL
- AwBw - Syn A 0.08 mg/mL + Syn B 0.6 mg/mL

Results

Comparison of different temperature transformation methods by CAT. Diamed testing was performed in DiaMed DiaClon ABO/D+ reverse grouping for patients, and BioVue testing was carried out in Ortho BioVue Anti-A/Anti-B/Anti-D/Control/Reverse diluent cassettes.

Cell	Transformation	DiaMed		BioVue	
		Anti-A	Anti-B	Anti-A	Anti-B
Aw	37°C/1 hour	2+	0	2+	0
Bw	37°C/1 hour	0	vw	0	1+
AwBw	37°C/1 hour	w+	0	2+	1+
Aw	21°C/5 hours	2+	0	3+	0
Bw	21°C/5 hours	0	vw-w+	0	2+
AwBw	21°C/5 hours	1+	vw	3+	2+
Aw	2-8°C/40 hours	2+	0	3+	0
Bw	2-8°C/40 hours	0	1+	0	2+
AwBw	2-8°C/40 hours	2+	1+	3+	2+

Conclusion

Transformation to Aw, Bw and AwBw can be achieved at 37°C for 1 hour and 21°C for 5 hours. Reactions for Bw with the DiaMed-IDMTS are negative or just detectable at these temperatures. The results for the transformation at 2-8°C for a 40 hour transformation period demonstrated desirable reactions for both the DiaMed-IDMTS and the Ortho BioVue system.

APPENDIX 8c

CSL KODE™ Stability Trial Report

CSL carried out experiments to evaluate the stability of KODE™ transformed RBCs. Abridged results of those experiments are presented here.

Method

The experiment was carried out over 16 weeks on three batches of independently transformed cells.

Preparation of transformation solutions

- The Syn A and Syn B stock solutions were filtered through a Millex-GV 0.22 μm filter unit under aseptic conditions.

Preparation of red cells

- The cells were washed using the continuous flow centrifuge method in Celpresol.
- The cells were suspended to >50% PCV in Celpresol, and the PCV measured.

Transformation

- The amount of Syn A and Syn B solutions to add was calculated using the following formula (concentrations used were Syn A 0.02 mg/mL and Syn B 0.18 mg/mL):

  To work out how much of the 1 mg/mL A solution (x) to add to every 1 mL of cell suspension (Note: initial PCV must be greater than 50%):
  x (mL) = PCV x final desired concentration
  (Note: PCV is expressed as a ratio of 1)

  To work out how much of the 5 mg/mL B solution (y) to add to every 1 mL of cell suspension:

  $$y \text{ (mL)} = \frac{\text{PCV x final desired concentration}}{5}$$

  Then, to work out what volume of cell preservative needs to be added to make up the volume (z):
  z (mL) = PCV − (1 − PCV) − x − y
  Multiply x, y and z by the number of millilitres of cell suspension you want to transform to get the final volumes to add.

- The cells were incubated for 3 hours at 20°C followed by an 18 hour incubation at 2-8°C under constant gentle agitation.
- The transformed cells were washed using the continuous flow centrifuge method in Celpresol.
- The PCV was measured and adjusted to 50% with Celpresol.

The results are presented in the tables below.

Tube serology stability trial results of three batches of KODE™ AwBw cells (Syn A 0.02 mg/mL and Syn B 0.18 mg/mL) over 14 weeks. Testing was performed using CSL Epiclone anti-A, anti-B and anti-AB.

Test	Time from manufacture		Serology								
			Batch 1			Batch 2			Batch 3		
	Day	Week	A	B	AB	A	B	AB	A	B	AB
1	1		2+	4+	4+						
2	4		1+	4+	3+	2+	4+	3+	2+	3+	3+
3	7	1	2+	3+	3+	3+	4+	3+	2+	3+	3+
4	14	2	2+	3+	3+	2+	3+	3+	2+	3+	3+
5	21	3	2+	3+	4+	3+	4+	3+	2+	4+	3+
6	28	4	2+	3+	3+	1+	3+	4+	2+	3+	3+
7	35	5	1+	3+	3+	3+	3+	3+	2+	3+	3+
8	49	6	1+	3+	3+	1+	2+	3+	2+	3+	3+
9	56	7	1+	3+	3+	1+	3+	3+	1+	3+	3+
10	63	8	1+	3+	3+	1+	2+	3+	1+	2+	3+
11	70	9	w+	3+	3+	1+	3+	4+	1+	3+	3+
12	77	10	1+	4+	4+	1+	4+	4+	1+	3+	3+
13	84	11	w+	4+	4+	w+	4+	3+	w+	3+	3+
14	91	12	w+	3+	3+	1+	3+	3+	w+	3+	3+
15	98	13	w+	3+	3+	1+	3+	3+	w+	3+	3+
16	105	14	2+	3+	3+	2+	3+	3+	1+	3+	3+

BioVue CAT serology stability trial results of three batches of KODE™ AwBw cells (Syn A 0.02 mg/mL and Syn B 0.18 mg/mL) over 14 weeks. Testing was performed in Ortho BioVue Anti-A/Anti-B/Anti-D/Control/Reverse diluent cassettes.

Test	Time from manufacture		Serology					
			Batch 1		Batch 2		Batch 3	
	Day	Week	A	B	A	B	A	B
1	1		1+	w+				
2	4		2+	1+	2+	1+	2+	1+
3	7	1	2+	1+	2+	1+	2+	1+
4	14	2	2+	1+	2+	1+	2+	1+
5	21	3	2+	w+	2+	w+	2+	w+
6	28	4	2+	1+	2+	1+	2+	1+
7	35	5	2+	1+	2+	1+	2+	1+
8	49	6	2+	1+	2+	1+	2+	1+
9	56	7	2+	1+	2+	1+	2+	1+
10	63	8	2+	1+	2+	1+	2+	1+
11	70	9	2+	2+	2+	2+	2+	2+
12	77	10	2+	1+	2+	1+	2+	1+
13	84	11	2+	1+	2+	1+	2+	1+
14	91	12	2+	1+	2+	1+	2+	1+
15	98	13	2+	1+	2+	1+	2+	1+
16	105	14	2+	1+	2+	1+	2+	1+

Note: Cells were between 10 and 23 days old (from the date of bleed) when they were transformed.

The CSL Stability Trial Report stated:

- There was no significant weakening of reaction strength (up to testing event 16).
- The product was stable using tube and column agglutination technology blood grouping and antibody screening immunohaematology techniques.
- No false positive or false negative reactions were obtained for the product (up to test event 10).
- The recommended date of expiry is greater than 8 weeks (56 days) from the date of bleed.
- Haemolysis was observed as a change in colour of the simulated plasma diluent, and haemolysis was consistent with the natural ageing of reagent red blood cells.
- Haemolysis was observed at a point beyond the recommended shelf-life of the product (of 8 weeks from the date of bleed).
- This observation had no impact on the quality of the product for a period of at least 56 days from the date of bleed.

Appendix 9

Agglutination Testing Methods

Cell agglutination was assessed using the Diamed-ID Micro Typing System (protocol 3.2) and conventional tube serology (protocol 3.1). The Diamed cards used were NaCl, Enzyme test and cold agglutinin cards, which are not pre-loaded with any antisera or other reagents. This allowed the selection of specific antisera in column as well as tube methodologies.

A trial was carried out between tube serology and the Diamed system to establish the comparability of the two systems. Cells were transformed with Nat A glycolipid at 25°C for 4 hours as set out in protocol 2.1 variation 3. Seraclone and Albaclone anti-A reagents were both used to gauge the equivalency of their performance in the two systems. The results are shown below.

Agglutination results comparing tube serology (protocol 3.1) with the Diamed column system (protocol 3.2). Cells were transformed at 25°C for 4 hours (protocol 2.1, v 3) and tested against two different anti-A sera – Albaclone and Seraclone.

Platform	Antisera	Nat A glycolipid (mg/mL)				
		10	5	2	1	0
Tube	Alba	3+	2+	0	0	0
	Sera	3+	2+	0	0	0
Diamed	Alba	2+	2+	0	0	0
	Sera	3+	2+	1+	w+	0

In this experiment, the Diamed column platform proved to be more sensitive to the weaker reactions, as seen with cells transformed with 1 and 2 mg/mL concentrations of Nat A, than manual serology. The Albaclone and Seraclone anti-A reagents are formulated differently, and are thus not expected to perform identically. However, the fact that Seraclone anti-A failed to detect positivity at natural glycolipid concentrations of less than 5 mg/mL in manual serology may be due to operator interpretation. Weaker reactions are notoriously difficult to accurately score, and the difference between 1+ and 0 can be difficult to discern in tubes – column technology is not subject to the level of interpretative variation inherent in the tube method. Overall, there was no significant difference between the Diamed system (protocol 3.2) and manual serology (protocol 3.1).

Appendix 10

Antisera panels

A antisera

	Manufacturer	Origin	Batch	Expiry
I	Alba-clone™, Diagnostics Scotland, Edinburgh, UK	Murine	132051	29.05.93
II	Alba-clone™, Diagnostics Scotland, Edinburgh, UK	Murine	Z0010680	01.02.03
III	BioClone™, Ortho Diagnostic Systems, New Jersey	Murine	BAA196A	03.11.99
IV	BioClone™, Ortho Diagnostic Systems, New Jersey	Murine	01102	16.05.02 (manuf)
V	Biolab, Auckland, New Zealand	Human	8606	Apr 87
VI	Biolab, Auckland, New Zealand	Human	8636	Oct 87
VII	Biolab, New Zealand	Human	8668	Feb 88
VIII	Epiclone™, CSL, Melbourne, Australia	Murine	20901	Nov 93
IX	Epiclone™, CSL, Melbourne, Australia	Murine	23302	Dec 98
X	Epiclone™, CSL, Melbourne, Australia	Murine	25202	27.09.02
XI	Epiclone™, CSL, Melbourne, Australia	Murine	026128301	18.02.07
XII	Gamma-clone®, Gamma Biologicals, Houston, US	Murine	AM30-1	19.07.93
XIII	Immucor, Georgia, US	Murine	1A6137A	22.04.93
XIV	Lorne Laboratories, UK	Murine	60086D	Aug 01
XV	Lorne Laboratories, UK	Murine	60090F	Sep 01
XVI	Novaclone, Dominion	Murine	NA00503	08.05.93
XVII	Organon Teknika BV, Boxtel NL	Murine	112Z15A	19.12.93
XVIII	Seraclone®, Biotest AG, Frankfurt	Murine	121087	Aug 89
XIX	Seraclone®, Biotest AG, Frankfurt	Murine	132051	29.05.93
XX	Seraclone®, Biotest AG, Frankfurt	Murine	1310401	12.04.03

B antisera

	Manufacturer	Origin	Batch	Expiry
I	Alba-clone™, Diagnostics Scotland, Edinburgh, UK	Murine	Z0110600	27.04.03
II	BioClone™, Ortho Diagnostic Systems, New Jersey	Murine	BBB589A	21.11.99
III	BioClone™, Ortho Diagnostic Systems, New Jersey	Murine	01103	16.05.02 (manuf)
IV	Biolab, Auckland, New Zealand	Human	8625	Jul 87
V	Biolab, Auckland, New Zealand	Human	8661	Dec 87
VI	Epiclone™, CSL, Melbourne, Australia	Murine	20801	Nov 93
VII	Epiclone™, CSL, Melbourne, Australia	Murine	23203	Dec 98
VIII	Epiclone™, CSL, Melbourne, Australia	Murine	23801	May 00
IX	Epiclone™, CSL, Melbourne, Australia	Murine	25302	22.10.02
X	Epiclone™, CSL, Melbourne, Australia	Murine	026628301	11.02.07
XI	Gamma-clone®, Gamma Biologicals, Houston, US	Murine	BM31-1	07.11.93
XII	Immucor, Georgia, US	Murine	IE6240-1	22.05.93
XIII	Lorne Laboratories Ltd, UK	Murine	61003A	Aug 01
XIV	Meditech Biotechnology, Taiwan	Murine	110199	Nov 01
XV	Novaclone™, Dominion Biologicals, Novascotia, Canada	Murine	00404	24.04.93
XVI	Organon Teknika BV, Boxtel NL	Murine	112X19B	16.12.93
XVII	Seraclone® Biotest AG, Frankfurt	Murine	114061	12.06.93

AB antisera

	Manufacturer	Origin	Batch	Expiry
I	Behringwerke AG, Marburg	Murine	001704A	30.06.88
II	BGA, Pennysylvania	Human	004	27.03.86
III	BioClone™, Ortho Diagnostic Systems, New Jersey	Murine		28.08.00 (manuf)
IV	Biolab, Auckland, New Zealand	Human	8604	Mar 87
V	Biolab, Auckland, New Zealand	Human	8667	Jan 88
VI	Salmond Smith Biolab, Auckland, New Zealand	Murine	MC9206	Jan 94
VII	Epiclone™, CSL, Melbourne, Australia	Murine	20902	Oct 93
VIII	Epiclone™, CSL, Melbourne, Australia	Murine	22702	Jul 98
IX	Epiclone™, CSL, Melbourne, Australia	Murine	026725701	21.10.06
X	Gamma-clone®, Gamma Biologicals, Houston, US	Murine	ABM29-1	06.09.93
XI	Immucor, Georgia, US	Murine	IC6405	15.04.93
XII	Novaclone™, Dominion Biologicals, Novascotia, Canada	Murine	00501	24.07.93
XIII	Novaclone™, Dominion Biologicals, Novascotia, Canada	Murine	01702	07.06.98
XIV	Seraclone®, Biotest AG, Frankfurt	Murine	121077	Oct 88
XV	Seraclone®, Biotest AG, Frankfurt	Murine	111089	31.08.91
XVI	Seraclone®, Biotest AG, Frankfurt	Murine	131021	28.02.93

Product Information

Securacell®

Quality Control System

Background

Immunohaematology Testing

Immunohaematology is significantly different from other pathology disciplines, whilst it involves a range of medical diagnostic tests, the prime role is to provide a therapeutic product – blood, for transfusion. Failure of any immunohaematology test including blood grouping, antibody screening or compatibility testing may lead to infusion of incompatible blood and subsequent serious morbidity or mortality. The mainstay of immunohaematology testing is the ABO RhD blood group procedure, which ensures that blood transfusion recipients do not have ABO antibodies that will react against donor red cells. These ABO antibodies are the most significant in transfusion medicine, as they appear very soon after birth, without immune exposure to heterologous red cells. Antibody screening is also performed to ensure blood transfusion recipients do not have antibodies against antigens other than ABO, as a result of exposure to red cells from previous blood transfusion(s) or pregnancy. These antibodies are termed "unexpected anti-erythrocytic alloantibodies" or more commonly just "alloantibodies". Pretransfusion testing is designed to detect clinically relevant antibodies that are present in prospective blood recipients that may react with infused donor red cells and cause adverse reactions, ranging from reduced lifespan of the infused red cells to sudden patient death as a result of intravascular haemolysis. Antenatal screening is designed to detect clinically relevant antibodies present in the maternal circulation that may cross the placenta and cause foetal morbidity or mortality.

Immunohaematology testing procedures almost invariably involve techniques using antibodies in serum, plasma or reagents and human red cells from patients, donors or reagents. The quality and freshness of the red cells used, and in particular, the cell surface antigen quality is critical to test performance. Useful control products containing fresh red cells have been difficult to obtain, because of the labile nature of red cells and the fact that technology is not available to preserve red cells satisfactorily for immunohaematology testing. Those that have been available have been relatively costly and are provided in very small volumes. More importantly, immunohaematology control products that simulate patient's samples have been unavailable. It has been common practice to use previously tested patient samples as controls. These "home made" controls are not characterised by an accredited external laboratory and will not detect and control all errors, especially if that error is repeated. Regulatory bodies are also becoming less amenable to such "home-made" tests and controls. Simulated patient sample controls are best termed "Process Controls" as they are able to undergo the complete sample testing process and can therefore, control all aspects of the testing process, whether manual techniques or automated instruments are used. Process Controls should be an integral part of laboratory Quality Control (QC) and are routinely used in other pathology disciplines. In many cases, they are mandatory.

Quality Control Principles

Quality Control (QC) may be defined as the management of the testing process. It includes controlling the testing environment to ensure that tests are reproducible, accurate and sensitive. QC of reagents should be a regular part of laboratory testing and should encompass tests for avidity, specificity and sensitivity. Reagent QC should challenge the limitations of the reagent and always include both Positive and Negative reactions. Controls should be tested in parallel with patient samples and control products that are used should be designed to test the inherent variables present throughout the entire testing procedure. While reagent QC is vital, it will not control and monitor the other aspects of the testing process, such as red cell dilution, result reading and interpretation. Hazard monitoring and reporting programmes such as the United Kingdom Serious Hazards of Transfusion (SHOT) scheme show that errors in transfusion laboratories are commonly due to transcription error. For QC to be effective, it should be capable of detecting, monitoring and correcting all types of possible errors and be an integral part of a managed Quality Assurance (QA) system. The 4th Edition of the Guidelines for Pretransfusion Testing - Australian & New Zealand Society of Blood Transfusion state: "All laboratories that perform pretransfusion testing shall participate in a Quality Assurance (QA) programme that is designed to ensure accurate and consistent performance in all aspects of the laboratory's work practices, from the collection of samples to the final release of blood for transfusion".

Product Design

CSL Securacell® is designed to be a stable control product with a wide range of applications in a single, cost effective product. The final design simulates a patient sample in both format and behaviour, and includes a significant volume of both red cells and

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simulated plasma to allow a large number of tests to be performed from each Sample in the kit. Approximately 400 blood groups and antibody screens can be performed with each Securacell® kit. The Samples are provided in commonly used blood collection tubes that can be processed in exactly the same way as patient samples with all routinely used methods and technologies, and are also suitable for use with all immunohaematology instrumentation. Securacell® can be tested in parallel with routine patient samples to control and ensure the process is capable of determining the correct ABO RhD Blood Group and is safely detecting, identifying and quantifying clinically relevant, unexpected antibodies. The inclusion of the four common blood groups and both RhD Positive and Negative Sample types allows complete and reliable control of blood grouping. Simulated plasma containing single and mixed alloantibodies and one plasma Sample that is screened as antibody negative, are included to control antibody screening and identification. Alloantibodies are chosen with a specificity that should be detected by systems designed to detect clinically relevant antibodies and are provided at a concentration that will give mid-strength reactions with commonly used techniques. One Securacell® Sample also contains a calibrated IgG anti-D antibody that provides a Secondary Standard and an Antibody Titre Precision Control.

KODE™ CAE (Kiwi Oz Designer Erythrocytes – Controlled Antigen Expression) is a novel and patented (patent pending) system that allows ABO antigens to be expressed on human erythrocytes in precisely controlled amounts. KODE™ CAE technology was developed by Kiwi Ingenuity Limited and the Biotechnology Research Institute at Auckland University of Technology. This technology has been further developed and commercialised by CSL IH Group. The practical application of KODE™ CAE is to convert Group O human red cells to A_{weak} and/or B_{weak} or in the case of CSL Securacell®, an $A_{weak}B_{weak}$ cell. These $A_{weak}B_{weak}$ cells are used to provide the world's first precise and reliable ABO blood grouping sensitivity control. KODE™ CAE technology is used to create cells with precisely controlled expressions of A and B antigens to mimic the reactions of A_x and B_x cells but with precisely controlled antigen expression, that is reproducible. This does not show the wide variation in natural weak ABO red cells. KODE™ CAE allows production of a consistent Group $A_{weak}B_{weak}$ sample that can be engineered to give weak reactions with a blood grouping system that is working correctly. The KODE™ CAE $A_{weak}B_{weak}$ red cells will therefore be able to detect any loss in analytical sensitivity by displaying a reduction or total loss of appropriate reactions.

Product Description and Specifications

Securacell® is a Quality Control System for immunohaematology laboratories. Securacell® is supplied as a kit with four 6mL collection tubes that are commonly used for routine patient sample collection. Fresh donor units are obtained from the Australian Red Cross Blood Service (ARCBS). The cells have been washed to remove plasma and any contaminating antibodies. Each of the four tubes contains 1.5mL of pooled human red cells and 4.5mL of simulated plasma. The Samples are labelled 1, 2, 3 and 4.The red cells are pooled and formulated to react during blood group testing as Blood Groups O, A, B and AB. The simulated plasma is formulated to include the appropriate ABO antibodies to match the blood group. This simulated plasma will provide reaction strengths similar to those of a patient sample. The red cells are phenotyped as antigen negative for any antibody present in the simulated plasma in the same tube. Alloantibodies such as anti-D, anti-K, anti-Fy^a, anti-c and anti-Jk^a are incorporated in the simulated plasma for antibody screening and identification. Securacell® contains Bovine Albumin in Celpresol™, an isotonic citrate buffer solution containing a purine, a nucleoside and glucose, Chloramphenicol and Neomycin Sulphate are included as antibacterial agents and Thiomersal is added as a preservative. Securacell® is suitable for all routine immunohaematology methods, including: Tile, Tube, manual and automated Microplate and Column Agglutination Technology (CAT) Systems.

Securacell® Sample 1 is always Group O RhD Negative, has appropriate reverse (serum) group antibodies and includes an anti-D titre control/standard. The other three Securacell® Samples are in random order. One of the three random Samples will always be a Group A Sample and one will be a Group B Sample , both containing the appropriate reverse group antibodies and will also include alloantibodies. The final random sample is an $A_{weak}B_{weak}$ red cell expressing controlled low levels of A and B antigen.

Securacell® is shipped on a four week shipping cycle and it has sufficient shelf life to allow overlapping expiries. While Securacell® is most often ordered on a standing order basis, CSL has an ordering policy for Securacell®, that also allows the placement of one off orders. As long as orders are placed within 2 to 3 weeks of the next scheduled shipment, order fulfilment will occur on the next scheduled shipment. Standing order cancellation will occur within 5 weeks of order cancellation advice.

Product	Catalogue No	Pack Size	Delivery Frequency
Securacell®	08710201	4 x 6mL	4 weekly

Securacell® Performance

Each Securacell® kit includes a sealed 'Results Sheet' with batch specific blood group results, $A_{weak}B_{weak}$ cell scores, antibody identity and indicative reaction scores derived from the 'CSL Reference Laboratory'. Blood grouping reactions and blood group interpretation are provided for Tube technology. $A_{weak}B_{weak}$ cell reaction scores are also provided for Tile, Tube, BioVue™ and DiaMed ID-MTS™ techniques. Antibody screening reactions and antibody identity are provided for Tube RAM, BioVue™ and DiaMed ID-MTS™ techniques. The anti-D Antibody Titre Control result using the 'NICE Method', is reported with the standard value measured in IU/mL, by the 'Technicon BG8 Method'. The reagents and CAT test identities, batch numbers and expiries used to test each Securacell® batch are also reported on the Result Sheet.

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Securacell® Field Trial and Validation

CSL conducted a stability trial by manufacturing three pre-production batches of Securacell® and fully performance testing the products during their shelf life. Securacell® has excellent stability and has been assigned a shelf life of 9 weeks from the earliest donor pack date of bleed. This allows a 4 weekly shipping frequency, with a significant safety margin of expiry overlap. The field trial was conducted in July 2002, in 18 Australian immunohaematology laboratories over a period of 14 weeks. Testing was also replicated in three separate CSL immunohaematology laboratories. This field trial was designed to validate the Securacell® product on all immunohaematology testing technologies and automated systems. The product design and format were examined to ensure they met user requirements and for any improvements that could be incorporated. The reaction strengths of the blood groups and antibodies were varied during the trial to "fine tune" these reactions to best meet customer needs and to ensure that Securacell® performs well on all testing platforms. A total of 1,360 samples were manufactured, 8,800 blood groups and1,979 antibody screens were performed and 60,000 data points were generated. An interesting outcome was that 30 clerical errors were identified during this trial. Some trial participants commented; "It works well and will greatly enhance the safety of our lab. When can we have it?" and "I am pleased this product is being put together. We do replicate testing and QC on our Reagent Red Blood Cells to make sure they detect weak antibodies, however having to rely on in-house antibodies is not the best option. I look forward to the formal release of Securacell". The Securacell® technology was further tested in conjunction with the RCPA in the form of the QAP Educational Exercise in March 2004. This exercise comprised a special Securacell® formulation with three A_{weak} cells and one B_{weak} cell being provided to all participants. This exercise provided interesting data, which has the capacity to be used to improve the quality of blood grouping in Australia.

Further validation work was performed to ensure the KODE™ CAE $A_{weak}B_{weak}$ cells performed satisfactorily in all technologies and that there were no clonal specificity issues. This testing compared reaction scores obtained for KODE™ cells using commonly available monoclonal reagents in tube methods. Weak A and B donor cells were also compared with KODE™ A_{weak} and B_{weak} cells in available CAT cards. This study found that KODE™ CAE red cells perform in exactly the same manner as donor red cells. All commonly used clones, reagents and CAT systems were confirmed to be capable of detecting KODE™ CAE red cells.

Examples of the KODE™ Specificity Data

Tube Testing Scores

KODE™ Cells	A_{weak}	B_{weak}
CSL Epiclone™ Anti-A	5	0
CSL Epiclone™ Anti-B	0	8
CSL Epiclone™ Anti-AB	5	5
Gammaclone™ Anti-A	5	0
Gammaclone™ Anti-B	0	5
Gammaclone™ Anti-AB	10	12
ORTHO Bioclone™ Anti-A	3	0

CAT Testing Scores

	Cell ID	Card/Cassette ID	Anti-A	Anti-B
Donor Cells	A_x	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	3	0
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	3	0
		BioVue™ Anti-A/Anti-B/Anti-D	5	0
	B_3	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	0	8
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	0	8
		BioVue™ Anti-A/Anti-B/Anti-D	0	8
	A_1B_3	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	12	10
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	12	10
		BioVue™ Anti-A/Anti-B/Anti-D	12	12
	A_2	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	12	0
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	12	0
		BioVue™ Anti-A/Anti-B/Anti-D	12	0

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CAT Testing Scores

	Cell ID	Card/Cassette ID	Anti-A	Anti-B
KODE™ CAE Cells	A_w	ID-MTS™ DiaClon Confirmation for donors ABD (VI+)	5	0
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	8	0
		Diaclon™ Confirmation for patients (VI-)	5	0
		BioVue™ Anti-A/Anti-B/Anti-D/Control/Reverse	10	0
	B_w	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	0	8
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	0	5
		Diaclon™ Confirmation for patients (Vi-)	0	5
		BioVue™ Anti-A/Anti-B/Anti-D/Control/Reverse	0	12
	A_wB_w	ID-MTS™ DiaClon ABD Confirmation for donors (VI+)	5	3
		ID-MTS™ DiaClon ABO/D+ Reverse grouping	8	5
		Diaclon Confirmation for patients (Vi-)	3	3
		BioVue™ Anti-A/Anti-B/Anti-D/Control/Reverse	10	8

Recommended Applications

CSL Securacell® is a multipurpose Quality Control System for immunohaematology laboratories. It is designed to control, standardise and validate all routine immunohaematology tests. These tests include blood grouping, antibody screening, antibody identification and antibody titres. This product should be processed as a routine patient sample. The integrity of the testing process is proven by the test results matching those of the published results on the accompanying 'Results Sheet'.

CSL Securacell® has seven primary applications:

1. ABO RhD Blood Grouping Control
2. Antibody Screening and Identification Control
3. Antibody Titre Standard
4. ABO Analytical Sensitivity Control
5. Routine Process Control for Manual and Automated Systems
6. Replicate Testing Control
7. Competency Assessment Control

1. ABO RhD Blood Grouping Control

ABO blood grouping is the most important immunohaematology testing procedure as mistakes can result in serious morbidity or fatalities. These blood group errors may occur during collection, testing and infusion and also includes technical errors, test failure, reagent or equipment failure and patient misidentification. Failure to recognise anomalous groups and subgroups may also cause grouping errors. It is worth pointing out that despite these errors and risks, blood grouping testing procedures are often taken for granted. This may in some part be due to the high performance of modern monoclonal reagents and technologies that almost always provide a reaction that is either completely negative or positive. Which leads to users expecting a simple yes or no answer. As a result, a tendency to take less care during blood group testing and in grading and interpreting reactions may occur. The high performance and potency of monoclonal reagents brings risks if the testing process is not controlled properly. Reagents are so potent that a very small amount of cross-contamination may cause incorrect grouping reactions. This contamination may occur due to errors such as incorrect dropper replacement in vials, incorrect dispensing procedures and misuse or reuse of pipettes. Incorrect blood group reactions in CAT systems have been observed due to column cross contamination as a result of aerosols (from monoclonal reagents) being produced during the removal of the foil seal and on pipette tips used for cell dispensing. In these events, probably as little as a few microlitres of a monoclonal reagent contaminating an adjacent well can cause incorrect grouping results. For this reason grouping controls that encompass all four of the common ABO blood groups should be used. Testing with only Group O and AB cells would not detect an Anti-A reagent contaminated with Anti-B and vice versa. All four groups are also required to reliably detect poor performing, incorrect or misplaced reagents. Controls should also be selected that have both an RhD Negative and a relatively weak expression of RhD (such as R_0r or R_1r) cell. While ABO RhD blood grouping may seem reliable and simple, reagent failure and technical errors can occur. These errors should be detected by checking procedures such as serum groups and repeat groups. More importantly, transcription errors can also be made and these errors are more insidious and dangerous, as they may not be picked up by checking procedures and will not be detected by reagent controls. For this reason, it is vital that process controls are used that mimic and are treated like patient samples. Routine use of process controls can greatly assist in the detection of transcription errors. It is also worth examining the entire testing process to reduce the number of potential error steps.

2. Antibody Screening and Identification Control

Antibody Screening controls should be chosen to challenge the test sensitivity and specificity. Controls should generally be stable, weak, clinically relevant antibodies. They should result in weak to mid-strength reactions, so that a loss or reduction in antibody detection scores indicates test performance failure. Any such failure

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should be investigated by examining the quality of additives and Reagent Red Blood Cells (RRBCs), inactivation of Anti-Human Globulin (AHG) reagent, poor temperature control, washing, timing and centrifugation. A negative control should also be used. Anti-D is often used because it is the most common clinically relevant alloantibody encountered. This is generally not considered to be sufficient by itself, as the RhD antigen is very robust and may not detect RRBC degradation. Anti-c, anti-e and anti-Fy^a are commonly used for this reason, but other antibody specificities may also be chosen. Multiple antibodies may be included and used as antibody investigation controls, to control antibody identification panel quality and specificity.

3. Antibody Titre Control

Antibody titre techniques are notoriously variable and imprecise. This is due to variations in techniques, volume, diluents, variations of antigen expression on indicator cells used and reaction grading. As antibody titre results are used to make significant clinical decisions, the precision and reliability of the technique is vital. Antibody titre controls have generally been unavailable, although a standard serum called "The British Standard for Anti-D (Rho) Antibodies, Human (code No 73/515)" is available. CSL has recently made an antibody titre control available as part of the Securacell® Quality Control System. This control serum will give a defined titre as stated in the accompanying 'Results Sheet' when tested by the 'NICE Method', using an end point as the last tube giving a score of 5 on the 12 scale (or 1 on the 4 scale). The concentration of this antibody is also stated in IU/mL and may be used as both an anti-D Secondary Standard and an Antibody Titre Precision Control. CSL strongly recommends the use of the standardised 'NICE Method' for antibody titre techniques, although the control can still be used with alternate techniques.

4. ABO Analytical Sensitivity Control

Modern, high quality Anti-A and Anti-B reagents have a high potency. In some cases, they can lose up to 95% of their analytical sensitivity due to damage or degradation, but still give normal strength reactions with A and B cells. The most commonly available red cells with a weak expression of the A antigen are A_2B cells. However, Group A_2B cells simply express too much A antigen for use as a weak A control and do not express low levels of B antigen and, as such, are unsuitable for use as a weak B control cell. Use of these types of control cells may give apparently satisfactory results, even when reagents may be significantly damaged or degraded and are actually incapable of detecting weak ABO subgroups, such as A_3 and B_3 cells. For this reason, A_x cells are generally agreed to be the gold standard for analytical sensitivity of Anti-A blood grouping reagents. The antigen expression of natural A_x cells, however, varies by up to 500% and are difficult for most laboratories to reliably obtain. While detection of natural A_x cells is not vital clinically in blood recipients, it is clinically important to detect A_x cells in blood donors as these cells are capable of causing haemolytic transfusion reactions. A_x blood donors should always be typed and labelled as Group A. It is also vital that the analytical sensitivity of any blood grouping procedure can be measured and monitored. In practice, this is difficult due to the unavailability of natural A_x cells and the inherent variation in the expression of the A antigens on A_x cells. Analytical sensitivity control of ABO grouping may be achieved by the use of the $A_{weak}B_{weak}$ cell available in the CSL Securacell® Quality Control System.

5. Routine Process Control for Manual and Automated Systems

Immunohaematology laboratories have for many years used reagent controls to prove reagent performance. More recently control kits have become available, but they are often provided in small volumes, have red cells provided as 3% suspensions and are unable to be processed like a real patient sample. They are more correctly termed 'competency assessment' or 'technical checking products'. Some manufacturers also provide controls for automated systems, but often these controls are barcoded, so the instrument's identify the sample specifically as a control, and then process the control differently to a routine sample. This approach defeats the purpose of the control, as it is not a blind test and does not undergo all the testing steps, it cannot, therefore, control the entire testing process. The concept of a 'Process Control' is to use a control that exactly simulates a patient sample and is processed through the entire testing process. This approach increases the control utility as it can detect errors during the entire testing process; from sample handling and dilution through to result recording or result data transmission by electronic interfaces. The concept of a process control applies to both manual and automated techniques, regardless of the testing technology used. As Securacell® is formatted and performs like a patient sample, it may be used as a routine process control by testing one or more Securacell® Samples with batches of patient samples. It should be processed in exactly the same manner as the patient samples including all preparation, dilution and testing steps. Internal patient sample barcodes may be used with Securacell®. CSL Securacell® has been designed as an immunohaematology process control to be used to measure the accuracy, precision and sensitivity of laboratory tests in clinical practice, so that automated systems are unaware that the control is not a routine patient sample.

6. Replicate Testing Control

Replicate testing is a term used to describe a programme where staff are required to perform immunohaematology testing (such as a blood group, antibody screen and antibody identification) on unknown, identical samples. Individual staff results can be examined and compared to ensure blood groups are correct and to monitor and correct variations in grouping and antibody screening technique and in reaction grading.

7. Competency Assessment Control

Competency assessment is a term used to describe a programme where staff are required to test samples and resolve any problems. Quite often this involves samples with complex alloantibodies and/or a simulated crossmatch. This process is designed to determine an individual staff member's ability to investigate and cope with complex clinical cases, and to make correct transfusion decisions. It also provides experience, skills and confidence in performing complex serological investigations.

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Advantages of CSL Securacell®

- Fresh – Manufactured using fresh red cells.
- Quality Assured – Proven performance, field trialed and validated product.
- Stable – Confirmed stability until expiry, consistently high level of sensitivity.
- Reliable – Developed and manufactured to CSL's strictest quality standards.
- CSL's History – An Australian publicly owned company, employing Australians to develop and produce our reagents.
- Innovative – Novel product design, utilising unique KODE™ CAE technology to create controlled antigen expression.
- Superior Performance – Seven primary purposes, a complete quality control system.

Precautions

The material from which this product was derived was found to be non-reactive for specified markers for HIV 1 and 2, Hepatitis B and C, HTLV and Syphilis by currently approved methods. However, no known method can assure that products derived from human blood will not transmit infectious agents. However, good laboratory practice requires safe handling procedures. For *in vitro* diagnostic use only. Securacell® contains Neomycin Sulphate and Chloramphenicol as antibacterial agents and Thiomersal as a preservative. Users should take appropriate precautions when handling and discarding this product.

Incorrect reactions may occur due to:

1. Failure to comply with the recommended procedures.
2. Variations in time and temperature of incubation, centrifuge speeds and reaction reading methods.
3. Contamination of test samples, reagents or supplementary materials.
4. Use of aged or expired samples or reagents.
5. Incorrect red cell suspension strengths.

Storage and Deterioration

Store at 2° to 8°C (Refrigerate. Do Not Freeze). Securacell® should be treated like other Reagent Red Blood Cell (RRBC) products, it should be refrigerated when not in use and should not be subjected to prolonged periods of high temperature. Take appropriate precautions to maintain sterility. Do not use if red cell suspensions show signs of gross haemolysis or the simulated plasma component is turbid. Do not use after the expiration date.

General Sample Handling Techniques

Securacell® is designed to be treated exactly like a patient sample. Before use, it should be left for a period of time to allow the red cells to separate or it may be centrifuged prior to use, using a method suitable for patient sample separation. If Securacell® is stored upright and handled gently, repeat centrifugation will not be required. Securacell® is not provided with barcode identification to allow the use of individual laboratory patient sample barcodes, in order that Securacell® is used exactly like a routine patient sample.

Summary

CSL Securacell® is designed as an immunohaematology process Quality Control System, to be used to measure the accuracy, precision and sensitivity of laboratory tests, in clinical practice. Securacell® can be applied to a standard internal Quality Control programme, as a routine control for manual or automated batch testing or as a training tool. Securacell® is provided with known blood group and antibody screen results. The design format of Securacell® is to allow immunohaematology laboratories to ensure improved safety of and control over pre-transfusion compatibility and antenatal testing.

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Technical Bulletin

QC in the Immunohaematology Laboratory

Principles and Methods in Quality Control in Immunohaematology (IH) Laboratories

This document is not intended to be a comprehensive quality control guide. It is generated from CSL's experiences and research. It is published to stimulate thoughtful consideration of immunohaematology QC systems and to promote discussion to improve blood transfusion safety.

Overview

Immunohaematology Testing

Immunohaematology is significantly different from other pathology disciplines, whilst it involves a range of medical diagnostic tests, the prime role is to provide a therapeutic product – blood, for transfusion. The mainstay of this testing is the ABO RhD blood group procedure that ensures that blood transfusion recipients do not have ABO antibodies that will react against donor red cells transfused. Antibody screening is performed to ensure blood transfusion recipients do not have antibodies against antigens other than ABO, as a result of exposure to red cells from previous blood transfusion(s) or pregnancy. These antibodies are termed "unexpected anti-erythrocytic alloantibodies" or more commonly "alloantibodies". Failure of any immunohaematology test, including blood grouping or antibody screening, may lead to the transfusion of incompatible blood and subsequent serious morbidity or mortality.

Regulatory Considerations

There is an increasing requirement for regulatory compliance across all disciplines in the pathology testing industry. As immunohaematology involves the safety testing of a life saving therapeutic product, it can be expected that the regulatory compliance requirements will continue to increase over time.

TGA

The Therapeutic Goods Administration (TGA) is a unit of the Australian Government's Department of Health and Ageing. The TGA carries out a range of assessment and monitoring activities to ensure therapeutic goods available in Australia are of an acceptable standard with the aim of ensuring that the Australian community has access, within a reasonable time, to therapeutic advances. The TGA regulates the activities of the Australian Red Cross Blood Service (ARCBS) and in some circumstances blood banks.

Human Blood and Tissues - Code of Good Manufacturing Practice

Blood, blood components and plasma derivatives are regulated under the Therapeutic Goods Act 1989. Under the Act 'blood' means whole blood extracted from human donors and 'blood components' means therapeutic components that have been manufactured from blood, including red cells, white cells, progenitor cells, platelets and plasma. Some blood and blood components are exempt from the TGA and these exemptions are generally considered to cover autologous and directed donations. Where storage occurs and supervision of that storage can not be guaranteed, the blood or blood components may not be exempt. Therapeutic Goods (Manufacturing Principles) Determination No 1 of 2000, determines that a manufacturer of blood or blood components must obtain a licence for the manufacture of blood, blood components and plasma. This means that institutions participating in cord blood banking are required to be TGA licensed. To meet the requirements of the Therapeutic Goods Act 1989, institutions that collect blood and tissue must meet the requirements of the Manufacturing Principles, which reference the Australian Code of Good Manufacturing Practice - Human Blood and Tissues. This code states: "The quality system should operate to ensure that all materiel, intermediate or finished product, or samples from any materiel or product relevant to product quality are tested to determine their release or rejection on the basis of their quality, and that facilities are chosen, personnel are competent, and procedures are in place, to ensure this quality". While general blood banks and immunohaematology laboratories are not currently required to comply with these regulations, the general principles of safe testing and blood issue should be understood and applied.

NPAAC

The National Pathology Accreditation Advisory Council (NPAAC) advises the Commonwealth, State and Territory Health Ministers on matters relating to the accreditation of pathology laboratories. NPAAC plays a key role in ensuring the quality of Australian pathology services and is responsible for the development and maintenance of standards and guidelines for pathology practices.

NATA

The National Association of Testing Authorities (NATA) is the Australian Government endorsed provider of accreditation for pathology laboratories and similar testing facilities. It is the peak authority in Australia for the accreditation of inspection bodies and is

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Australia's GLP compliance monitoring authority for Good Laboratory Practice (GLP). In 1986, accreditation of pathology laboratories became mandatory for the payment of Medicare benefits, and NATA was appointed by the Commonwealth as the national accreditation agency responsible for conducting assessments. Laboratories continue to be assessed to the Standards set by NPAAC, as well as, ISO/IEC 17025 - 1999 (General Requirements for the Competence of Testing and Calibration Laboratories) and further specific requirements relating to pathology laboratories.

RCPA

The Royal College of Pathologists of Australia (RCPA) is the national leading medical diagnostic organisation. It promotes the science and practice of pathology and provides the RCPA Transfusion Quality Assurance Programme (QAP). This QAP has three programs available. The major (A) survey assesses all aspects of transfusion techniques required for the provision of compatible blood six times per year. The minor (B) survey assesses blood grouping and antibody screening four times per year. A FMH (Foetomaternal Haemorrhage) survey is also available.

ANZSBT

The Australian and New Zealand Society of Blood Transfusion (ANZSBT) aims to advance knowledge in blood transfusion, transfusion medicine and to promote improved standards in the practice of blood transfusion. The ANZSBT publish a number of important guidelines in key areas of transfusion practice that are utilised and followed by most Australasian laboratories. Guideline topics include:

- Guidelines for the Administration of Blood Components.
- Blood Grouping and Antibody Screening in the Antenatal and Perinatal Setting.
- Gamma Irradiation of Blood Components.
- Laboratory Assessment of Foetomaternal Haemorrhage.
- Pretransfusion Testing.
- Autologous Blood Collection.
- Irradiated Blood Products.
- Leucocyte Depletion of Blood & Blood Components.
- Pre-operative Autologous Blood Collection.

AIMS

While the Australian Institute of Medical Scientists (AIMS) is not a regulatory body, it does publish Competency Standards for Australian medical scientists and also operates the useful Australian Professional Acknowledgment Continuing Education (APACE) scheme. APACE is a voluntary programme that recognises continuing education. It provides formal courses and a wide range of professional activities which contribute to professional education.

Principles of Quality Control and Quality Assurance

Quality Control (QC) may be defined as the management of the testing process. It includes controlling the testing environment to ensure that tests are reproducible, accurate and sensitive. QC of reagents should be a regular part of immunohaematology laboratory testing and should encompass tests for avidity, specificity and sensitivity. Reagent QC should challenge the limitations of the reagent and always include controls that give both positive and negative reactions. In most cases positive controls should be weak to demonstrate that tests meet sensitivity requirements. Controls should be tested in parallel with patient samples and control products should be designed to test the inherent variables present throughout the entire testing procedure. While reagent QC is vital, it will not control and monitor all aspects of the testing process, such as red cell dilution, result reading and interpretation. Hazard monitoring and reporting programmes, such as the United Kingdom Serious Hazards of Transfusion (SHOT) scheme show that errors in transfusion laboratories are commonly due to transcription error. For QC to be effective, it should be capable of detecting, monitoring and correcting all potential errors and be an integral part of a managed Quality Assurance (QA) system.

QA may be defined as the management, monitoring and control of the entire process of providing a service. In blood transfusion, this includes all of the processes from the initial medical decision to transfuse and the collection of the sample, through to the final outcome of transfusion. QA is a control, review and action process and should be integrated into medical and laboratory policy. QA should involve participation in an external QA programme to allow laboratory performance to be compared with other external institutions. This should complement an internal QA system that involves the use of process controls, replicate testing and testing for known antibodies. This QA system should control test sensitivity and specificity. Internal proficiency testing may also be used to monitor staff ability, consistency and to standardise the reading and scoring of tests within the laboratory. Monitoring these testing processes is essential to ensure, detect and correct process problems that may affect results. QA is particularly important in an environment where staff, methods and equipment are changing. Effective quality systems ensure change is controlled and does not impact on patient care and safety.

The 4th Edition of the Guidelines for Pretransfusion Testing - Australian and New Zealand Society of Blood Transfusion states: "All laboratories that perform pretransfusion testing shall participate in a Quality Assurance (QA) programme that is designed to ensure accurate and consistent performance in all aspects of the laboratory's work practices from collection of samples to the final release of blood for transfusion". Participants in External QA programmes can expect to test the ability of their laboratory to investigate complex clinical cases and compare their performance with other participating laboratories. While external QA programmes are a vital component of QA, they cannot be processed and used by all staff in large laboratories and are not designed to be used as daily or routine controls. They are also generally not formatted as a patient sample, thus they are not treated and processed in the same manner as routine samples.

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QA programmes should be implemented to examine and control the following aspects of laboratory operation:

- Personnel skill.
- Reagent quality.
- Analytical test performance.
- Equipment performance.
- Patient and Donor Sample compliance and quality.
- Blood and blood product management.
- Record control.
- Reliability of computer / information management system.

Internal control products are an integral part of QC and are routinely used in other pathology disciplines and in many cases, are mandatory. True process control products that simulate patient samples are standard in most laboratory disciplines, but have generally been unavailable for immunohaematology use. Previously tested patient samples have therefore been commonly used. These "home made" controls are not characterised by an accredited external laboratory and will not detect and control all errors, especially if that error is repeated. Control products used should be treated exactly like a patient sample and control all parts of the testing process, including manual techniques and automated instruments.

Quality Documentation

As immunohaematology involves the safe provision of blood, the quality system is not only relevant to operations within the laboratory but should include all the processes involved in blood transfusion. This encompasses the initial medical decision to transfuse, through the safe collection and identification of the patient sample, immunohaematology testing and the final outcome; blood transfusion.

It is not enough for blood bank laboratories to document their internal procedures only, they should also include blood collection and infusion policies and procedures. These documented procedures of the quality system are required to enable the effective implementation of the quality objectives. An overall quality system document is usually developed that defines the quality system, policy(s) and quality objectives. All blood collection, testing and infusion processes should be documented, available to all staff and most importantly, complied with at all times by all staff (whether employed directly by the laboratory or not). This may be difficult in some institutions, as enforcing procedural compliance by non-immunohaematology staff can be challenging. In these cases, the authority and support of a transfusion committee and senior management has an important role to play in managing staff education, communication and compliance.

Quality documentation should also encompass emergency responses, as these situations can be a significant source of error and potentially unsafe practice. Procedures and processes should be designed, tested and practiced, to safely cope with these situations when they arise.

For a quality system to be effective, the following operational aspects should be considered:

- Documents must be reviewed and updated as part of a continuing improvement process.
- Document access must be controlled to ensure all relevant staff have access to current and correct procedures.
- Procedures must be complied with at all times. This may sometimes be difficult and controversial with some aspects of blood transfusion, such as compliance with sample labelling requirements.
- Management should recognise and prevent pressure being applied on immunohaematology staff by other medical personnel to bypass or circumvent procedures, such as the use of insufficiently labelled specimens.
- Procedural compliance should be measured, usually by regular audits.
- Non-compliance must be detected and corrective action taken to prevent error recurrence.
- Management of the required documentation is time consuming and as such, this activity should be resourced appropriately.

Personnel Issues

Regardless of the level of automation utilised in modern laboratories, the critical aspects of laboratory safety relate to personnel. All immunohaematology staff require a clear and accurate job description and should be assigned duties appropriate to their level of education, training and experience. Management should ensure that adequate numbers of qualified, dedicated staff are employed. Resources should be adequate to enable an appropriate response to all situations, including emergencies. New staff should be provided with an orientation programme and an ongoing, supervised training programme with appropriate support materials. Written laboratory techniques and procedures must be provided to all staff and the level of understanding and compliance should be assessed on a regular basis. All staff should participate in continuing education programmes and a proficiency testing programme should evaluate and monitor technical ability and competency of all staff who perform testing. Internal quality assurance may involve the use of process controls, replicate testing, mixed cell populations and known antibodies. This QC system should also monitor the detection of weak reactions and standardise the reading and scoring of reactions.

Change Control

Quality systems and processes should be continuously improved to provide the safest possible transfusion service. This requires managing the change to systems and processes. Any change to current systems should be planned and implemented in a controlled fashion, as uncontrolled change brings significant problems with document control and staff training. The reasons for the change, changes to documents and staff communication and training should be recorded. A system must be established and maintained to identify, document, review and approve all process and protocol

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changes. The results of the review must be recorded and any changes or modifications approved by the Quality Assurance Manager, or nominee, before implementation.

Patient Sample and Donor Unit Quality Control

It can be argued that the most significant errors in immunohaematology laboratories are due to human mistakes. The majority of severe transfusion reactions reported are due to clerical, rather than technical errors. Many are due to incorrect patient identification, patient sample labelling and matching the correct donor unit to the blood recipient. Policies should be in place to ensure confirmation of patient identity at the time of sample collection and to detail the minimum acceptable requirements for sample labelling. The test request form and the sample label must carry identical patient identification information and the blood collector's signature. In most cases the labelling policy requires the patient's full name, date of birth, hospital record number, date and time of collection and signature of the collector. Rejection criteria for unacceptable specimens received by the laboratory should be advertised and rigorously applied. The blood group of donor units provided by the ARCBS is provisional and must be confirmed. Any allocated donor unit should have the intended recipient clearly identified and the label should incorporate donation number details, blood groups of the donor and recipient and date of compatibility testing.

Reagent Quality Control

Reagent performance may be affected by delays in delivery, inappropriate storage conditions, inappropriate use and bacterial or cross contamination. Reagent Quality Control (QC) should be a part of routine laboratory testing. Reagent QC should encompass tests for avidity, sensitivity, specificity, challenge the limitations of the test and provide both Positive and Negative reactions. Anti-Human Globulin (AHG) reagents should be controlled by cells weakly sensitised with an IgG antibody.

General considerations for reagent QC:

- Read and follow the manufacturer's instructions.
- Examine for abnormal appearance, ie. turbidity or haemolysis.
- Store reagents in an appropriate manner, refrigerate where indicated and keep out of direct light.
- Understand the limitations of a reagent.
- Do not use beyond the expiry date.
- Inform staff of any changes in reagent instruction.

Technique Quality Control

Techniques in use should be published in a written instruction or method manual. Methods should be examined and authorised by the laboratory manager or staff member with the appropriate level of authority. Standard Operating Procedures (SOPs) are a requirement of accreditation organisations worldwide. They should contain guidelines for all laboratory and administration procedures. Procedures should include all processes undertaken in the laboratory with techniques cross-referenced to the method manual. All staff must adhere strictly to these methods, policies and procedures. Any personal variations are unlikely to be sanctioned in legal proceedings. Manuals must be accurate, current and reflect prevailing laboratory practices. Techniques should be subject to controls that test the limitations of the procedure. The controls should be tested in parallel with patient samples and be designed to measure any variation present throughout the entire procedure. For example, the Indirect Antiglobulin Test (IAT) should be monitored by a positive control test, run in parallel with each batch of patient samples comprising a known weakly reacting antibody. The control should ideally only be detected by the technique under test.

The critical risk in immunohaematology techniques is transcription errors. Steps should be taken to minimise the potential for these kinds of errors. All tests and reagents must be labelled properly and this should be a non-negotiable rule for all laboratory staff. Reagents made or modified in-house should be labelled with identity, date of manufacture or modification, and identity of the operator. Test materials such as Tiles, Tubes and Microplates should be clearly labelled to ensure reagents added and sample identity and placement are clear to all staff.

Equipment Quality Control

Equipment and automated instruments should undergo regular maintenance programmes. Records should be kept of all routine maintenance, repairs and the results from all QC testing. Refrigerators and freezers used for storing of blood and blood products must conform to the Australian Standard (AS 3864-1997).

Equipment maintenance and QC should include:

- Pipettes/diluters – accuracy and reproducibility.
- Waterbaths/incubators – water level, temperature.
- Thermometer – calibrated against a credentialled thermometer.
- Cell washers – saline delivery volume, residual volume, replicate testing.
- Blood fridges/freezers – temperature monitoring and recording, high/low temperature alarm checks, power failure response.
- Reagent storage refrigerator – temperature monitoring and recording, high/low temperature alarm checks.
- Centrifuges – speed, timing.
- Timers – accuracy.
- Automated processing instruments – mechanical checks and calibration, fluidic carryover, data interface validation, use of process controls that simulate real samples.

Blood Product Quality Control

Many blood bank laboratories store, handle and issue blood products. Documented procedures for the handling and administration of blood and blood products should address the storage conditions for individual blood components, transport and temperature monitoring during transport of blood products, maintain the storage specification for the duration of transport and provide transport packaging that prevents spillage should any leaks occur. The thawing of frozen products should be carried out in a manner that minimises contamination risks, be at an appropriate

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temperature. The storage temperature and expiry following thawing must be controlled. Handling of blood products that have been returned unused should be controlled and appropriately recorded.

Record Management

Laboratories must maintain appropriate documentation for a period of time as recommended by NPAAC. This documentation includes:

- Reagent QC records.
- Equipment maintenance records.
- Superseded procedures, manuals and publications.
- Proficiency testing surveys.
- Temperature records of blood and blood product storage.
- Staff training records.
- Donation or batch numbers and descriptions of all blood products handled by the laboratory.
- The fate of all blood and blood components (issued, returned, expired, transfused or transferred).
- Blood recipient details.
- Error, accident, adverse events and complaints log, which includes action(s) taken.

Computer System Quality Control

Laboratory Information Management Systems (LIMS or LIS) are not infallible. They should be validated and controlled to ensure that no electronic transcription errors are made or are able to be made. All software must be validated and re-validated if any modifications are made to the software. All transactions should be logged and traced to an individual staff member. Hierarchical security levels must restrict the ability to modify results, software modules or algorithms, to authorised staff only.

Additional Quality Control Considerations

Active blood utilisation management must be employed and managed by laboratories and systems and policies are used to measure and monitor blood usage. These include a transfusion committee that is responsible for the assessment of the transfusion service. A Maximum Blood Ordering Schedule (MBOS), Crossmatch-to-Transfusion (C:T) Ratio and expired unit review, are used as an indication of the effectiveness of stock control procedures within the laboratory. For the purposes of accreditation, laboratories are expected to be far more proactive in the area of QA in terms of implementation of document control procedures and regular audits of laboratory practices.

Specific Test Procedure – QC

1. Control of ABO RhD Blood Grouping

ABO blood grouping is the most important immunohaematology testing procedure as mistakes can result in serious morbidity or fatalities. These blood group errors may occur during collection, testing and/or infusion and also includes, technical errors, test failure, reagent or equipment failure and patient misidentification. Failure to recognise anomalous groups and subgroups may also cause grouping errors. It is worth pointing out that despite these errors and risks, the blood group testing procedures are often taken for granted. This may in some part be due to the high performance of modern monoclonal reagents and technologies that almost always provide a reaction that is either completely negative or positive. As a result, this leads to users expecting a simple yes or no answer. This may lead to a tendency to take less care during blood group testing and in the grading and interpretion of reactions. The high performance and potency of monoclonal reagents brings risks if the testing process is not controlled properly. Reagents are so potent that a very small amount of cross-contamination may cause incorrect grouping reactions. This contamination may occur due to errors such as incorrect dropper replacement in vials, incorrect dispensing procedures and misuse or reuse of pipettes. Incorrect blood group reactions in Column Agglutination Technology (CAT) systems, have been observed due to column cross contamination as a result of aerosols (of the monoclonal reagents), produced during the removal of the foil seal and on pipette tips used for cell dispensing. In these events, probably as little as a few microlitres of a monoclonal reagent contaminating an adjacent well can cause an incorrect grouping results. For this reason grouping controls that encompass all four of the common ABO blood groups should be used. Testing with only Group O and AB cells would not detect an Anti-A reagent contaminated with Anti-B and vice versa. All four groups are also required to reliably detect poor performing, incorrect or misplaced reagents. Controls should also be selected that have both an RhD Negative and a relatively weak expression of RhD (such as R_0r or R_1r) cell.

While ABO RhD blood grouping may seem reliable and simple, reagent failure and technical errors can occur. These errors should be detected by checking procedures, such as reverse (serum) groups and repeat groups. More importantly, transcription errors can also be made and these errors are more insidious and dangerous as they may not be picked up by checking procedures and will not be detected by reagent controls. For this reason it is vital that process controls are used that mimic and are treated like patient samples. Routine use of process controls can greatly assist detection of transcription errors. It is also worth examining the entire testing process to reduce the number of potential error steps. Here is an example:

In the past CSL witnessed a laboratory that used the following process. The laboratory computer system was used to generate numbered blood group worklists. Patient sample tubes were then labelled by marker pen with the worklist number; ie. a large black 1, 2, 3, etc was written on the collection tube label over the patient details. Laboratory staff were using a variety of grouping techniques and a total of 8 different grouping methods using different reagents (tiles or tubes were in common use). No consistent method identifying the grouping reagents was written in the method manual. The patient blood

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group result was then written on the paper worklist and the result later entered into the computer by clerical staff. A separate antibody screening worklist was then generated and the patients were listed in a different numerical order (that staff had no control over.) The first number on the patient sample tubes was crossed out and the new number written; ie. sample 2 now becomes sample 13 and sample 13 becomes sample 23. The antibody screening result was written on the paper worklist and the result entered into the computer by another clerical staff.

Take a minute to count the number of potential transcription error steps inherent in this process. It is a recipe for disaster and even the most diligent staff member is likely to make mistakes under these conditions.

2. Control of Antibody Screening and Identification

Antibody Screening controls should be chosen to challenge the test sensitivity and specificity. Controls should generally consist of stable, weak, clinically relevant antibodies. They should result in weak to mid-strength reactions, so that a loss or reduction in antibody detection scores indicates test performance failure. Any such failure should be investigated by examining the quality of additives and Reagent Red Blood Cells (RRBCs), inactivation of Anti-Human Globulin (AHG) reagent, poor temperature control, washing, timing and centrifugation. A negative control should also be used. Anti-D is often used because it is the most common clinically relevant alloantibody encountered. This is generally not considered to be sufficient by itself, as the RhD antigen is very robust and may not detect RRBC degradation. Anti-c, anti-e and anti-Fy^a are commonly used for this reason, but other antibody specificities may also be chosen. Multiple antibodies may be included and used for antibody investigation controls, to control antibody identification panel quality and specificity.

3. Control of Antibody Titres

Antibody titre techniques are notoriously variable and imprecise. This is due to variations in techniques, volume, diluents, variations of antigen expression on indicator cells used and reaction grading. As antibody titre results are used to make significant clinical decisions, the precision and reliability of the technique is vital. Antibody titre controls have generally been unavailable although a standard serum called "The British Standard for Anti-D (Rh_o) Antibodies, Human (code No 73/515)" is available. CSL has recently made an antibody titre control available as part of Securacell® Quality Control System. This control serum will give a defined titre as stated in the accompanying 'Results Sheet' when tested by the 'NICE Method', using an end point as the last tube giving a score of 5 on the 12 scale (or 1 on the 4 scale). The concentration of this antibody is also stated in IU/mL and may be used as both an anti-D Secondary Standard and an Antibody Titre Precision Control. CSL strongly recommends the use of the standardised 'NICE Method' for antibody titre techniques, although the control can still be used with alternate techniques.

4. Control of ABO Analytical Sensitivity

Modern, high quality Anti-A and Anti-B reagents have a high potency. In some cases they can lose up to 95% of their analytical sensitivity due to damage or degradation, but still give normal strength reactions with Group A and Group B cells. The most commonly available red cells with a weak expression of the A antigen are Group A_2B cells. Group A_2B cells simply express too much A antigen for use as a weak A control and do not express low levels of B antigen and, as such, are unsuitable for use as a weak B control cell. Use of these types of control cells may give apparently satisfactory results, even when reagents may be significantly damaged or degraded and are actually incapable of detecting weak ABO subgroups, such as A_3 and B_3 cells. For this reason, A_x cells are generally agreed to be the gold standard for analytical sensitivity of Anti-A blood grouping reagents. The antigen expression of natural A_x cells, however, varies by up to 500% and are difficult for most laboratories to reliably obtain. While detection of natural A_x cells is not vital clinically in blood recipients, it is clinically important to detect A_x cells in blood donors, as these cells are capable of causing haemolytic transfusion reactions. A_x blood donors should always be typed and labelled as Group A. It is also vital that the analytical sensitivity of any blood grouping procedure can be measured and monitored. In practice, this is difficult due to the unavailability of natural A_x cells and the inherent variation in the expression of the A antigen on A_x cells. Analytical sensitivity control of ABO grouping may be achieved by the use of the $A_{weak}B_{weak}$ cell available in the CSL Securacell® Quality Control System.

5. Control of Manual and Automated Processes

Immunohaematology laboratories have for many years used reagent controls to prove reagent performance. More recently control kits have become available, but they are often provided in small volumes, have red cells provided as 3% suspensions and are unable to be processed like a real patient sample. They are more correctly termed 'competency assessment' or 'technical checking products'. Some manufacturers also provide controls for automated systems, but often these controls are barcoded so the instrument's identify the sample specifically as a control and then process the control differently to a routine sample. This approach defeats the purpose of the control, as it is not a blind test and as it does not undergo all testing steps, it cannot therefore control the entire testing process. The concept of a 'Process Control' is to use a control that exactly simulates a patient sample and is processed through the entire testing process. This approach increases the control utility, as it can detect errors during the entire testing process; from sample handling and dilution through to result recording or result data transmission by electronic interfaces. The concept of a process control applies to both manual and automated techniques,

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regardless of the testing technology used. As Securacell® is formatted and performs like a patient sample, it may be used as a routine process control by testing one or more Securacell® Samples with batches of patient samples. It should be processed in exactly the same manner as the patient samples including all preparation, dilution and testing steps. Internal patient sample barcodes may be used with Securacell®. It has been designed as an immunohaematology process control to be used to measure the accuracy, precision and sensitivity of laboratory tests in clinical practice, so that automated systems are unaware that the control is not a routine patient sample.

6. Replicate Testing Control

Replicate testing is a term used to describe a programme where staff are required to perform immunohaematology testing (such as a blood group, antibody screen and antibody identification) on unknown, identical samples. Individual staff results can be examined and compared to known results, to ensure blood groups are correct and to monitor and correct variations in grouping and antibody screening technique and in reaction grading.

7. Competency Assessment Control

Competency assessment is a term used to describe a programme where staff are required to test samples and resolve any problems. Quite often this involves samples with complex alloantibodies and/or a simulated crossmatch. This process is designed to determine an individual staff members ability to investigate and cope with complex clinical cases, and to make correct transfusion decisions. It also increases confidence and provides experience using the skills required to perform complex serological investigations.

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