PRODUCTION OF SOLUBLE RECOMBINANT COMPLEMENT RECEPTOR 1 (CR1) ANTIGENS TO DETECT OR INHIBIT ANTIBODIES TO KNOPS (KN) BLOOD GROUP SYSTEM ANTIGENS

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ABSTRACT

The purpose of this study was to produce a reagent to use in investigation of antibodies directed against the Knops blood group system antigens. A novel reagent based on sr-proteins was produced and used in a new test to inhibit these antibodies.

Current investigation of patients with alloantibodies directed against Knops blood group system antigens can be a difficult, time-consuming process and the provision of blood for transfusion of these patients can often be delayed. This is because these antibodies are hard to identify and the most commonly found anti-Knops antibodies react with most reagent or donor cells that they are tested with because the corresponding Knops antigens are found at high frequency in most populations. The presence of Knops related antibodies can mask underlying antibodies that are clinically significant.

The Knops antigens are carried on Complement Receptor 1 (CR1) located on the red blood cell membrane. Two DNA constructs encoding different parts of CR1 termed long homologous repeat (LHR) C and D were used to transfect human embryonic kidney (HEK293) cells. The cells were grown in different culture systems. Cell culture supernatant containing soluble recombinant (sr)-LHRC or sr-LHR-D was harvested and purified by affinity gel chromatography. The production and purification processes were optimised in terms of protein yield and cost.

The resulting purified sr-LHR-C and sr-LHR-D proteins were used to create a novel reagent containing both proteins. This reagent was used in a new inhibition test based on an indirect antiglobulin technique using commercial gel cards. Using the reagent all examples of previously identified Knops antibodies were inhibited. In addition once these antibodies had been inhibited, underlying antibodies were then detected and identified in some samples.

For the first time Knops specific antibodies can be detected and identified using one unique test. Any underlying clinically significant antibodies will be rapidly identified if present due to inhibition of the KN antibodies. Introduction of the inhibition test into nine NHSBT patient testing laboratories will reduce the time taken for investigation of these patients and make provision of blood for patients a safer, faster process.

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ABBREVIATIONS

AET	2-aminoethylisothiouronium
AHG	Anti-human globulin
AQP1	Aquaporin 1
BCA	Bicinchoninic acid
BITS	Bristol Institute for Transfusion Sciences
BMS	Biomedical Scientist
BSA	Bovine serum albumin
CAT	Column indirect antiglobulin test
CMV	Cytomegalovirus
CR1	Complement receptor 1
DMEM	Dulbecco's modification of Eagles medium
DTT	Dithiothreitol
ECD	Extracellular domain
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
G418	Geneticin sulphate
HAMA	Human anti-mouse antibodies
HCL	Hydrochloric acid
HDFN	Haemolytic disease of the foetus and newborn
HEK	Human embryonic kidney
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
HTLA	High titre low avidity
HTR	Haemolytic transfusion reaction

IAT	Indirect antiglobulin technique
IBGRL	International Blood Group Reference Laboratory
KNIR	KN inhibition reagent
LG	L-Glutamine
LHR	Long homologous repeat
LHR-C	Long homologous repeat C
LHR-D	Long homologous repeat D
MAIEA	Monoclonal antibody-specific immobilization of erythrocyte antigens assay
MALDI-TOF	Matrix-assisted laser desorption / ionization – Time of Flight
MEL	Mouse erythroleukaemic
MWCO	Molecular weight cut off
Neo	Aminoglycoside phosphotransferase II gene
NHSBT	National Health Service Blood and Transplant
OPD	O-phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PBSTM	PBST with milk
PPT LS	Preprotrypsin leader sequence
PS	Penicillin and streptomycin
PVDF	Polyvinylidene difluoride
R-protein	Recombinant protein
RBC	Red blood cell
RCI	Red Cell Immunohaematology
RCR	Red Cell Reference

RT	Room temperature, 18º to 25ºC
SCR	Short complement regulator
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
sr	Soluble recombinant
sr-LHR-C	Soluble recombinant LHR-C
sr-LHR-D	Soluble recombinant LHR-D
Sr-protein	Soluble recombinant protein
TBS	Tris buffered saline

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1.0 INTRODUCTION

1.1 Blood group antigens

Blood group antigens are inherited markers found on the external surface of red blood cells. Currently, over 300 blood group antigens are recognised and they may be located on proteins, glycoproteins or glycolipid structures that protrude from the red cell membrane (Daniels and Reid, 2010). These structures have various functions that have now been mostly elucidated. For instance, the Colton blood group system is carried on the water channel aquaporin 1 (AQP1) (Murakata *et al.*, 2000). The glycoprotein carrying Kell antigens is an active endopeptidase enzyme that processes big endothelin-3 to endothelin-3, a potent vaso-constrictor (Turner *et al.*, 2001) and Knops antigens featured in this study are carried on complement receptor 1 (CR1) a complement receptor protein that binds immune complexes (Moulds, 2010). Nomenclature of the antigens and their corresponding antibodies has been inconsistent. Many antigens have been named after the first person found to have the antigen or more often the antibody e.g. Knops (abbreviated to Kn, first two letters) and Duffy (abbreviated to Fy, last two letters) or some have been named from part of a name like Kell (Kelleher), or even from initials like JMH, from John Milton Hagen. In 1980 an International Society of Blood Transfusion (ISBT) Working Party on Terminology for Red Cell Surface Antigens was set up to devise a uniform naming system that should be eye and machine readable (Garratty *et al.*, 2000). Since 1980 the Working Party has published guidance on nomenclature and in 2004 the terminology was described and tabulated (Daniels *et al.*, 2004). With machine readable i.e. numeric notation, a blood group antigen system is identified by a three-digit number e.g. ABO is 001, and then each ABO antigen is also given a three-

digit number so that the A antigen is 001 and the B antigen is 002. These are put together to form six digit numbers for each antigen, 001001 for the A antigen or 001002 for the B antigen. Leading zeros may be dropped so that the A antigen becomes 1.1 and the B antigen 1.2. However, more readily accessible symbols have also been agreed so that ABO is retained as a symbol and for example Duffy becomes FY, Kell becomes KEL and Knops becomes KN.

All antigens now fall into one of four classifications: systems, collections, low-incidence antigens (700 series) or high-incidence antigens (901 series). The majority of antigens belong to one of 33 systems (ISBT, 2014; Table 1.1). Each system is genetically discrete from the other systems but they have been grouped together because they all contain one or more antigens controlled at a single gene locus or by two or more closely linked homologous genes. In addition the gene encoding the antigen must have been identified and sequenced and the chromosomal location must be known.

Table 1.1 The 33 blood group systems

System number	System name	System symbol	Gene name	Chromosomal location	CD numbers
001	ABO	ABO	*ABO*	9q34.2	*
002	MNS	MNS	*GYPA, GYPB, GYPE*	4q31.21	CD235
003	P	P1		22q11.2-qter	*
004	Rh	RH	*RHD, RHCE*	1p36.11	CD240
005	Lutheran	LU	*LU*	19q13.32	CD239
006	Kell	KEL	*KEL*	7q34	CD238
007	Lewis	LE	*Fut3*	19p13.3	*
008	Duffy	FY	*FY*	1q23.2	CD234
009	Kidd	JK	*SLC14A1*	18q12.3	*
010	Diego	DI	*SLC4A1*	17q21.31	*
011	Yt	YT	*ACHE*	7q22.1	*
012	Xg	XG	*XG, MIC2*	Xp22.33, Yp11.3	CD99
013	Scianna	SC	*ERMAP*	1p34.2	*
014	Dombrock	DO	*DO*	12p12.3	*
015	Colton	CO	*AQP1*	7p14.3	*
016	Landsteiner-Wiener	LW	*ICAM4*	19p13.2	CD242
017	Chido/Rodgers	CH/RG	*C4A, C4B*	6p21.3	*
018	H	H	*FUT1*	19q13.33	CD173
019	Kx	XK	*XK*	Xp21.1	*
020	Gerbich	GE	*GYPC*	2q14.3	CD236
021	Cromer	CROM	*DAF*	1q32.2	CD55
022	Knops	KN	*CR1*	1q32.2	CD35
023	Indian	IN	*CD44*	11p13	CD44
024	Ok	OK	*BSG*	19p13.3	CD147
025	Raph	RAPH	*CD151*	11p15.5	CD151
026	John Milton Hagen	JMH	*SEMA7A*	15q24.1	CD108
027	I	I	*GCNT2*	6p24.2	*
028	Globoside	GLOB	*B3GALT3*	3q26.1	*
029	Gill	GIL	*AQP3*	9p13.3	*
030	Rh-associated glycoprotein	RHAG	*RHAG*	6p12.3	CD241
031	FORS	FORS	*GBGT1*	9q34.13	*
032	JR	JR	*ABCG2*	4q22	*
033	LAN	LAN	*ABCB6*	2q36	*

*The symbols, gene name(s), chromosomal locations and cluster of differentiation (CD) numbers (ISBT, 2014) for the known blood group systems are shown. CD numbers were assigned to certain blood group systems during the 7th Human Leucocyte Differentiation Antigens (HDLA) workshop (Zola et al., 2003). An * indicates that no CD number has been assigned.*

There are seven blood group collections (Table 1.2). These do not fit the criteria needed to be allocated as systems but the antigens in each collection have been shown to be related in some way either serologically, biochemically or genetically.

Table 1.2 Blood group collections

Number	*Name*	*Symbol*
205	Cost	COST
207	Ii	I
208	Er	ER
209	*	GLOB
210	*	Le^c, Le^d
212	Vel	VEL
213	MNCHO	MNCHO

*These cannot be allocated to systems but the antigens in each collection have been shown to be related either biochemically, serologically or genetically; an * indicates that no name has been assigned (ISBT, 2014).*

The 700 series contains low-incidence antigens (Table 1.3). These have an occurrence of fewer than 1% in most populations but they cannot be assigned to any existing system or collection.

Table 1.3 The 700 series of low frequency antigens

Number	Name	Symbol	Number	Name	Symbol
700002	Batty	By	700040	Rasmusen	RASM
700003	Christiansen	Chr[a]	700044	*	JKV
700005	Biles	Bi	700045	Katagiri	Kg
700006	Box	Bx[a]	700047	Jones	JONES
700017	Torkildsen	To[a]	700049	*	HUK
700018	Peters	Pt[a]	700050	*	HOFM
700019	Reid	Re[a]	700052	*	SARA
700021	Jensen	Je[a]	700054	*	REIT
700028	Livesay	Li[a]			
700039	Milne	**			

*These antigens have a frequency of less than 1% in most populations tested (ISBT, 2014). An * indicates that no name has been assigned and ** indicates that there is no symbol for this antigen.*

Likewise, the 901 series of high-frequency antigens cannot be assigned to systems or collections (Table 1.4). These antigens occur at an incidence of greater than 90% in most populations (Reid *et al.*, 2012).

Table 1.4 The 901 series of high frequency antigens

Number	Name	Symbol
901002	Langereis	Lan
901003	August	At[a]
901008	*	Emm
901009	Anton	AnWj
901012	Sid	Sd[a]
901014	*	PEL
901016	*	MAM

These antigens have a frequency of greater than 90% in most populations tested (ISBT, 2014). *An * indicates that no name has been assigned*.

Knowledge of gene sequence has allowed the amino acid sequence of molecules carrying blood group antigens to be determined and their structures

within the red blood cell (RBC) membrane predicted. Figure 1.1 shows a variety of blood group antigen systems. The antigens may be carried by integral blood cell membrane proteins with a single transmembrane domain. These are further categorised as type I, like MNS or KN with the N-terminus lying outside the membrane or type II, like KEL where the N-terminus lies inside the membrane. Other antigens have multiple transmembrane domains e.g. RH and FY. Others are glycosylphosphatidylinositol (GPI) linked proteins e.g. Cromer (CROM) (Reid *et al.*, 2012). Carbohydrate antigens of the ABO system are defined by immunodominant sugars attached to different types of oligosaccharide chains carried on glycosphingolipid and glycoprotein molecules (Daniels, 2013). Many antigenic proteins are glycosylated and potential sites of glycosylation are shown in Figure 1.1. N-linked glycosylation can occur at any extracellular asparagine in the sequence asparagine -X- serine / threonine except where X is proline. O-linked glycosylation occurs at serine or threonine. However, not all of the potential sites will be glycosylated (Reid *et al.*, 2012).

Figure 1.1 Model of red blood cell membrane components. These carry blood group antigens. Potential sites of glycosylation are shown as branches off the main antigen structure (Reid et al., 2012). See Tables 1.1 to 1.4 for antigen nomenclature.

RBC antigens are generally carried on extracellular domains and allelic variation within blood group systems is determined predominantly by single nucleotide polymorphisms (SNPs), for example the Fy^a/Fy^b (FY1/FY2) blood group polymorphism is determined by a 125G>A nucleotide change resulting in the presence of glycine for Fy^a or aspartic acid for Fy^b at amino acid 42 in the N-terminal extracellular domain (Dean, 2005).

1.2 KN blood group antigens

The nine antigens of the KN system (also known as Knops) are carried on complement receptor 1 (CR1) a type I membrane glycoprotein. The antigens are all associated with nucleotide changes in CR1. There are three antithetical antigen pairs: Kn^a and Kn^b, McC^a and McC^b, Sl^a and Vil. The other three antigens are: Sl3, KCAM and Yk^a (Covas *et al.*, 2007 and Veldhuisen *et al.*, 2011). The antigens, nucleotide SNPs and resulting amino acids are shown in Table 1.5.

Table 1.5 Antigens of the KN blood group system

ISBT terminology	*Original antigen names*	*SNP (Counted from initiation codon AUG)*	*SNP (without 27 nucleotide leader sequence)*	*Amino acid*
KN1 / KN2	Kn^a / Kn^b	4681 G>A	4708 G>A	Val1561Met
KN3 / KN6	McC^a / McC^b	4768 A>G	4795 A>G	Lys1590Glu
KN4 / KN7	Sl1 (Sl^a) / Sl2 (Vil)	4801 A>G	4828 A>G	Arg1601Gly
KN8	Sl3	4801A 4828T	4828A 4855T	Arg1601 Ser1610
KN9	KCAM+ / KCAM-	4843 A>G	4870 A>G	Ile1615Val
KN5	Yk^a	4223C	4250C	Thr1408

The KN system has nine antigens. The SNPs and resulting amino acid changes are shown. Current publications use SNP positions counted from the initiation codon but older publications use the positions without the 27 nucleotide leader sequence.

All the KN polymorphisms are associated with SNPs in exon 29 of the *CR1* gene located on chromosome 1 (1q32). There is a wide variation in the frequency of KN antigens in different populations as shown in Table 1.6. Kn^a, McC^a and Yk^a antigens are found at high frequency in Caucasian and Black populations. McC^b and Sl2 antigens are extremely rare in Caucasian populations but occur at frequencies of 45% and 80% respectively in

individuals of African descent (Tetteh-Quarcoo *et al*., 2012). This uneven distribution of KN antigens implies that they might be under some selection pressure and it has been speculated that the distribution of McC^b and Sl2 antigens reflect a survival advantage where malaria caused by the *Plasmodium falciparum* parasite is endemic (Rowe *et al*., 1997). However, the situation is far from clear as a study in 2012 (Tetteh-Quarcoo *et al*.) found no evidence that these Knops blood group antigens could protect red blood cells from invasion by *P.falciparum*. The CR1 receptor, Knops antigens and malaria are discussed further in section 1.9.

Table 1.6 Frequency of KN antigens in different ethnic groups (Moulds, 2010)

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1.3 Antibody detection and identification

All blood group antigens are defined serologically by alloantibodies. Most alloantibodies are formed after people lacking particular antigens are exposed to them either through blood transfusion or pregnancy. However, some alloantibodies are naturally occurring such as anti-A or anti-B of the ABO blood group system. These are thought to be caused by exposure to oligosaccharides commonly occurring in nature, so that, for example, blood group A or O individuals become immunised to type-B carbohydrate antigen

present on bacteria (Spalter *et al.*, 1999). Immune antibodies are usually IgG whereas naturally occurring antibodies like anti-A are usually IgM (Figure 1.2).

Figure 1.2 The basic structural unit of immunoglobulins and an IgM molecule. The basic structural unit has two heavy and two light chains. If the heavy chains in the basic structural unit were gamma chains then this would be IgG. IgM structural units have mu heavy chains and five of these form the IgM pentamer (Flynn, 1998).

The basic structural unit for IgG and IgM molecules is the same; it is formed of two heavy and two light chains. Light chains are common to both IgG and IgM molecules and these are always of the same type in each molecule i.e. both are kappa light chains or both are lambda. The heavy chains are unique to each type of immunoglobulin. Heavy chains are gamma in IgG molecules and mu in IgM molecules. The heavy and light chains can be further divided into variable and constant domains. Both chains have terminal variable regions that are involved in antigen binding. IgM molecules are pentamers formed of five IgM monomers joined together by disulphide bonds and a joining or J chain that joins two of the monomers (Czajkowsky and Chao, 2009). If the basic structural unit is cleaved by use of proteolytic enzymes

then the antibody binding regions can be released. These are termed fragment of antigen binding or Fab fragments. The remaining tail piece or Fc fragment (c for crystallisable) are responsible for effector functions such as complement activation (Moulds, 2009).

Detection of both types of antibody in the laboratory relies on haemagglutination techniques. Red cells are negatively charged so when they are suspended in solutions such as saline they attract positive ions which surround the cells and cause them to repel each other (Fernandes *et al*., 2011). The large molecular size of IgM antibodies means that they can bridge the gap between red cells and if they bind to antigens on different red cells can cause them to clump together or agglutinate (Flynn, 1998). This agglutination can be seen by the naked eye and can be brought about by simple incubation of plasma containing the antibody and cells with the corresponding antigen, or alternatively plasma and cells may be mixed in tubes and immediately centrifuged and the tubes are examined for agglutination of the red cells indicating a positive reaction (Daniels and Bromilow, 2007). These direct agglutination techniques, however, are not suitable for detection of IgG antibodies. IgG exists as a single molecule and so being much smaller than IgM molecules they cannot bridge the gap between antigens on different red cells to bring about agglutination without an enhanced technique (Flynn, 1998; Figure 1.3).

Figure 1.3 Sensitisation and agglutination of red blood cells. IgM blood group antibodies bring about direct agglutination of red cells They can bridge the gap between adjacent red cells which are held apart by a positive charge that surrounds them. IgG antibodies cannot bring about direct agglutination as they are too small to bridge the gap. IgG blood group antibodies can bind to the red cell surface and cause them to be sensitised (Flynn, 1998).

The most widely used technique in blood group serology is the indirect antiglobulin technique (IAT) for detection of IgG antibodies. Traditionally, the IAT is a two stage test where patient's plasma and phenotyped reagent red cells are first incubated together to induce sensitisation of the cells with antibody if the corresponding antigen is present on the cells (Figure 1.4). The second stage involves washing away any unbound antibodies and plasma and then addition of anti-human globulin reagent (AHG) containing anti-IgG and anti-complement component C3d followed by centrifugation. Positive reactions will be observed where the AHG has combined with IgG or complement molecules attached to the red cell antigens and caused the red

cells to agglutinate. The wash stage ensures that the antiglobulin reagent is not neutralised by unbound antibodies or plasma (Klein and Anstee, 2005).

Figure 1.4 Principle of the indirect antiglobulin test. In the first stage sensitisation of red cells occurs if antibodies bind to their corresponding antigens on the red cells (bound antibodies shown in blue). This is followed by washing to remove any unbound antibodies (shown in red and yellow). In the second stage AHG is added (shown in green) and agglutination of the red cells is brought about by centrifugation (Daniels and Bromilow, 2007).

IAT reactions have traditionally been performed in glass tubes but now commercial systems are available where a plastic card with columns containing gel or glass microbeads pre-mixed with anti-IgG and C3d are used. Plasma and reagent red cells are added to the top of the column, incubated and then the cards are centrifuged using pre-set parameters. Red cells coated with antibody are agglutinated and unable to pass through the gel. The agglutinated cells appear as layers of cells on the top of the column or as agglutinates dispersed through the gel matrix. In negative reactions unagglutinated cells can pass through the gel or beads and appear as buttons of cells at the bottom of the column (Figure 1.5). As plasma proteins are less dense than the gel or beads they do not pass through the column and so do

not neutralise the AHG and thus a washing step is not needed (Lapierre *et al.*, 1990 and Reis *et al.*, 1993).

Figure 1.5 Principle of the gel indirect antiglobulin test. AHG is incorporated in the gel matrix. Red cells and plasma containing antibodies are incubated together at the top of the column. On centrifugation, sensitised cells react with the AHG in the gel. Agglutinates form that cannot pass through the gel thus giving a positive reaction. A negative reaction is seen when non-sensitised cells are centrifuged to the bottom of the column (adapted from Daniels and Bromilow, 2007).

Generally, screening tests using IAT may reveal the presence of an antibody in a patient's plasma sample. Then identification of the antibody is essential as many are clinically significant, with the potential to cause haemolytic transfusion reactions (HTRs) and haemolytic disease of the foetus and newborn (HDFN) (Daniels *et al.*, 2002). Identification involves testing patient plasma against panels of reagent red cells prepared from donor blood. These panel cells must be selected to express a minimum defined set of RBC antigens (National Blood Service, 2013). However, mixtures of antibodies can make detection and identification of clinically significant antibodies difficult

when using panel reagent cells with multiple antigen expression. Detection of clinically significant antibodies can sometimes present a challenge as they are often found in antibody mixtures; levels of these antibodies can often become undetectable in plasma by IAT or the antibodies may only react with red cells having homozygous expression of the antigen. Also, clinically significant antibodies may be masked by the presence of antibodies to high frequency antigens that will react with most or all panel reagent cells tested and the presence of these antibodies can make crossmatching difficult (Moulds and Rowe, 1996).

1.4 Haemolytic transfusion reactions

Before donor blood can be transfused, the compatibility of donor and recipient must be assessed. Antibodies of the ABO system, anti-A, anti-B and anti-A,B, are naturally occurring, IgM antibodies, so that if mismatched blood is transfused reaction between recipient antibodies with the corresponding donor antigen occurs during or within 24 hours of the transfusion. These acute HTRs are caused by activation of the complement cascade of proteins by bound antibodies (Stowell *et al.*, 2012).

The complement system consists of over 30 soluble or membrane bound proteins and can be activated via three pathways: the alternative pathway; the classical pathway or the lectin pathway. The alternative and lectin pathways play a key role in immunity against bacterial molecules. The alternative pathway is activated by recognition of pathogen associated molecular patterns on bacterial surfaces; the lectin pathway by detection of

bacterial sugars (Berentson and Sundic, 2014). It is the classical pathway that is activated by antigen-antibody complexes. All pathways converge on formation of an enzyme that converts complement component C3 into the active products C3a and C3b (Figure 1.6). The classical pathway, initiated by antibody binding with antigen, can be activated when red blood cell antibodies bind to their corresponding antigen on the red cell surface (Stowell *et al.*, 2012). This pathway starts with activation of complement component C1, which consists of three proteins, C1q, C1r and C1s. When C1q binds to the Fc portions of immunoglobulins (Daha *et al*, 2011) it causes a conformational change in C1r allowing it to cleave C1s, resulting in an active C1s protease. This complex activates components C2 and C4 generating red cell bound C2a and C4b and release of soluble C2b and C4a. Bound C4b and C2a form C3 convertase that cleaves C3 to C3a and C3b (Stoermer and Morrison, 2011). C3b facilitates continuation of the complement cascade when it attaches to the target cell membrane. Bound C3b facilitates conversion of C5 to C5a and C5b. Soluble components C3a and C5a are anaphylatoxins and promote chemotaxis and regulate a wide variety of systemic factors in immunity. Bound C5b continues the cascade and together with components C6, C7, C8 and C9 forms the membrane attack complex (MAC) that results in the insertion and polymerization of C9 in the target membrane and intravascular haemolysis (Goldfarb and Parillo, 2005).

Figure 1.6 The complement cascade. On complement activation the complement components interact with each other sequentially causing a cascade of reactions involving enzymatic cleavage of inert components into active fragments which then contribute to activation of the next component. Ultimately, the membrane attack unit (C5b6789) may be formed (Stowell et al, 2012).

In the case of activation by red blood cell antibodies, red blood cells would be destroyed rapidly in the circulation resulting in the presence of free haemoglobin in the plasma (haemoglobinaemia) and urine (haemoglobinuria) and these reactions may be fatal (McLelland, 2001).

Other clinically significant antibodies tend to be immune and IgG in nature. These usually cause delayed HTRs typically 5-8 days post transfusion (Strobel, 2008). These occur because antibody present in the recipient due to

previous sensitisation is not detected prior to transfusion sometimes because it is at such low levels that it is not detected by serological techniques. A secondary immune response follows transfusion of incompatible units where antibody levels rise and transfused red cells become coated with antibody (Garratty, 2008). Extravascular haemolysis occurs when macrophages with receptors for the Fc region of immunoglobulin molecules, particularly in the spleen, trap and ingest the antibody coated red cells thus removing them from the circulation. The interaction between IgG and Fc receptors is modulated by the structure of the IgG N-linked glycans (Aricescu and Owens, 2013). Sensitised RBCs may be completely or partially ingested by the macrophages. Partial ingestion leads to the formation of spherocytes, which may undergo further destruction during subsequent passages through the spleen (Berentsen and Sundic, 2014). Also, part or all of the RBC destruction may occur external to the macrophage by antibody-dependant cellular cytotoxicity (Garratty, 2008). Delayed HTRs are characterised by a fall in haemoglobin levels, jaundice and fever (Murphy and Pamphilon, 2001). CR1 as a member of the complement control protein super family helps to regulate complement activation as it can bind and transport complement coated immune complexes, parasites or bacteria to the spleen and liver where they can be removed from the circulation. Failure to remove such complexes can result in tissue injuries (Tham *et al.*, 2011).

1.5 Haemolytic disease of the foetus and newborn

Also, in the Red Cell Immunohaematology (RCI) laboratory, it is important to identify antibodies that can cause HDFN. This is caused by maternal IgG

antibodies that are actively transported across the placenta during pregnancy (Mathiesen *et al.*, 2013). If the foetus carries the corresponding antigen, inherited from its father, then maternal antibody can sensitise foetal red cells causing them to be destroyed by foetal macrophages and thus causing foetal anaemia. The anaemia may be so severe that the foetus could die from heart failure unless transfused *in utero* or, if less severe, then immediately after birth (Ross and de Alarcon, 2013). Exchange transfusion may be required if haemoglobin levels are low or bilirubin levels from the breakdown of red cells are particularly high (McLelland, 2001). Anti-D, formed against the RhD antigen can cause severe and fatal HDFN. Antibodies directed against other blood group antigens e.g. those of the KEL, FY and JK (Kidd) systems are all normally IgG and can cause HDFN and transfusion reactions of varying severity (Garratty, 2012).

1.6 Clinical significance of antibodies

Whether a red cell antibody is clinically significant or not depends on several factors. Thermal amplitude is important as the antibody must react at body temperature i.e. 37ºC. Activation of complement depends on immunoglobulin type and subclass. Activation of C1 requires changes in two separate CH2 domains on antibody immunoglobulin heavy chains. As IgM is a pentameric molecule it has multiple antigen binding sites and only one bound molecule of IgM is needed to activate C1. However, two of the smaller IgG molecules are needed for complement activation and these must attach to epitopes within 20-30 nm of each other to activate C1 (Stowell *et al*, 2012). In addition IgG has four subclasses that have different hinge regions that affect the

conformational flexibility of the IgG heavy chain. This affects the ability of the CH2 domain to activate complement (Stowell *et al*, 2012). IgG_1 and IgG_3 are good activators of complement, IgG_2 is a poor activator and IgG_4 does not activate complement at all (Moulds, 2009).

1.7 KN blood group antibodies

Not all IgG antibodies are clinically significant and these include a group of antibodies that tend to give weak and variable reactions by IAT with the majority of red cells, reactions which are not always reproducible (Ensley, 2012). These antibodies were previously known as high titre, low avidity (HTLA) antibodies. This was because if they are titrated i.e. doubling dilutions of plasma are prepared and tested by IAT against antigen positive cells, then they often react at high dilutions e.g. > 1 in 64 despite giving weak reactions (Ensley, 2012). Most other red cell antibodies that react weakly when undiluted will generally be non-reactive when diluted (Moulds and Rowe, 1996). Antibodies to the KN system of antigens fall into this HTLA group. KN antigens can occur at high frequency on RBCs so the antibodies will react with nearly all reagent or donor red cells by IAT (Moulds, 2010). They can mask the presence of clinically significant antibodies that may also be present. Approximately 50% of patient samples containing KN antibodies also contain other alloantibodies (Daniels, 2013). Investigation of patients with these antibodies can be difficult and time consuming. Crossmatching, the process for ensuring that patient plasma is compatible with donor units, can take a lot longer if KN antibodies are present as nearly all donor units will be

incompatible and often incompatible units may have to be issued to avoid delays in supply of units to the patient (personal experience).

Typically, the specificity of antibodies to high frequency antigens can be identified by testing patient plasma with rare cells that are negative for high frequency antigens. If, for instance, no reactions were obtained by reacting patient plasma with cells that were negative for the high frequency Kn^a (KN1) antigen then it would be suspected that the antibody specificity was anti-Kn^a. However, these rare cells are not always available. Chemical reagents and enzymes are often used to destroy some blood group antigens. This chemical or enzyme modification of intact red cells often aids in the identification of antibody specificity (Reverberi and Reverberi, 2008). The effects of proteinase enzymes such as papain, ficin, trypsin and sialidase and chemicals such as 2-aminoethylisothiouronium (AET) and dithiothreitol (DTT) on blood group antigens are well known and documented (Daniels, 1992 and Fernandes *et al*., 2011). KN antigens are variable in their susceptibility to enzyme treatment but they are weakened or destroyed by the disulphide bond reducing agents AET and DTT (Reid, 2012). If the presence of KN antibodies is suspected then reagent red cells treated with either AET or DTT can be tested with patient's plasma by IAT and negative or weakened reactions would be obtained if the antibody had KN specificity. Unfortunately, other antigens are also destroyed by AET and DTT and these can be clinically significant e.g. KEL antigens and the high frequency antigens Gy^a and Hy (part of the Dombrock, (DO) blood group system) so interpretation of results needs to be done with experience and caution (Costa *et al.*, 2010). Even if a

specificity can be defined in this way there is still the problem of being able to distinguish the non-clinically significant anti-KN antibodies from potentially clinically significant antibodies that may also be present (Torres *et al.*, 2010).

If RCI laboratories cannot identify an antibody specificity then samples can be referred to the Red Cell Reference (RCR) laboratory, part of the International Blood Group Reference Laboratory (IBGRL), based at National Health Service Blood and Transplant (NHSBT), Filton. RCR can repeat the testing that RCI laboratories have performed but with the advantage of having access to more rare cells negative for high frequency antigens and patient's plasma referred from all over the world containing rare antibodies.

RCR can also perform a monoclonal antibody-specific immobilization of erythrocyte antigens assay (MAIEA) that can confirm that an antibody is directed at specific red cell membrane proteins (Petty *et al.*, 1997). As shown in Figure 1.6, two antibodies are used; one a human alloantibody and the other a non-human, usually mouse monoclonal antibody. These antibodies whilst being specific for the same protein recognise epitopes on different regions of the protein. The antibodies are incubated with intact human red cells. Then the red cells are haemolysed and red cell membranes solubilised. In a positive reaction a trimolecular complex of the two antibodies and membrane protein is formed. This complex is immobilised on a multi-well plate coated with anti-mouse immunoglobulin and detected by use of peroxidase-conjugated goat anti-human immunoglobulin. However, this test takes between two to three days to perform (Nicole Thornton, Head of RCR

personal communication) so is not suitable for investigation of urgent samples from patients requiring transfusion.

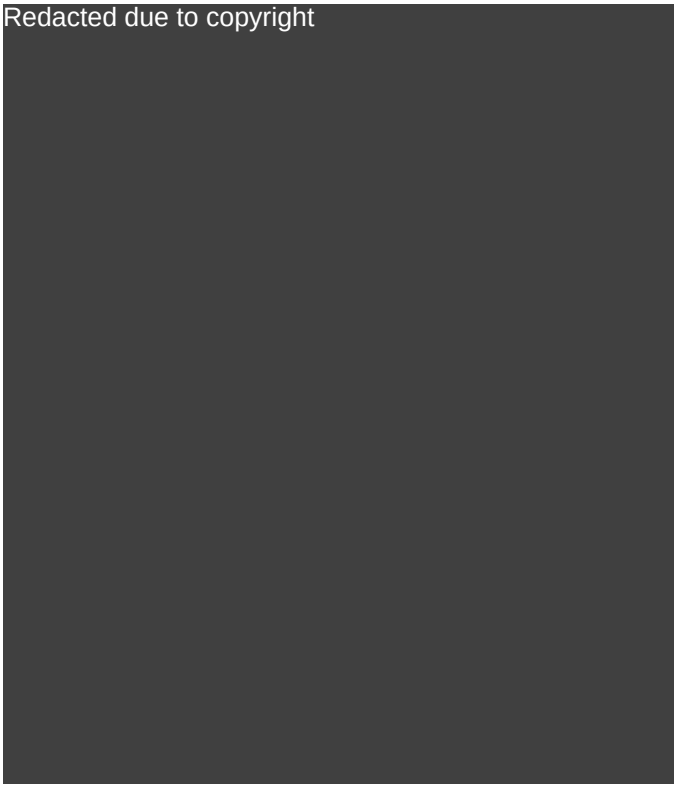

Figure 1.7 Principle of the monoclonal antibody-specific immobilization of erythrocyte antigens assay. Y *= mouse IgG,* Y *= human IgG,* Y *= anti-mouse IgG,*  *= peroxidase conjugated anti-human IgG. 1. Human and mouse antibodies binding to different epitopes on the same red cell membrane. 2. Solubilisation of the membrane to release the trimolecular complex. 3. Capture of the complex by immobilised anti-mouse IgG. 4. Detection of the complex with peroxidase conjugated anti-human IgG (from Petty et al., 1997).*

Other antibodies in the 'HTLA' group include anti-CH/RG antibodies. CH/RG antigens are found on complement components that bind to the RBC surface (Kahwash *et al.*, 2005). Complement is found in soluble form in plasma and thus incubation of the antibody with plasma can inhibit the antibody. Inhibition of these antibodies in this way is a useful way of ensuring that there are no

other underlying antibodies present that could be clinically significant. KN antigens are found in soluble form in plasma but only at very low levels so plasma cannot be used to inhibit these antibodies (Moulds, 2010). However, the increasing availability of soluble recombinant (sr) blood group antigens does offer the possibility of inhibiting antibodies whose antigens are not available in any other soluble form. Inhibition of any KN specific antibodies present would allow detection and identification of any underlying clinically significant antibodies that might also be present. This would give the opportunity to provide safe blood for patients with problematic antibodies (Denomme and Flegel, 2008).

1.8 Recombinant antigens

Detailed knowledge of the molecular structure and genetic background of antigens has enabled soluble recombinant blood group antigens to be developed that retain their antigenic activity but are free of the RBC membrane. These include: soluble recombinant (sr)-FY antigens (Sheffield *et al.*, 2006 and Ridgwell *et al.*, 2007); sr-K antigens (Ridgwell *et al.*, 2007); sr-LU antigens (Ridgwell *et al.*, 2007 and Seltsam *et al.*, 2008) and sr-JMH antigen (Seltsam *et al.*, 2008).

Use of recombinant antigens, in a soluble form, offers new opportunities for blood transfusion science. Current antibody detection and identification techniques rely on the availability of panels of human red cells that must be selected to express a minimum defined set of RBC antigens (National Blood Service, 2013). Positive reactions indicate that an antibody to one or more

antigens on the cells is present in the patient's sample. Analysis of patterns of reactivity against up to ten different cells contained in the panel indicates what the specificity of the antibody might be. Some workers argue that if single soluble recombinant RBC antigens were available, then antibody identification would be simpler with a positive reaction unambiguously confirming the specificity of the antibody. Panels of single specificity sr-RBC antigens could be assembled and would replace the need for donor blood to create panels. This would have the advantage of releasing donor units for transfusion (Seltsam and Blasczyk, 2009). However, to use recombinant antigens in this way they must be available in soluble form and it is the 3D structure of the proteins within the red cell membrane that largely determines whether soluble versions can be generated (Seltsam *et al.*, 2008). For some single pass proteins with a single large extracellular domain (ECD), the ECDs have been expressed as soluble recombinant molecules. Soluble recombinant KEL and Lutheran (LU) proteins have been produced and shown to retain blood group antigen expression (Ridgwell *et al.*, 2007). KEL antigens are carried on a single-pass type II red blood cell membrane glycoprotein and LU antigens on a single-pass type I membrane glycoprotein (Figure 1.1). FY1 and FY2 sr-antigens have also been produced (Sheffield *et al.*, 2006 and Ridgwell *et al.*, 2007). The FY antigens are carried on a type III membrane glycoprotein which spans the RBC membrane seven times. However, the FY1/FY2 polymorphism is located on the N-terminal extracellular domain (Sheffield *et al.*, 2006). It has proved difficult to produce soluble recombinant (sr)-proteins with blood group specificity for multipass proteins or those with a complex

structure of the extracellular domain or antigens requiring significant post-translational modification (Seltsam, 2009).

Soluble recombinant KEL, LU and FY antigens that have been produced were used in a solid-phase enzyme-linked immunosorbent assay (ELISA) technique to detect corresponding antibodies in human samples (Ridgwell *et al.*, 2007). Recombinant Semaphorin 7A, the protein carrying high-frequency antigens of the JMH system, was produced and coupled onto superparamagnetic particles. These coated particles were used in a particle gel immunoassay to detect anti-JMH antibodies (Seltsam *et al.*, 2008). Semaphorin 7A is a glcosylphosphatidylinositol-linked protein (Figure 1.1). Of the sr-proteins with blood group specificity that have been produced only KEL and FY would be included in a panel for antibody identification based on current guidelines (National Blood Service, 2013) as their corresponding antibodies are clinically significant. Use of these proteins, as published, would involve developing techniques not routinely used in blood group serology currently e.g. solid-phase ELISA assays (Ridgwell *et al*., 2007). Another way that sr-RBC antigens can be used in blood group serology now is to use them to inhibit haemagglutination reactions. Use of sr-antigens could be implemented easily as part of the haemagglutination process. Patient's plasma could be pre-treated by incubation with sr-antigen before the IAT test was performed. Sr-recombinant CR1 protein that carries the KN antigens was used (Moulds and Rowe, 1996) to inhibit anti-KN antibodies. There is no need to identify these antibodies if they can be inhibited as they are not clinically significant so development of a solid-phase assay using these proteins to detect and

identify them would not be cost effective or particularly useful. However, if they can be "removed" from patient's plasma this offers a quick and simple way of identifying whether any clinically significant antibodies are also present in the patient's plasma.

1.9 Complement receptor 1

As discussed in section 1.5, CR1 as a complement control protein can regulate complement activation. As well as clearing up complement coated immune complexes it can also regulate further complement activation by accelerating the decay of C3 and C5 convertases and it can act as a co-factor for cleavage of C3b and C4b (Yazdanbakhsh, 2005).

CR1 exists in four forms with the most common designated as CR1*1. The ECD can be divided into 30 short complement regulator (SCR) domains (also known as complement control protein modules) each having 60 to 70 amino acids. The first 28 SCRs are arranged into four longer regions termed long homologous repeats (LHRs) called LHR-A, LHR-B, LHR-C and LHR-D. They consist of seven SCRs each (Tham *et al*., 2011) (Figure 1.8).

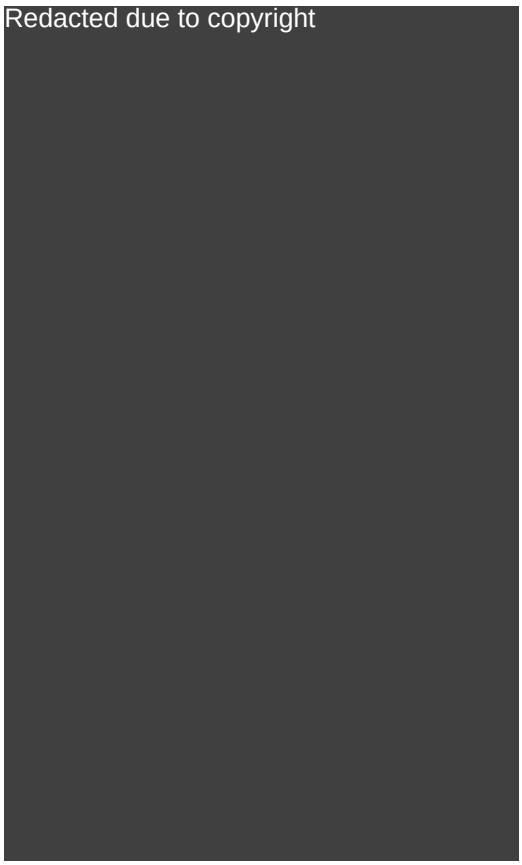

Figure 1.8 A best-fit scattering model for complement receptor 1. The four LHR regions are shown with the 28 SCR domains numbered (seven in each LHR). The final two SCRs are 29 and 30, these lie next to the red blood cell membrane (from Furtado et al., 2008).

CR1 is a membrane bound glycoprotein found on most human peripheral blood cells. As red blood cells are the most numerous cells in blood, they carry most of the CR1 in the body. However, the number of CR1 molecules varies from between 300 to 800 per red blood cell. This accounts for the variability in reactions seen in haemagglutination tests when KN antibodies are tested with reagent cells and also because CR1 can be lost from the red cell surface during storage (Moulds, 2010). CR1 has a major role in removing immune complexes from circulation. Immune complexes opsonised by C3b can bind to CR1 on the red cell surface. When red cells circulate through the

liver and spleen the immune complexes are removed by macrophages and red cells may also lose CR1 molecules during the process. In conditions where a high level of immune complexes are processed such as haemolytic anaemia, insulin-dependent diabetes mellitus or human immunodeficiency virus (HIV) infection, Knops antigens are found to be depressed due to loss of CR1 (Stoute, 2011).

Three sites have been identified on CR1 where complement components are bound. These are on LHR-A, LHR-B and LHR-C, whilst Knops antigens are located on LHR-D (Figure 1.9).

Figure 1.9 A diagrammatic representation of the short consensus repeats (SCRs) in complement receptor 1 (Stoute, 2011). The three binding sites for complement components C3b and C4b are shown. The Knops antigens are located in SCR25 of LHR-D.

The biological significance of blood group polymorphisms is not known but they probably arose due to a past selective advantage which may now have disappeared (Daniels, 2013). However, glycoproteins that carry blood group antigens are often used as receptors by pathogenic micro-organisms for attachment to red cells and subsequent invasion (Lisowska, 2002). The diversity in the distribution of Knops antigens Sl2 and McC^b (Table 1.6) was

speculated to be due to a survival advantage in places where *P.falciparum* was endemic (Rowe *et al*. 1997). However, the Knops antigens are carried on LHR-D and so do not occupy the known functional sites of the CR1 molecule (Tetteh-Quarcoo *et al*., 2012). The *P.falciparum* adhesin protein, PfRh4, binds to red cells but only at short concensus repeats (SCRs) 1 to 3 (the C4b binding site), which is remote from the site of the Knops antigens in LHR-D (Tham *et al*., 2011). It is possible that the distribution of Knops variants could arise from less well understood interactions between CR1 and pathogenic organisms. It is known that CR1 on red cells binds immune complexes formed during invasion of bacteria such as *Mycobacterium tuberculosis*, the main cause of tuberculosis in sub-Saharan Africa. The presence of McC^b and Sl2 antigens appears to confer resistance to tuberculosis infection so could explain the selection of these antigens in this population (Noumsi *et al*., 2011).

1.10 Sr-protein expression systems

There are several steps required for expression of a foreign gene in a host organism so that recombinant protein (r-protein) is produced. The first step generally requires the insertion of that gene into an expression vector. Either full length or modified versions of the gene have been used for expression. With blood group antigens expression of the ECD is often used as antigens are present on this part of the molecule. To enhance solubility and facilitate purification of the protein, DNA that encodes for a fusion partner (such as the FLAG peptide) may be present in the vector. Recombinant proteins produced from a FLAG vector carry a short peptide, amino acid sequence: Asp-Tyr-Lys-Xaa-Xaa-Asp, encoded by the expression vector at their amino or carboxyl termini. This peptide, known as an affinity tag, provides a means

of isolating and purifying the protein, for instance by using anti-FLAG monoclonal antibodies to capture FLAG tagged proteins in an agarose affinity gel column system (Terpe, 2003). The 3XFLAG tag developed from the FLAG tag has three adjacent FLAG epitopes. The p3XFLAG-cytomegalovirus (CMV)™-13 expression vector is designed to establish stable expression of C-terminal 3XFLAG-tagged proteins in mammalian cells (Figure 1.8). Presence of the preprotrypsin leader sequence (PPT LS) results in secretion of the 3XFLAG fusion protein into the culture medium. The promoter regulatory region (CMV promoter) of the human cytomegalovirus drives transcription of 3xFLAG-fusion constructs. FLAG expression vectors encode the aminoglycoside phosphotransferase II gene (neo^r) that confers resistance to aminoglycosides such as geneticin sulphate (G418). This vector also contains the pMB1 origin of replication in bacterial cells which is a derivative of pBR322, the β-lactamase gene for ampicillin resistance selection in bacteria (amp^r), hGH polyA and the f1 origin.

Figure 1.9 The p3xFLAG-CMV-13 vector. This vector contains the strong CMV promoter for high-level expression in mammalian cells. Three adjacent FLAG epitopes, Asp-Tyr-Lys-Xaa-Xaa-Asp (3xFLAG) are downstream of the multiple cloning site (MCS). The preprotrypsin leader sequence (PPT LS) directs secretion into the culture medium. Neo^r and amp^r confer resistance to aminoglycosides and ampicilin respectively (Sigma-Aldrich, 2010).

The expression vector must be integrated into a host cell's genome to produce a stable recombinant cell line. Mammalian cell lines such as human embryonic kidney (HEK293) and COS-7 have been used successfully to express recombinant blood group antigens and have the advantage that they can perform post-translational modifications needed, such as glycosylation and di-sulphide bond formation, needed for appropriate protein folding (Sweich *et al.*, 2012). This is important as recombinant blood group antigens

must mimic the structure and conformation of RBC antigens so that they can be detected by human alloantibodies (Yazdanbakhsh, 2007). Glycosylation is an important post-translational modification. It can be extremely complex with mono, oligo or long and complex polysaccharides attached to proteins at multiple sites (Ellis and Noid, 2014). Glycosylation is important for protein folding. Some proteins do not fold properly unless they are glycosylated whilst some are not stable unless they contain N-linked polysaccharides (Aricescu and Owens, 2013). It is also important for protein mobility and signal transduction (Roth *et* al., 2012). However, mammalian expression systems are the most expensive to use and protein yields can be significantly less than in other systems (Seltsam *et al.*, 2007). Other expression systems available include insect cells, bacteria and yeast. These may produce higher yields than mammalian cells but they cannot perform all the necessary post-translational modifications, in particular they cannot perform glycosylation and blood group antigens are often glycosylated (Sweich *et al.*, 2012).

Adherent cells like HEK293 need a surface on which they can grow. At its simplest this involves growing the cells in plastic flasks or dishes that provide a rigid substrate (McAteer et al., 2002). Cells also need to be grown in a medium such as Dulbecco's Minimal Eagle's Medium (DMEM) that provides nutrients, salt and pH control. Supplements are often added to facilitate growth; the amino acid glutamine is an important energy source and provides amines essential for the synthesis of nucleic acids and other compounds. Added protein helps buffer the culture system. Protein is normally added in the form of albumin contained in foetal calf serum (FCS), which also provides

growth factors. The most frequently used antibiotics are mixtures of penicillin and streptomycin because of their combined action against gram-positive and gram-negative bacteria. A pH indicator like phenol red can be added to DMEM to indicate whether the pH is within optimum range (normally pH7.0 – 7.5 for most cells). At this range the colour is red but any change to yellow indicates that the medium has become more acidic (Cartwright and Shah, 2002) which could be due waste products produced by the cells and is an indication that the medium needs to be replaced. Mammalian cells have efficient systems for secreting proteins into the culture medium, this makes harvesting the r-protein a relatively simple process (Brown, 2010). Purification of the protein can then be based on an attached affinity tag, such as the 3xFLAG tag, which can result in sr-protein of high purity (Lichty *et al.*, 2005).

1.11 Previous work on soluble CR1

As high frequency antibodies of the Knops system cannot be inhibited by serum, saliva or urine as other antibodies are, Moulds and Rowe (1996) obtained some soluble recombinant CR1, lacking the transmembrane and cytoplasmic domains, to assess whether this could be used to inhibit them. The sr-CR1 had been produced using a cDNA sequence encoding the CR1*1 allotype modified by introduction of a stop codon at the junction of the extracellular and transmembrane regions. They labelled the sr-CR1 with ^{125}I and used examples of human and monoclonal antibodies with anti-KN specificities to immunoprecipitate complexes which they eluted and ran on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels under non-reducing conditions. All antibodies tested immunoprecipitated

a protein of approximately 190 kDa, corresponding to the predicted molecular mass of the sr-CR1 molecule. An inhibition technique was developed where human sera containing examples of Knops system antibodies were incubated with sr-CR1 prior to testing with KN antigen positive cells in a standard IAT technique. Samples containing individual KN antibodies e.g. anti-Kn^a and anti-Sl^a and samples containing mixtures of KN antibodies e.g. anti-Kn^a plus anti-McC^a were tested. Only one example of anti-Kn^a was not inhibited by this technique. No examples of non-Knops related antibodies also tested were inhibited. It was concluded that use of sr-CR1 offered a valuable alternative to the current serological techniques.

Yazdanbakhsh *et al.* (2000) speculated that mouse erythroleukaemic (MEL) cells might be used to express individual blood group antigens that could be used to replace RBCs to absorb specific antibodies as part of the antibody identification and detection process before compatibility testing. This was because MEL cells had been used as an erythroid tissue culture model. They used a whole CR1 cDNA sequence to transfect MEL cells. Stable clones of transfected cells were established and surface expression of CR1 was confirmed by flow cytometry using a monoclonal anti-CR1 antibody. Absorption of sera containing Knops antibodies with the transfected mouse cells did lead to inhibition of anti-Knops antibodies.

Truncated CR1 proteins were produced as a step towards mapping the epitopes of Knops system antigens (Tamasauskas *et al.*, 2001). As part of this study the ability of the individual LHRs they had produced to inhibit antibodies with anti-KN specificity was assessed. PCR primer pairs were

developed to amplify the four LHR regions from the same sr-CR1 ECD construct used by earlier workers (Moulds and Rowe, 1996). Human embryonic kidney cells (HEK293) were transfected with the individual LHRs and culture supernatants harvested and purified. The recombinant proteins produced were used to inhibit sera containing anti-KN antibodies. They found that three out of six sera containing anti-Kn^a were almost completely inhibited by soluble recombinant (sr)-LHR-C and the other three by soluble recombinant (sr)-LHR-D. Two sera containing anti-McC^a were inhibited by LHR-D, whilst seven out of eight examples of anti-Sl^a showed reduced activity with LHR-D only. They concluded that McC^a, Sl^a and Yk^a antigens reside within LHR-D and that some of the Kn^a epitopes are within LHR-C and some within LHR-D. Their study also suggested that the sr-CR1 ECD was sometimes not as efficient as that of sr-LHR-D in inhibiting Knops system antibodies. They concluded that it might prove more cost effective and be more efficient to express the smaller LHRs rather than the whole CR1 molecule. In 2005 Yazdanbakhsh, one of the co-authors of the above work speculated that a mixture of sr-LHR-C and sr-LHR-D could be sufficient for KN antibody inhibition.

In 2007, Dr K. Ridgwell obtained full length human CR1*1 from Dr K. Marchbank, University of Cardiff. The CR1*1 was cloned into the pCDM8 vector (Invitrogen, Figure 1.9). Dr Ridgwell designed PCR primers to amplify the LHR-C and LHR-D regions of CR1. These regions were ligated into p3xFLAG-13 expression vectors (Sigma-Aldrich) (Figure 1.8). HEK293 cells were transfected and cultured. Proteins purified from the cell culture supernatant were used to perform some preliminary investigations into their

ability to inhibit samples containing antibodies directed against KN system antigens. It was shown that Knops antibodies were inhibited, some with sr-LHR-C and some with sr-LHR-D. This work was promising but not pursued at the time due to other commitments, including producing other sr-blood group antigens as part of a commercial contract.

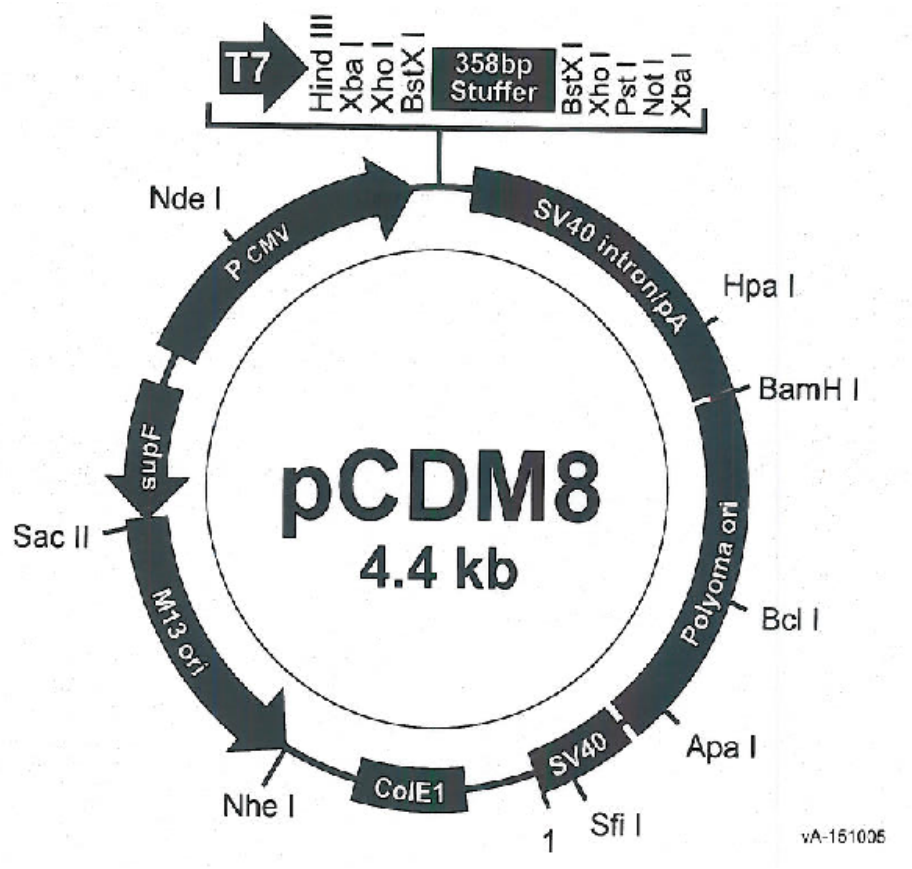

*Figure 1.10 The pCDM8 vector. The ECD region of CR1*1 was cloned into a pCDM8 vector (Invitrogen) which was obtained from Dr K Marchbank.*

There is now a growing interest in developing the use of sr-blood group antigens for RCI and recognition that blood bank scientists increasingly need to be familiar with molecular theory and techniques (Denomme and Flegel, 2008). This study was devised to produce and investigate the use of both sr-

LHR-C and sr-LHR-D for inhibition of Knops system antibodies. This approach has been suggested by several workers as described above but there is no reagent currently available for the reference laboratories of NHSBT to use. Although KN antibodies are not clinically significant they are not uncommon and are often difficult to identify. This makes them a nuisance as they can cause an incompatible crossmatch and must ideally be identified before transfusion of incompatible blood can be recommended. This means that investigation of patient samples containing KN antibodies can be a time consuming process and can cause delays in provision of blood for transfusion. This can potentially put patients' lives at risk if provision of blood is delayed or if investigation has not identified underlying clinically significant antibodies then the patient is at risk of suffering a haemolytic transfusion reaction.

1.12 Aims and objectives

The aims of this research were to:

- Produce sr-LHR-C and sr-LHR-D proteins to be used in NHSBT laboratories for investigation of patient samples and in the crossmatch procedure.
- Optimise a haemagglutination inhibition technique for use in the above laboratories using the two sr-LHR proteins.
- Analyse the cost benefit of this approach.

The objectives to achieve these aims were to:

- Transfect HEK293 cells to produce sr-LHR-C or sr-LHR-D protein
- Clone transfected cells to select those producing the most protein and set up frozen cell banks for future use

- Optimise the protein purification process
- Develop an inhibition test and an ELISA to test sr-protein antigenicity using the purified sr-LHR proteins
- Calculate the cost of the process to provide sufficient reagent to nine NHSBT laboratories
- Investigate CE marking the product for distribution more widely

1.13 Assay validation

An assay for use in RCI laboratories would need to be fully validated before use. Within NHSBT a change control process would need to be established and full validation script written before the reagent could be distributed. This would be so that intra- and inter- assay variability was minimised.

2.0 MATERIALS AND METHODS

2.1 DNA techniques

2.1.1 Preparation of recombinant plasmid DNA for transfection

A plasmid DNA preparation of p3xFLAG-LHR-D was kindly donated by Dr K Ridgwell for this study but a plasmid DNA preparation of p3xFLAG-LHR-C was not available. However, a frozen sample of p3xFLAG-LHR-C transfected *E.coli* preserved in glycerol was obtained from Dr K Ridgwell. In this study, the transfected *E.coli* cells were streaked onto an agar plate containing 100 μg mL^{-1} ampicillin. After overnight incubation at 37 ºC, colonies had grown and these were used to inoculate 5 mL 2.5% Luria broth. The broth samples were incubated overnight at 37 ºC with shaking. From these samples some cells were frozen in glycerol for stock by adding 500 μL cell culture to 125 μL 75% glycerol (final glycerol concentration 15%) in a 2 mL cryovial and freezing at -80 ºC. Plasmid DNA was purified from the remaining cell sample using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to manufacturer's instructions. Briefly, this involved production of a cleared cell lysate and then binding the plasmid DNA onto a DNA binding resin in a mini column collection tube. The DNA was then washed and eluted from the column and suspended in nuclease-free water. To check that LHR-C and LHR-D inserts of the correct size were present, plasmid DNA was cut using restriction enzymes, *Cla1* and *Kpn1* (Sigma-Aldrich). These enzymes had been used previously to clone the CR1 inserts into the 3XFLAG vector. For 10 μL restriction enzyme digest, 2 μL DNA was used with 0.5 μL of *Cla1* (5 units) and 0.5 μL of *Kpa1* (5 units), 1 μL 10x concentration Bovine Serum Albumin (BSA) and 1 μL 10x concentration restriction enzyme SL buffer

(Sigma-Aldrich). SL buffer contained 100 mM Tris-hydrochloric acid (HCl) pH 7.5, 100 mM $MgCl_2$ and 10 mM 1,4-Dithioerythritol (DTE). The restriction mixture was incubated at 37 ºC for 1.5 hours. This was followed by 1% agarose gel electrophoresis of cut and uncut samples (Method 2.1.2).

2.1.2 Agarose gel electrophoresis

Agarose gel was prepared by dissolving 0.2 g agarose (Invitrogen) in 20 mL 0.5x Tris-acetate EDTA buffer (2 M Tris-acetate and 50 mM EDTA) to make the required 1% gel. SYBR® Safe DNA gel stain (Invitrogen) was added (2 µL of x10,000 concentrate). Samples were suspended in blue/orange 6x loading dye (Promega). This ready to use mixture contained: 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0). Samples were loaded onto gels together with a Smart Ladder molecular size marker (Eurogentec). This marker gave 14 bands from 200 to 10,000 base pairs spaced in multiples of 100 base pairs. Gels were run at 100 V for approximately 20 minutes on a Mupid®-eXu submarine electrophoresis system (Advance Co Ltd) or until the coloured markers had reached the bottom of the gel and then DNA bands were visualised with UV transillumination (302 nm) read on a Bio-Doc-It™ Imaging System (Ultra-Violet Products Ltd).

2.1.3 Spectrophotometric analysis of p3xFLAG-LHR-C plasmid DNA

DNA sample purity was measured by reading absorbance at 260 and 280 nm on a spectrophotometer (Multiskan EX, Thermo Scientific) and the ratio A260/A280 determined. DNA concentration was determined by multiplying

A260 obtained by 50 µg mL^{-1} and sample dilution (based on an absorbance of 1 equal to 50 µg mL^{-1} DNA).

2.1.4 3xFLAG-LHR-C and 3xFLAG-LHR-D plasmid DNA sequencing

To confirm that LHR-D and LHR-C had been correctly inserted into the p3xFLAG-CMV vector, DNA samples of p3xFLAG-LHR-D (from KR) and p3xFLAG-LHR-C (current research) were mixed with appropriate primers prior to Sanger di-deoxy chain termination sequencing. Two vector sequencing primers, N-CMV-30 (Sigma-Aldrich) and C-CMV-24 (Sigma-Aldrich) and an in-house designed primer, CR1-F3 were used. To a final concentration of 10 µM for each primer, was added 1 µg DNA. N-CMV-30 (sequence 5'-AAT GTC GTA ATA ACC CCG CCC CGT TGA CGC-3') was used to sequence at the 3' end of the LHR insert within the vector. Reverse primer, C-CMV-24 (sequence 5'-TAT TAG GAC AAG GCT GGT GGG CAC-3') was used to sequence at the 5' end of the LHR insert. CR1-F3 (5'-AAG GTG TTT GAG CTT GTG GGG-3') was designed to detect the LHR inserts within the vector and was complimentary to sequences within both LHR-C and LHR-D. The samples were sent to Peter Martin (Bristol Institute for Transfusion Sciences (BITS)/IBGRL) for sequencing. The DNA sequences, when received, were analysed on SeqScape software (Invitrogen) and compared by eye to the sequences of LHR-C and LHR-D within CR1 GenBank accession number Y00816.

2.1.5 Transfection of HEK293 cells with p3xFLAG-CMV-LHR-C or p3xFLAG-CMV-LHR-D

HEK293 cells were obtained from Mathew Hazell (BITS/IBGRL). In this study, these cells were transfected with p3xFLAG-CMV-LHR-C or p3xFLAG-CMV-LHR-D recombinant vector DNA preparations using the FuGENE® HD Transfection Reagent (Promega). The manufacturer's standard protocols were used and reactions were set up in a 6-well plate. HEK293 cells (2 mL; 2 x 10^4) in cell culture medium DMEM (Sigma-Aldrich) containing 10% FCS (Hyclone®, Fetalclone®, Thermo Scientific), 1% penicillin-streptomycin (PS) (stock solution containing 10,000 units penicillin and 10 mg streptomycin per mL, Sigma-Aldrich) and 1% L-Glutamine (LG, stock solution containing 200 mM L-Glutamine, Sigma-Aldrich) were added to each well of the two 6-well plates the day before transfection. For transfection the reagent was warmed to room temperature (RT) and mixed well before use. Serum free medium (to make a final volume of 100 µL) was added to Eppendorf tubes and DNA or water was added. The transfection mixture was added and incubated at RT for 25 minutes. Two different ratios of transfection reagent to DNA were used for both LHR-C and LHR-D. These were 3:1 (6 µL:2 µg) and 1.5:1 (3 µL: 2 µg). The medium was gently removed from the HEK293 cells and 1 mL of fresh medium was added to each well. The transfection reagent was added to each well and the plate mixed carefully prior to incubation in a 7% CO_2 incubator at 37 ºC. After 24 hours, G418 (Gibco) at a final concentration of 400 µg mL^{-1}, was added to the wells. This was to select for cells containing transfected DNA as the vector contained the aminoglycoside phophotransferase gene that conferred resistance to G418. At regular intervals (3 to 4 days) cell culture supernatant was removed from the wells

and retained and fresh culture medium containing 200 µg mL^{-1} G418 was added. An ELISA (see Method 2.3.1) was performed on the supernatants to determine if the cells were producing sr-LHR-C and sr-LHR-D protein. After 4 weeks the selected cells were removed from the appropriate well by trypsin treatment (see Method 2.2.1) and transferred to individual T-75 flasks (BD Falcon™).

2.2 Cell culture techniques

2.2.1 Cloning of stably transfected HEK293 cells producing sr-LHR proteins

When the cells in the flasks were confluent i.e. cells adhered onto the bottom of the flask with few or no gaps between them, the cells were dislodged from the bottom of the flask by trypsin treatment. Trypsin-EDTA solution (0.5 M, Sigma) had been pre-warmed to 37 ºC. Culture medium was decanted from the flask and residual culture medium in the flasks was removed by washing with phosphate buffered saline (PBS). Trypsin-EDTA was added just to cover the cell layer in the base of the flask (3 to 5 mL). The flasks were incubated at room temperature for 2 to 5 minutes until the cells could be dislodged by tapping the flask. When the cells had been dislodged the trypsin was neutralised by adding 3 to 5 mL cell culture medium. The cell suspension was mixed gently and then decanted into sterile centrifuge tubes and centrifuged at 470 g for three minutes. A sample was prepared for cell counting. The neutralised cells were resuspended in 10 mL cell culture medium. To 50 µL of this cell suspension, 50 µL 0.2% Tryphan blue was added. The preparation was applied to a haemocytometer and cells were counted in duplicate. In order to be able to select individual cell colonies increasing dilutions of cells

were prepared in cell culture medium, from 1/10 to 1/1,000,000. These were used to seed six petri dishes (20 mm, Falcon BD). Over several days as the colonies developed, the petri dishes were examined under a microscope in a flow cabinet and individual cell colonies were selected. These were picked using a sterile pipette tip and transferred to individual wells of a 24-well plate. From these plates the six colonies producing the highest amounts of sr-LHR-C and sr-LHR-D as measured by ELISA (see Method 2.3.1) were transferred to 6-well plates and then into T-75 flasks. This cloning process was repeated twice more. After the third round of cloning the cells producing the most sr-LHR-C and sr-LHR-D as seen from the ELISA results were selected and master and working Cell Banks were prepared and frozen at -80 °C (see Method 2.2.2).

2.2.2 Freezing cloned stably transfected cells for liquid nitrogen storage

Sterile freezing medium (90% FCS, 10% dimethyl sulphoxide) was prepared and pre-chilled on ice. Freezing medium was added to a pellet of HEK293 cells and they were mixed gently. The cell suspension was transferred to properly labelled freezing vials that had been pre-cooled on ice. Filled vials were frozen gradually, over 2 to 3 hours, at -80 ºC in a 'Mr Frosty' freezing container (Nalgene) which was filled with 100% isopropyl alcohol, with a cooling rate of -1 ºC minute^{-1}, and then stored in liquid nitrogen.

Stably transfected HEK293 cells from two flasks of confluent T-175 flasks were suspended in sterile freezing medium and 1 mL was added to each of 10 vials and frozen as detailed above to form the Master Cell Bank. Each vial

contained approximately 3 x 10^7 cells. The contents of three confluent T-175 flasks were suspended in freezing medium and 1 mL was added to each of 12 vials to form the Working Cell Bank, with a cell count of approximately 4 x 10^7 per vial.

2.2.3 Recovery of cells from liquid nitrogen storage

Cell culture medium was pre-warmed at 37 ºC. One vial each of HEK293-LHR-C and HEK293-LHR-D cells from the Working Cell Bank were taken from liquid nitrogen storage and immediately thawed at 37 ºC in a waterbath. In a flow cabinet the thawed cells were transferred to 10 mL of warmed culture medium in a sterile 25 mL Universal container, mixed and centrifuged at 470 g for three minutes. Supernatant was discarded and the cells were resuspended, in residual cell culture medium remaining in the tube, by agitation. A 20 mL volume of warm culture medium containing G418 (200 µg mL^{-1}) was added and the cell suspension transferred to a T-75 culture flask. Flasks were incubated in a 7% CO_2 incubator at 37 ºC.

2.2.4 Production of sr-LHR-C and sr-LHR-D in HEK293 cells

Cloned cells were recovered from frozen storage as described in Method 2.2.3 and were cultured in three different growing systems: T-175 flasks, spinner flasks (Integra Biosciences) and CellMax™ Hollow Fiber Bioreactors (Spectrum Europe BV). Supernatant was collected and stored frozen at -30 ºC until sufficient volumes had been obtained for protein extraction (Methods 2.2.5, 2.2.6 and 2.2.7).

2.2.5 Transfected HEK293 cell culture in T-175 flasks

When cells in one T-75 flask were 70 to 80% confluent (approximately 6 x 10^7 cells) they were expanded into three T-175 flasks (four different sized culture flasks are in Figure 2.1). The cells were removed by trypsin treatment as described in Method 2.2.1. The cells from the T-75 flask were transferred into three T-175 flasks (cell count approximately 2 x 10^7 per flask). Culture medium containing 10% FCS, 1% PS, 1% LG was added to 25 mL and G418 antibiotic was added (final concentration 200 µg mL^{-1}) and the flasks placed in a 7% CO_2 incubator at 37 ºC.

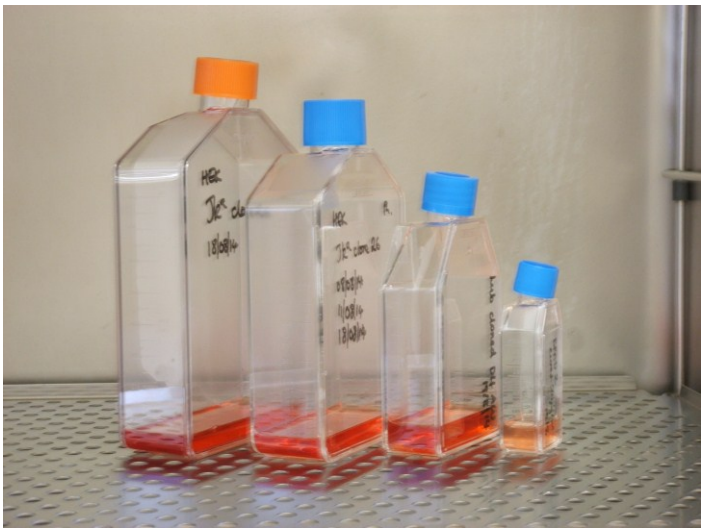

Figure 2.1 Four cell culture flasks of various sizes in a flow cabinet. From left to right, T-225, T-175, T-75 and T-25 flasks containing cell culture supernatant.

Flasks were checked regularly until cells were confluent, this was about 5 to 6 days. At this point supernatant was harvested, centrifuged to remove debris and stored frozen at -30 ºC. The cells in the flasks were trypsin treated (Method 2.2.1) then cells were pooled from the three flasks and split between

five T-175 flasks (approximate cell count per flask 3 x 10^7). Five flasks were maintained and supernatant was collected every 5 to 6 days when cells were confluent. The cells were pooled, centrifuged and approximately 20% were used to reseed five flasks with the rest being discarded. Harvested supernatant was collected and stored frozen at -30 ºC until sufficient volumes had been obtained for protein extraction. Prior to extraction by using anti-FLAG agarose affinity gel columns, supernatant of the same type i.e. containing sr-LHR-C or sr-LHR-D was pooled and filtered using a 0.22 µm vacuum filtration unit (Millipore) to remove any remaining cells and cell debris.

2.2.6 Transfected HEK293 cell culture in spinner flasks

The cells harvested from three confluent T-175 flasks were transferred to a Cellspin 250 spinner flask (approximately 2 x 10^7 cells) and the volume made up to 250 mL with culture medium containing 10% FCS, 1% PS, 1% LG and G418 (200 µg mL^{-1}). Two spinner flasks on a stirrer platform are shown in Figure 2.2.

Figure 2.2 Two spinner flasks shown on a stirrer platform. (from www.bioresearchonline.com).

The screw tops on the side ports were loosened slightly and the spinner flasks were incubated at 37 ºC on a Cellspin stirrer platform (stirring at 20 rpm) (Integra Biosciences) for approximately 14 days when the cell culture medium had just started to turn from red to yellow. Then supernatant was harvested in a flow cabinet. HEK293 cells were allowed to settle to the bottom of the flask. Supernatant was removed with a pipette and filtered through a 0.22 µm vacuum filtration unit (Millipore) to remove cells and cell debris. The spinner flask was topped up with culture medium and antibiotic as before and returned to the incubator. Harvested supernatant was frozen at -30 ºC until protein extraction could take place.

2.2.7 Transfected HEK293 cell culture in Hollow Fiber Bioreactors

Polysulphone cartridges (12 mL) with a 30 kDa molecular weight cut-off and a surface area of 1,700 cm^2 were used in the CellMax™ Hollow Fiber Bioreactor system (Spectrum Europe). Prior to use the bioreactor cartridge was flushed

through with culture medium containing 10% FCS, 1% PS, 1% LG and G418 (200 µg mL^{-1}) following the manufacturer's instructions. This priming operation was done on two successive days before cells were introduced to the system. The confluent cells from five T-175 flasks (approximately 2 x 10^8 cells) were washed, trypsin treated, neutralised and resuspended to 30 mL in cell culture medium containing 10% FCS, 1% PS, 1% LG and G418 (200 µg mL^{-1}). The bioreactor was isolated by closing the inlet and outlet slide clamps and the cells were collected in a syringe and introduced into the isolated bioreactor via the left hand port on the cartridge (shown in Figure 2.3). The cell suspension was flushed backwards and forwards three to four times through the extra capillary space to uniformly distribute the cells. This was done by fixing an empty syringe to the outlet port and gently pushing the fluid between the inlet and outlet ports. A fresh bottle of culture medium containing 5% FCS, 1% PS, 1% LG and G418 (200 µg mL^{-1}) was attached to the bioreactor.

Figure 2.3 A CellMax bioreactor. This is shown prior to being primed with cell culture medium. Inlet and outlet ports of the cartridge are shown in blue (from www.fisher.co.uk).

Two bioreactors containing HEK293 cells transfected with LHR-C and LHR-D were set up and were connected to a CellMax(R) Pump System and placed at 37 ºC in a 7% CO_2 incubator (Figure 2.4 shows a bioreactor set up in a flow cabinet). Supernatant (30 mL) was harvested at regular intervals according to the manufacturer's instructions and stored frozen at -30 ºC. Prior to protein extraction an ELISA was performed to determine the samples with the highest protein concentration (Method 2.3.1). Selected supernatant samples of the same type i.e. containing sr-LHR-C or sr-LHR-D were pooled and filtered using a 0.22 µm vacuum filtration unit (Millipore) to remove cells and cell debris.

Figure 2.4 A Bioreactor system. This system has two cartridges (one on the other side, not seen) linked to cell culture medium. Bioreactors and cell culture medium bottles are located on a platform with pump so that the medium is constantly pumped through the cartridge. NB the cartridge seen here is from NBS Biologicals. These have now replaced the CellMax bioreactors which are no longer available.

2.3 Protein detection and purification techniques

2.3.1 ELISA for sr-LHR-C and sr-LHR-D protein detection

The wells of a high protein binding 96 well microtitre plate (Immulon 2HB, Thermo Scientific) were coated with 100 µL goat anti-mouse IgG (Fc specific) (Sigma) at a concentration of 1 µg mL^{-1} in carbonate coating buffer pH 9.6 (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$). After overnight incubation at 4 ºC the plate was washed once with PBS pH 7.2 containing 0.05% Tween® 20 (PBST) (Sigma) by filling up the wells with PBST and then flicking it off into a sink. The plate was then blocked with PBST containing 5% low fat milk powder (PBSTM), by adding 100 µL to each well, and incubated with shaking for one hour at 37 ºC. The plate was washed once with PBST as before. Mouse monoclonal anti-CD35 (anti-CR1, clone E-11, Santa Cruz Biotechnology Inc) was added at a concentration of 10 µg mL^{-1}, 100 µL per well and the plate was incubated with shaking for one hour at 37 ºC. The plate was washed four times with PBST. Culture samples were added (100 µL) and the plate incubated with shaking for one hour at 37 ºC. The plate was washed four times in PBST and then 100 µL of rabbit anti-Flag antibody (Sigma) was added to each well at a concentration of 1 µg mL^{-1} in PBSTM and the plate incubated with shaking for one hour at 37 ºC. After being washed four times in PBST, horse radish peroxidase goat horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Dako) was added at a concentration of 1 µg mL^{-1} and the plate incubated with shaking at 37 ºC for one hour. After four washes in PBST, 100 µL of developer was added to each well (0.1% O-phenylenediamine dihydrochloride (OPD) in pH 5.0 citrate buffer (0.024 M citric acid, 0.051 M Na_2HPO_4) with 0.015% hydrogen peroxide added just before use. Reactions were stopped by adding 100 µL 1 M HCl

when colour in the wells had developed sufficiently (10 to 15 minutes). The absorbance of each well at 492 nm was measured using a microplate photometer (Multiskan EX, Thermo Scientific). Standards were prepared by diluting purified sr-LHR-C and sr-LHR-D in culture medium containing 10% FCS, 1% PS and 1% LG.

2.3.2 Immunopurification of sr-3X-FLAG fusion proteins using anti-FLAG agarose affinity gel column

The column was prepared by adding 1 mL anti-FLAG M2 agarose affinity gel (Sigma) to a glass column (length 100 mm, internal diameter 10 mm) (Amersham Biosciences). It was washed with a minimum of 25 column volumes of Tris-buffered saline (TBS) wash buffer (0.5 M Tris pH 7.4, 1.5 M NaCl). Any residual, unbound antibody was removed from the column by washing with five column volumes of 0.1 M glycine HCl at pH 3.5 and then washed with at least 10 column volumes of TBS. Pooled, filtered culture supernatant was added to the column coupled to a peristaltic pump using a flow rate of 1 mL minute^{-1}. Flow through from this stage, containing unbound material, was retained for further purification. The column was washed with at least 10 column volumes TBS.

2.3.2.1 Acid elution

3xFLAG fusion protein was eluted under acid conditions using 0.1 M glycine-HCl at pH 3.5, followed by one column volume of TBS to wash through the acid, under gravity flow. The eluate was neutralised by addition of 200 µL Tris buffer (0.5 M, pH 7.4). The column was neutralised with 25 column volumes of TBS.

2.3.2.2 3xFLAG elution

Competitive elution was performed using five column volumes of 3xFLAG peptide (Sigma-Aldrich) at 100 µg mL^{-1} in TBS. After application of the 3xFLAG peptide, the gel was washed through with 1 mL TBS. After the peptide elution step the gel was washed with 25 mL TBS and then flushed through with 10 mL glycine-HCl pH 3.5 to remove all traces of peptide and regenerate the column. This was followed by washing the column with 25 mL TBS.

Use of TBS for immunopurification was recommended by the manufacturer (Sigma-Aldrich, 2010). After elution from the affinity column the TBS buffer was exchanged with PBS. This was because all serological techniques are performed in PBS rather than TBS. Buffer exchange was achieved by washing the eluates from both elution methods three times in PBS in a 30,000 kDa molecular weight cut off (MWCO) Vivaspin ultracentrifugation unit (Sartorius). The Vivaspin tubes were centrifuged at 4000 g for 25 minutes each time and concentrated to a minimum volume in PBS.

2.3.3 Bicinchoninic acid protein assay

The protein content of the eluate from the affinity column was determined by QuantiPro bicinchoninic acid (BCA) assay (Sigma) following the manufacturer's instructions and was then stored frozen at -30 ºC. This assay is similar to the Lowry procedure, where formation of a Cu^{2+} protein complex under alkaline conditions is followed by reduction of Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the amount of protein present. BCA

forms a purple complex with Cu^{1+} in alkaline conditions so the amount of purple colour produced can be measured on a spectrophotometer at 562 nm and by comparison with a known standard the amount of protein present can be calculated (Noble *et al*., 2007).

2.3.4 SDS-PAGE analysis of sr-proteins

Proteins were assessed using SDS-PAGE. The gels were either stained for total protein or western blot was performed. Commercial Mini-PROTEAN precast gradient gels (BIO-RAD) were used. Samples for analysis were prepared by mixing sample (2 µg) with sample buffer (5% SDS, 5 mM EDTA, 20 mM Tris pH 8.0, 10% glycerol with 6% mercaptoethanol added just prior to use) and water to a volume of 18 µL and then heated at 100 ºC for 2 minutes.

Gels were mounted in the Mini-PROTEAN Tetra Cell (BIO-RAD) and running buffer was added to the inner and outer chambers. Samples and a protein standard, ProSeive® (Invitrogen) were loaded into the wells. The gel was run at 100 V until the tracking dye in the sample buffer had reached the bottom of the gel (approximately one hour).

2.3.5 Semi-dry electro blotting (western blotting) from SDS-PAGE gels

Sr-protein samples run on SDS-PAGE gels were transferred onto 0.2 µM polyvinylidene difluoride (PVDF) membrane, using the Trans-Blot™ Turbo™ Mini PVDF Transfer System (BIO-RAD) and Trans-Blot™ Turbo™ Transfer Packs (BIO-RAD). For each blot one PVDF membrane was soaked in 100% methanol until transparent (2-3 minutes) and was then transferred to transfer buffer solution (200 mL 5x concentrate transfer buffer (BIO-RAD) added to

600 mL H_2O and 200 mL ethanol). Two stacks of four pieces of filter paper were soaked in transfer buffer for 3-4 minutes until wet. One stack of filter paper was placed on the anode side of the semi-dry apparatus and PVDF membrane was placed on top. The SDS-PAGE gel (see Method 2.3.4) was placed on top of the membrane and the second filter paper stack placed on top, any bubbles were rolled out at this point and then the cathode assembly was placed onto the transfer pack and placed into the Transfer System.

2.3.6 Development of western blots

After removing from the transfer system, the PVDF membrane was blocked using PBSTM (PBS containing 0.2% Tween20 and 5% milk powder) for 30 minutes at RT or at 4 ºC overnight. It was then rinsed in PBST (containing 0.2% Tween20) and HRP-conjugated antibody was added. In this study HRP-conjugated anti-mouse IgG (Dako), HRP-conjugated anti-bovine serum albumin (BSA) (Immune Systems Ltd) and anti-Flag-M2-peroxidase (HRP) (Sigma-Aldrich) were used at a concentration of 1 µg mL^{-1} in PBSTM. The membrane was incubated at RT with mixing on an automated rocker for one hour (Gyro-Rocker STR9, Bibby Stuart). The membrane was placed in PBST to wash for 10 minutes at RT. The wash stage was repeated twice more before the membrane was put into developing reagent (300 µg mL^{-1} 3, 3'-diaminobenzidine (DAB, Sigma), 0.03% H_2O_2 (Sigma, 1 in 1000 dilution of 30% stock, added just before use). When brown bands had developed in the marker positive control sample (up to 15 minutes) the PVDF membrane was washed in water to stop the reaction and dried on tissues. Anti-CD35 clones E-11 and J3D3 (Santa Cruz Biotechnology Inc.) were also used in a two stage

process. First anti-CD35 was added as a primary antibody in the process above at a concentration of 1 μg mL^{-1} in PBSTM. After the incubation and washing stages as described, HRP-conjugated anti-mouse was added as a secondary antibody (1 μg mL^{-1} in PBSTM) and the incubation and washing stages repeated before addition of the DAB developing reagent.

2.3.7 Protein staining of SDS-PAGE gels and western blots

SDS-PAGE gels were washed in deionised water three times and then immersed in SimplyBlue™ SafeStain (Invitrogen) for one hour at room temperature. They were then washed a further three times in deionised water and examined for protein bands. Blotted PVDF membranes were washed in water and then immersed in G-250 membrane stain (3 g Coomassie brilliant blue G-250 (Molekula) dissolved in 450 mL ethanol, 100 mL acetic acid added and made to 1 L with water) for 3 to 4 minutes and washed in water and then ethanol.

2.3.8 Mass spectrometry analysis of sr-proteins

Stained samples from SDS-PAGE gels were cut out with a scalpel and placed in Eppendorf tubes and sent to Dr K Heesom at Bristol University for Matrix-Assisted Laser Desorption / Ionization – Time of Flight (MALDI-TOF) assessment. This method uses a soft ionisation technique which is useful for the analysis of proteins as they tend to be fragile and fragment easily. The samples submitted in-gel were subjected to tryptic digestion and the resulting peptides analysed by MALDI-TOF to generate a peptide mass fingerprint and peptide sequence information. These were searched against the SwissProt

human databases to identify the protein present (personal communication from Dr K Heesom).

2.4 Patient testing

2.4.1 Clinical material

Plasma samples were provided by several RCI reference laboratories and RCR/IBGRL laboratory. These samples had been referred to these laboratories from hospital transfusion laboratories for further testing and in some cases for provision of compatible blood. All of the plasma samples had either been confirmed as containing KN related antibodies or they had reacted in such a way during investigation that laboratories suspected they might contain KN related antibodies. The samples were stored frozen at -30 ºC. Each sample had been labelled with the NHSBT sample number and sample date. These identifiers were then used to collect details, from the NHSBT diagnostic laboratory computer system, of the antibody specificities where known.

2.4.2 Ethical considerations

Ethical approval was not applied for under advice from NHSBT National Research and Development Manager as the testing performed was a continuation of the clinical investigation of these samples. Consent is implicit when samples are initially referred from hospital laboratories to NHSBT for testing. Plasma samples are not regulated by the Human Tissue Act, 2004, as they do not contain human cells (Royal College of Pathologists, 2009).

Investigation of one sample during this study did lead to a changed diagnosis of antibody specificity. However, the patient did not need blood transfusion and a modified report was written after the sample had been referred to IBGRL for further testing. Samples may be referred to IBGRL if an RCI laboratory cannot identify antibody specificity and in these cases reports may need to be updated if the IBGRL findings are different to RCI initial findings.

2.4.3 Antigenicity ELISA

Sr-LHR-C and sr-LHR-D were diluted in carbonate coating buffer pH 9.6 (see Method 2.3.1 for ELISA buffer compositions; sr-protein concentration 10 µg mL^{-1}) and 100 µL was added to the wells of a Maxisorp 96 well plate (Nunc). The plate was incubated for one hour at 37 ºC with shaking and then washed four times with PBST. The plate was blocked by addition of 100 µL PBSTM per well and incubation at 37 ºC with shaking for 30 minutes. After washing the plate four times with PBST, 100 µL per well of human patient plasma, diluted 1 in 5 in PBSTM was added. The plate was incubated for one hour at 37 ºC with shaking, followed by four washes in PBST and then 100 µL of HRP-anti-human IgG (stock concentration 5.5 mg mL^{-1}, Sigma-Aldrich) diluted 1/1000 in PBSTM was added to each well. The plate was again incubated for one hour at 37 ºC with shaking. The plate was washed four times in PBST and 100 µL OPD developer (1 µg $µL^{-1}$) was added to each well. The plate was incubated at RT for 5 to 15 minutes until yellow colour had developed. Reactions were stopped by adding 1M HCl and the plate absorbances read at 492 nm. A positive result was indicated by a ratio of greater than 2:1 of mean

test absorbance value to negative control absorbance. This would indicate the presence of a KN related antibody.

2.4.4 Monoclonal antibody-specific immobilization of erythrocyte antigens assay (MAIEA)

This test as described by Petty *et al.* in 1997 was used by IBGRL staff to determine anti-KN specificity of patient samples used in this study. The principles of the method are described in the Introduction, section 1.4.

2.5 Patient testing using serological techniques

2.5.1 Red cell reagents in CellStab

In this study red cell reagents provided by NHSBT Reagents, Liverpool were used. These are manufactured in accordance with guidelines published by the UK Blood Transfusion Services (National Blood Service, 2013). An identification (ID) panel and 3 Cell Screening set, both in the diluent CellStab (glycine buffered saline containing sugars, trimethroprim and sulfanethaxazol as preservatives supplied by BIO-RAD), were used. Both panels are made from donated red cells and are provided as 0.8% cell suspensions in CellStab (0.8% refers to the volume of packed red cells in relation to the diluent). Both panels have an expiry date of three weeks from manufacture which is stated on the forms that accompany them when they are issued from Reagents. They must not be used beyond the expiry date. The ID panel consists of 10 cell samples that between them cover all the clinically significant antigens with homozygous and heterozygous expression. The clinically significant antigens are defined as the Rh antigens D, C, E, c, e and Fy^a, Fy^b, Jk^a, Jk^b, S, s, M, Kp^a and C^w. In addition cell samples 1-2 must cover K+, k+, Fy^a, Fy^b, Jk^a, Jk^b, S

and s. Also included on the panel are Lu^a+, Kp^a+, MM and NN cells. Ideally, if available then k- and / or Le^{a-b-} cells should be included. The 3 Cell Screening set consists of O $R1^wR1$, O R_2R_2 and Orr cells that cover the Rh antigens D, C, E, c and Cw in both homozygous and heterozygous form. The set must also have homozygous expression of Fy^a, Fy^b, Jk^a, Jk^b, S and s. Between the cells the following antigens must be expressed: C^w, M, N, P_1, K, k, Kp^a, Le^a and Le^b.

2.5.2 Column indirect antiglobulin test

An appropriate number of IAT cards (BIO-VUE) were selected and 50 µL 0.8% cell suspension of reagent cells in cellstab (BIO-VUE) was pipetted into the top of each required column. After addition of 25 µL plasma to each column the cards were incubated for 15 minutes at 37 ºC. The cards were then centrifuged in a BIO-VUE gelcard centrifuge with preset parameters. Cards were read by eye and results graded (Figure 2.5).

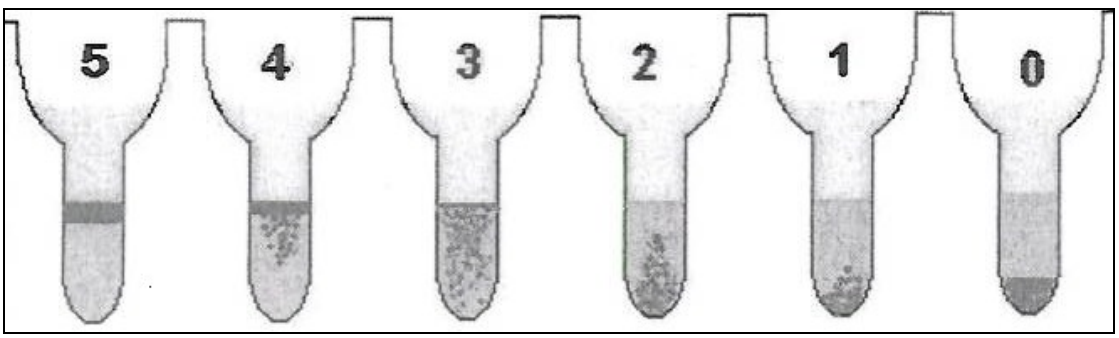

Figure 2.5 Grading of reactions on a gel IAT card. 0 = negative, where unagglutinated cells have passed through the column. Positive reactions are

graded 1 to 5 in order of increasing strength of reaction and hence size of agglutinates that either all remain on top of the gel when too large to pass through (grade 5) or are dispersed through the gel to varying degrees, grade 4 to grade 1. (Adapted from NHSBT, RCI, INF335 Grading of reactions).

2.5.3 Inhibition of KN antibodies

A serological inhibition technique was developed using the column indirect antiglobulin test (CAT) described in Method 2.5.2. Prior to performing the CAT test, 1 in 20 dilutions of approximately 1 mg mL^{-1} sr-LHR-C, sr-LHR-D, a 1 in 10 dilution of a 1:1 mixture of sr-LHR-C and sr-LHR-D (KN inhibition reagent (KNIR)) or PBS were prepared with patient or donor plasma and incubated for 30 minutes at RT. The CAT test was then performed using 50 µL 0.8% reagent red cells and 25 µL of treated patient plasma as described above. After incubation and centrifugation as described in Method 2.5.2, the reactions were read by eye and graded as described in Figure 2.5.

2.6 Statistical methods used in this study

Two statistical tests were used in this study. These were the one-way Analysis of Variance (ANOVA) and Student's *t*-tests (Kirkman, 1996 and Ashcroft and Pereira, 2003). ANOVA was used to compare the yield of protein from three different culture methods in Protein Production section 3.3.3. Student's t-test was used to compare the yield of protein using two different elution techniques in Protein Purification section 4.3.1. These parametric statistical tests were chosen with the assumption that a normal distribution of results would be expected from the culture techniques used and from the two elution techniques.

3.0 SR-LHR-C AND SR-LHR-D PROTEIN PRODUCTION IN HEK293 CELLS

3.1 Introduction

The aim of this part of the study was to produce sufficient protein to develop a reagent for use in NHSBT laboratories. The first objective to achieve this aim was to transfect HEK293 cells to produce sr-LHR-C and sr-LHR-D protein. If these proteins were produced then a further objective would be to clone the transfected cells to select those producing the most protein so that frozen cell banks could be set up for future use. In addition comparison of different culture systems for growing the HEK293 cells, would allow selection of the best system for protein production in terms of ease of use, cost and yield of protein, which would be important factors for future reagent production.

The HEK293 cell line is derived from human embryonic kidney cells. It is one of the most widely used cell lines for research purposes as, unlike bacterial or yeast expression systems, it can express human proteins with complex post-translational modifications. The cells are also easy to grow, are highly transfectable and can secrete proteins into the cell culture medium, which can then be collected and purified to isolate the required protein (Burnouf, 2011).

Cloned, stably transfected HEK293 cells expressing 3xFLAG tagged sr-LHR-C or sr-LHR-D proteins were produced by drug selection using G418. Those cells that had taken up the expression vector had G418 resistance conferred by the aminoglycoside phosphotransferase II gene in the expression vector. Stably transfected cells were then subjected to a cloning process (Materials

and Methods section 2.2.1) where the colonies producing the highest amount of sr protein were selected using an ELISA technique (Materials and Methods section 2.3.1).

Although HEK293 is an adherent fibroblast cell line, the cells can be grown in suspension in cell culture medium (Chapple *et al.*, 2006). In this study the cloned cells were grown in spinner flasks, T-175 disposable flasks, and in a commercial system CellMax® Duo Hollow Fiber Cell Culture System (Spectrum Labs). In T-175 flasks, cells can adhere to the bottom of the flasks whilst in spinner flasks the cells are suspended in the cell culture medium and continuously agitated. In the CellMax® system, hollow fibres provide a large surface area on which the cells can grow. The cell culture supernatant was collected at various intervals, pooled and purified on affinity gel columns. The yield of protein, ease of use and cost of production were assessed for each culture system. The purified protein was assessed for antigen activity, mobility on SDS-PAGE and submitted for MALDI-TOF analysis (described in Materials and Methods section 2.3.8).

3.2 Materials and Methods

3.2.1 Primer design

Plasmid DNA, p3xFLAG-LHR-C and p3xFLAG-LHR-D, was sequenced to ensure that cDNA encoding LHR-C or LHR-D had been correctly inserted. The method and primers used are described in Materials and Methods section 2.1.4. A new primer was designed to sequence LHR-D around the SNP encoding the Kn^a / Kn^b polymorphism as when sequenced with the original

primers (C-CMV-24, N-CMV-30 and CR1-F3) this region was missed (Figure 3.1). The LHR regions are highly homologous so the potential primer was checked for matching sequences using LALIGN (part of the FASTA package of sequence analysis program, European Bioinformatics Institute) and EMBOSS Water (European Bioinformatics Institute). Using both packages no other matching sequences were found in LHR-D.

4201	CGTGCTGGTC	ACTGTAAAAC	CCCAGAGCAG	TTTCCATTTG	CCAGTCCTAC	GATCCCAATT
4261	AATGACTTTG	AGTTTCCAGT	CGGGACATCT	TTGAATTATG	AATGCCGTCC	TGGGTATTTT
4321	GGGAAAATGT	TCTCTATCTC	CTGCCTAGAA	AACTTGGTCT	GGTCAAGTGT	TGAAGACAAC
4381	TGTAGACGAA	AATCATGTGG	ACCTCCACCA	GAACCCTTCA	ATGGAATGGT	GCATATAAAC
4441	ACAGATACAC	AGTTTGGATC	AACAGTTAAT	TATTCTTGTA	ATGAAGGGTT	TCGACTCATT
4501	GGTTCCCCAT	CTACTACTTG	TCTCGTCTCA	GGCAATAATG	TCACATGGGA	TAAGAAGGCA
4561	CCTATTTGTG	AGATCATATC	TTGTGAGCCA	CCTCCAACCA	TATCCAATGG	AGACTTCTAC
4621	AGCAACAATA	GAACATCTTT	TCACAATGGA	ACGGTGGTAA	CTTACCAGTG	CCACACTGGA
4681	CCAGATGGAG	AACAGCTGTT	TGAGCTTGTG	GGAGAACGGT	CAATATATTG	CACCAGCAAA
4741	GATGATCAAG	TTGGTGTTTG	GAGCAGCCCT	CCCCCTCGGT	GTATTTCTAC	TAATAAATGC
4801	ACAGCTCCAG	AAGTTGAAAA	TGCAATTAGA	GTACCAGGAA	ACAGGAGTTT	CTTTTCCCTC
4861	ACTGAGATC	TCAGATTTAG	GGGTTTGTCA	ATGTCAGCCC	TGGTAGGGTC	CCACACTGTG

Figure 3.1 The primer designed to detect the Kn^a/Kn^b SNP. Part of the nucleotide sequence for LHR-D is shown (taken from CR1 reference sequence, GenBank accession number: Y00816). Kn^a / Kn^b SNP at position 4708 (shown in blue) matches reference sequence, G at 4708 for Kn^a. The new primer is shown in red 4495 to 4511.

3.2.2 Development of an ELISA for detection of sr-3xFLAG tagged sr-LHR-C and sr-LHR-D protein

N.B. All volumes stated in these methods are per well.

3.2.2.1 ELISA development method one

A high protein binding 96 well microtitre plate (NUNC Maxisorp, Sigma-Aldrich) was coated with 100 µL mouse monoclonal anti-CD35 (E-11; Santa Cruz Biotechnology Inc, Dallas, Texas) at a concentration of 20 µg mL^{-1} in carbonate coating buffer pH 9.6 (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$) and then incubated with shaking for one hour at 37 ºC. Wells were washed four

times with PBS pH 7.2 containing 0.05% Tween® 20 (PBST; Sigma-Aldrich). Either 100 µL purified srLHR-D or sr-LHR-C or cell culture supernatants were added, prior to incubation with shaking for one hour at 37 ºC followed by again washing four times with PBST. Goat HRP-conjugated anti-FLAG M2 antibody (Sigma) was added, at a concentration of 1 µg mL^{-1} in PBSTM (PBST containing 5% low fat milk powder) and the plate incubated with shaking for one hour at 37 ºC. After being washed four times in PBST, plates were developed with 100 µL 0.1% OPD in pH 5.0 citrate buffer (0.024 M citric acid, 0.051 M Na_2HPO_4) with hydrogen peroxide at a final concentration of 0.015% added just before use. Reactions were stopped by adding 100 µL 1 M HCl when colour in the wells had developed sufficiently (15 minutes). The absorbance of each well at 492 nm was measured using a microplate photometer (Multiskan EX, Thermo Scientific). The basic principle of the ELISA method is shown in Figure 3.2.

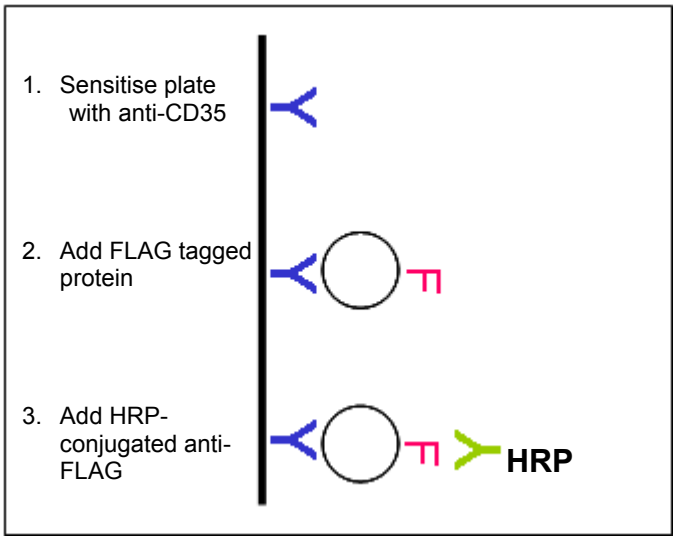

Figure 3.2 ELISA development method one. Coloured antibodies denoted Y: blue is mouse monoclonal anti-CD35, green is goat HRP-conjugated anti-FLAG.

3.2.2.2 ELISA development method two

This was the same as method one except after addition of the sr-FLAG tagged protein and four washes in PBST, 100µL rabbit anti-FLAG (Sigma-Aldrich) at a concentration of 1 µg mL^{-1} diluted in PBSTM was added. The plate was incubated for one hour at 37 ºC with shaking, washed four times in PBST and then 100 µL goat HRP-conjugated anti-rabbit immunoglobulin at a concentration of 1 µg mL^{-1} in PBSTM was added. After incubation with shaking at 37 ºC for one hour, the plate was washed four times in PBST, OPD developer was added and the plate read in the same way as method one (Figure 3.3).

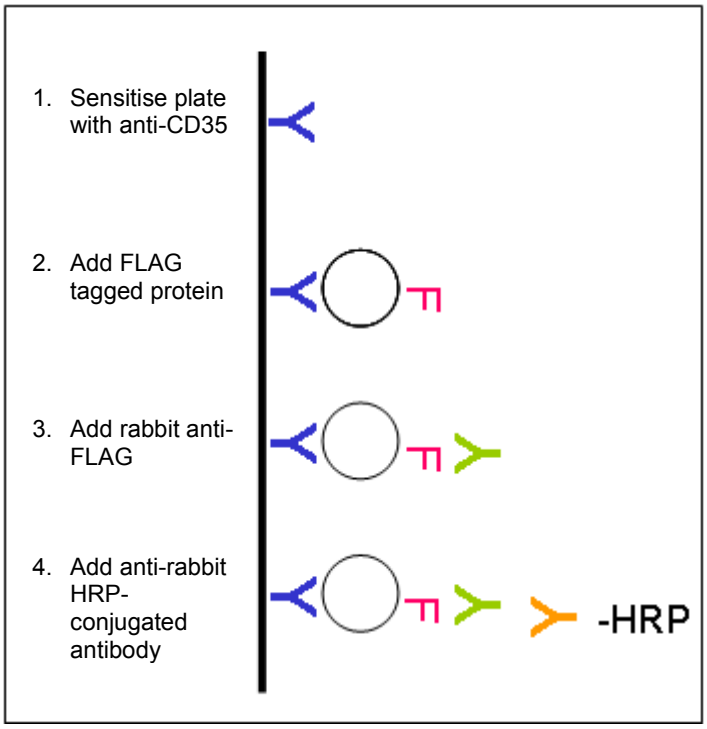

Figure 3.3 ELISA development method two. Coloured antibodies denoted Y: Blue is anti-CD35, green is rabbit anti-FLAG, orange is goat HRP-conjugated anti-rabbit Ig.

3.2.2.3 ELISA development method three

High protein binding microtitre plates (Immulon™ 2HB) were first sensitised with 100 µL goat anti-mouse IgG (Sigma-Aldrich) at a concentration of 1 µg mL^{-1} in pH 9.6 carbonate coating buffer. Plates were left at 4 ºC prior to use (minimum 12 hours), before washing once in PBST followed by incubation with 100 µL PBSTM for one hour at 37 ºC with shaking. The plates were then washed once in PBST and anti-CD35 added. Then method two was followed (Figure 3.4).

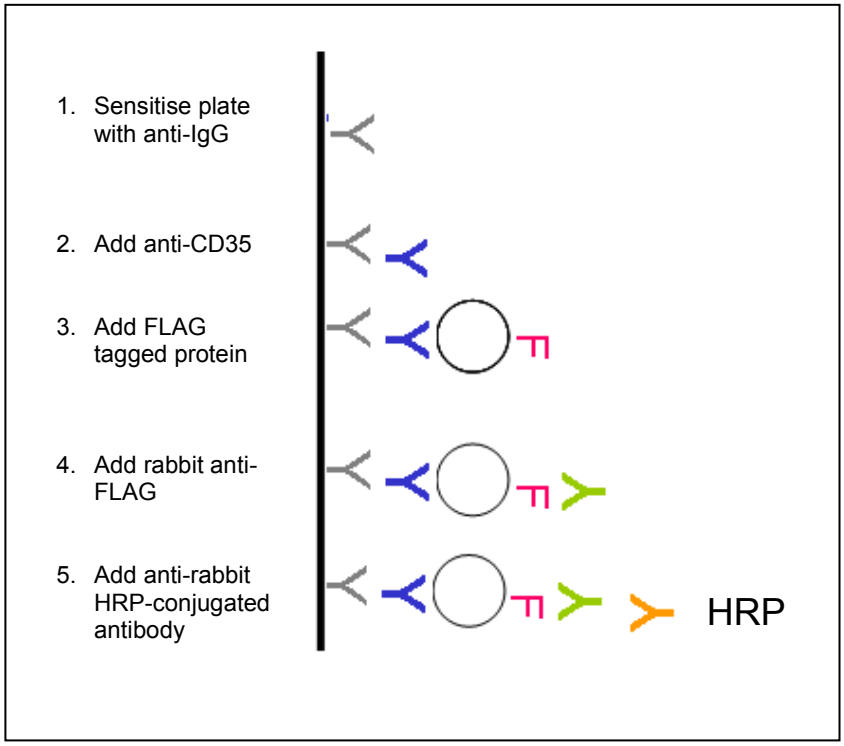

Figure 3.4 ELISA development method three. Coloured antibodies denoted Y: grey is goat anti-mouse IgG, blue is mouse monoclonal anti-CD35, green is rabbit anti-FLAG, orange is goat HRP-conjugated anti-rabbit Ig.

Method three was also used with anti-CD35 (clone J3D3; Santa Cruz Biotechnology Inc, Dallas Texas) an alternative mouse monoclonal antibody raised against full length native CD35 of human origin.

3.2.3 Comparison of different cell culture methods

HEK293 cells stably transfected with 3xFLAG -LHR-C were grown in three cell culture systems. The intention was to grow them in all systems for the same amount of time. Five T-175 flasks were seeded with stably transfected HEK293 cells and cultured over a period of 35 days. Supernatant was harvested when the cells were approximately 80% confluent (between 5 to 7 days) and then flasks were reseeded (Materials and Methods section 2.2.5). Harvested supernatant was stored frozen at -30 ºC. Prior to purification the supernatant was thawed and 0.2 µm filtered. A total of 650 mL supernatant was collected and pooled.

A spinner flask containing HEK293 cells stably transfected with 3xFLAG-LHR-C was cultured over a period of 34 days (following the method described in Materials and Methods section 2.2.6). Cell culture supernatant was harvested at approximately 11 day intervals and 0.2 µm filtered. The flask was topped up with fresh culture medium after each harvest. A total of 800 mL cell culture supernatant was collected and stored frozen at -30 ºC. Prior to purification the supernatants were thawed and pooled.

The CellMax® system once primed with stably transfected HEK293 cells was cultured for 29 days (Materials and Methods section 2.2.7) and then had to be stopped due to a fungal infection in the system, probably due to a loose tube connection. Whilst it was in operation, cell culture supernatant was harvested twice weekly and stored frozen at -30 ºC. It was thawed, 0.2 µm filtered and pooled prior to use, giving a total volume of 720 mL. Three 100 mL aliquots of

pooled cell culture supernatant from each of the systems were purified on 1 mL anti-FLAG affinity gel columns, at a flow rate of 1 mL per minute, and eluted with 3xFLAG peptide (Materials and Methods section 2.3.2). The yield of protein (µg) produced from each culture system was calculated by BCA protein assay (Materials and Methods section 2.3.3). The approximate molecular weight (Mr) of the purified protein was assessed by SDS-PAGE (Materials and Methods section 2.3.4) followed by western blotting onto PVDF membranes (Materials and Methods section 2.3.5). The membranes were Coomassie blue protein stained (Materials and Methods section 2.3.7) or immunoblotted with anti-FLAG M2-Peroxidase or anti-CD35 clones E-11 and J3D3 (Materials and Methods 2.3.6). Protein purity was assessed by immunoblotting with anti-mouse-HRP and anti-BSA-HRP (Materials and Methods section 2.3.6). For all the blots described, 2 µg of purified protein was used. Protein antigenicity i.e. whether it expressed functional KN antigens was measured by its ability to inhibit anti-KN antibodies using the CAT inhibition test (described in Materials and Methods section 2.5.3).

3.3 Results

3.3.1 Sequencing of p3xFLAG-LHR-C and p3xFLAG-LHR-D plasmid DNA used to transfect HEK293 cells

Sequencing results for both p3xFLAG-LHR-C and p3xFLAG-LHR-D were as expected and matched the CR1 reference sequence (GenBank accession number Y00816). The nucleotides giving rise to KN antigens were as expected as shown in Table 3.1 below.

Table 3.1 The KN antigens in the CR1 reference sequence

Position	*Nucleotide*	*KN antigen*
4708	G	Kn^a (KN1)
4795	A	McC^a (KN3)
4828	A	Sl^a (KN4)
4223	C	Yk^a (KN5)
4870	A	KCAM (KN9)

Nucleotides giving rise to KN antigens in the CR1 reference sequence GenBank accession number Y00816, are shown.

A primer was designed to sequence p3xFLAG-LHR-D around the Kn^a / Kn^b polymorphism. The Kn^a SNP that was detected by the newly designed primer is shown in the sequence fragment for p3xFLAG-LHR-D illustrated in Figure 3.5. G is seen at position 4708 which codes for the Kn^a antigen (the full sequence analysis is included in Appendix 1).

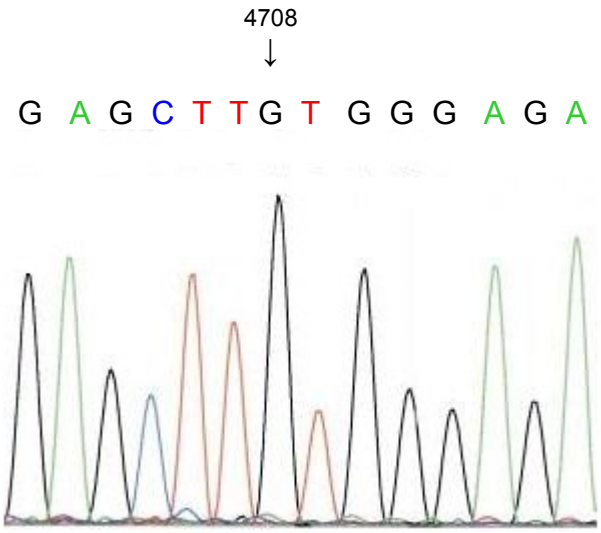

Figure 3.5 Sequence fragment showing the Kn^a/Kn^b SNP. This sequence fragment for p3xFLAG-LHR-D shows G at position 4708 coding for the Kn^a antigen (chromatogram and sequence analysed using SeqScape software).

3.3.2 ELISA for detection of 3xFLAG tagged sr-LHR-C and sr-LHR-D protein

The three ELISA methods described were compared for sensitivity so that protein production by transfected HEK293 cells could be measured during cloning to ensure that the highest protein producing clones were selected. No reactions were obtained with sr-LHR-C by either method one or two. Monoclonal anti-CD35 E-11 was used in both methods as this was reported to react with both sr-LHR-C and sr-LHR-D (Nickells *et al.*, 1998). Using method three, reactions were obtained with both sr-LHR-C and sr-LHR-D protein. Although the reactions with sr-LHR-C were not as strong as for sr-LHR-D they were still strong enough to be able to distinguish the highest sr-LHR-C secreting clones during the cloning process. This ELISA method was used during the cloning process to test cell culture supernatants. During the cloning process typical OD 492 values for sr-LHR-C were around 0.2 and for sr-LHR-D 0.5 with a background of 0.05. These values were sufficiently different from the background to be distinguishable.

Purified protein was used to establish standard curves so that the initial concentration of protein in the cell culture supernatants could be calculated. Some success was achieved with sr-LHR-D. Figure 3.6 shows a curve for sr-LHR-D based on duplicate samples, diluted from 0 to 600 ng mL^{-1} sr-LHR-D. However, the linear part of the curve was within a very narrow range. One sr-LHR-D cell culture supernatant tested on this plate, in duplicate, had a mean OD 492 reading of 0.381 which, from the graph gives a value of approximately 80 ng mL^{-1}. This supernatant had a volume of 100 mL so therefore appeared to contain 8 µg protein. After purification the protein yield was estimated by

BCA assay as 42 µg which is much higher than expected. It was found with several cell culture supernatants that this ELISA method was not quantitative.

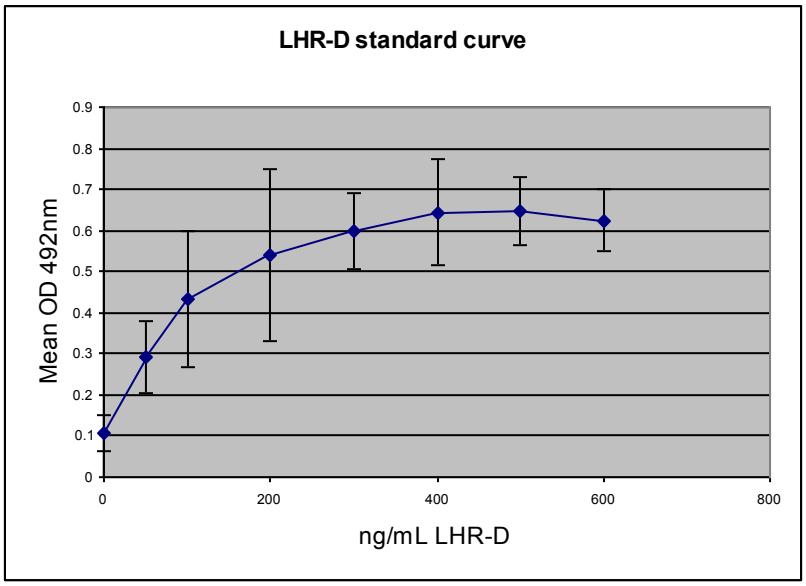

Figure 3.6 Standard curve for sr-LHR-D tested by ELISA method three (n=3). Error bars represent standard deviations calculated from the the data.

After the cloning had been performed another monoclonal anti-CD35, clone J3D3, was obtained. This was reported to react with LHR-C but not LHR-D (Nickells *et al.*, 1998). A comparison of the two monoclonal anti-CD35 reagents and reactions with sr-LHR-C proteins was performed by ELISA (Figure 3.7). Although slightly stronger reactions were obtained using anti-CD35 antibody J3D3 the OD 492 readings were still very low.

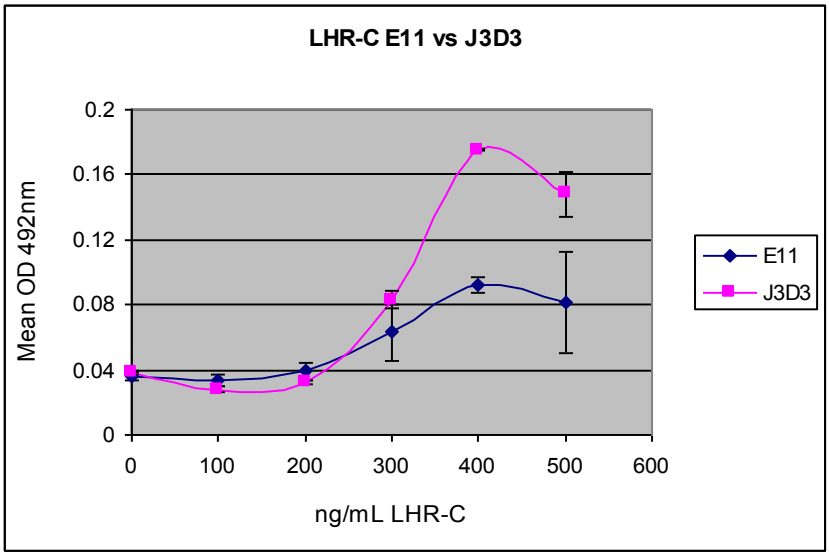

Figure 3.7 Standard curves for sr-LHR-C. Two clones of anti-CD35, E-11 and J3D3 were tested against sr-LHR-C flag tagged protein by ELISA method 3 (n=3). Error bars represent standard deviations calculated from the the data.

Western blotting with both anti-CD35 reagents gave very weak reactions with purified sr-LHR-C protein. The same amount of purified sr-LHR-D was also used (i.e. 2 µg) and much stronger reactions were obtained with both anti-CD35 reagents (Figure 3.8). It appeared that anti-CD35 clone J3D3 had reacted with sr-LHR-D. This is contrary to the report by Nickells *et al.*, (1998) which stated that anti-CD35 clone J3D3 did not react with LHR-D. However, when compared to reactions with anti-CD35 E-11, no band can be seen at approximately 65-80 kDa which is probable sr-LHR-D. Bands were seen with both anti-CD35 reagents on the sr-LHR-D samples at approximately 20-25 kDa and 50 kDa. It was thought that these could be due to mouse immunoglobulins in the eluate reacting with HRP-conjugated anti-mouse secondary antibody used to detect the mouse derived anti-CD35 reagents. The mouse immunoglobulins could be anti-FLAG M2 antibodies eluted from the affinity gel column during the purification process. During the purification

process using an affinity gel column, the sr-LHR-D had been eluted by acid whereas the sr-LHR-C had been eluted with 3XFLAG peptide. A difference was seen in that mouse IgG appeared to be only in samples eluted with acid. This was investigated further (section 3.3.3) and also in the Protein Purification Results section 4.3.1.

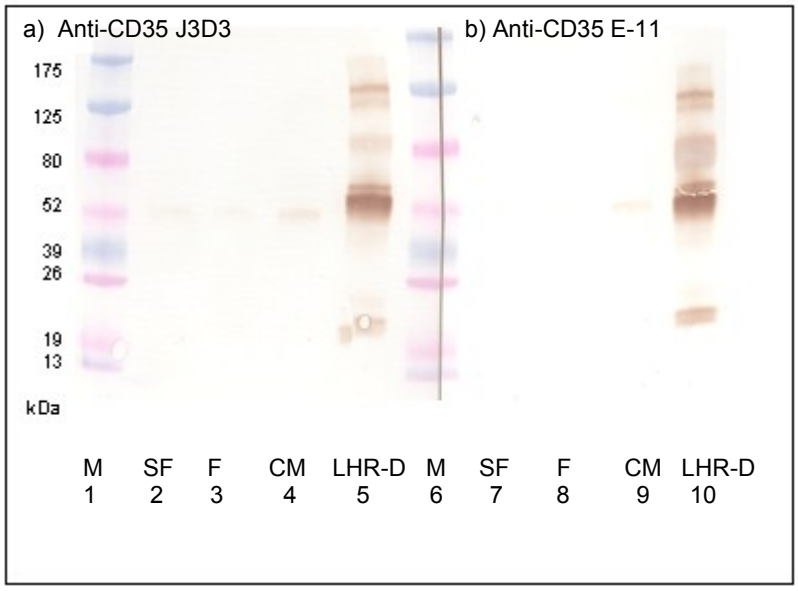

Figure 3.8 Western blots probed with two different clones of anti-CD35. Proteins were immunostained with a) anti-CD35 clone J3D3 and b) anti-CD35 clone E-11. Purified sr-LHR-C protein from supernatants harvested from: spinner flasks (SF) are in lanes 2 and 7; flasks (F) in lanes 3 and 8 and CellMax® (CM) in lanes 4 and 9. A purified pooled sr-LHR-D sample was also tested for comparison (LHR-D) (lanes 5 and 10). M= molecular marker in lanes 1 and 6.

3.3.3 Comparison of different culture methods

Figure 3.9 shows the sr-LHR-C protein yield from cell culture supernatant grown in spinner flasks, T-175 flasks and in the CellMax® system. A 1 mL affinity gel column was used for each method to purify three 100 mL aliquots of pooled material from each culture system. It was recognised that the number of uses of the gel may possibly affect yield but the same regime was used for all three systems so that the gels were used a total of 3 times for each system and the mean protein yield (µg) was calculated.

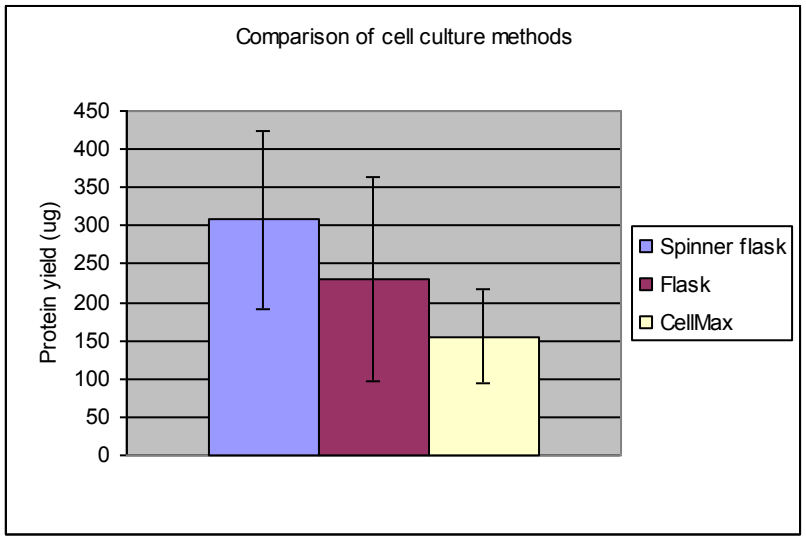

Figure 3.9 The yield of protein from three different culture methods. The protein yield obtained from culture supernatants grown in spinner flasks, T-175 flasks and the CellMax system, after purification by affinity gel chromatography, is compared. Error bars represent standard deviations calculated from the data (n=3).

Mean protein yield appeared to decrease from spinner flasks (308 µg) to T-175 flasks (230 µg) to CellMax® (156 µg). An ANOVA statistical test was performed on the data (Kirkman, 1996) assuming the null hypothesis that there was no difference in protein yields between the culture methods used.

Based on between samples variance (df_B) of 2, within samples variance (df_w) of 6, confidence interval of 0.05, the probability of this result, assuming the null hypothesis, was 0.304. This is less than the critical value of 5.14 (from tables in Ashcroft and Pereira, 2003), so the null hypothesis cannot be rejected i.e. there is no statistical significance between yields for the three techniques used.

Protein staining and an immunoblot with HRP-conjugated anti-FLAG are shown in Figure 3.10. The protein detected had an approximate Mr of 65-80 kDa which is larger than the estimated Mr of approximately 50 kDa for LHR-C based on amino acid sequence but not accounting for glycosylation.

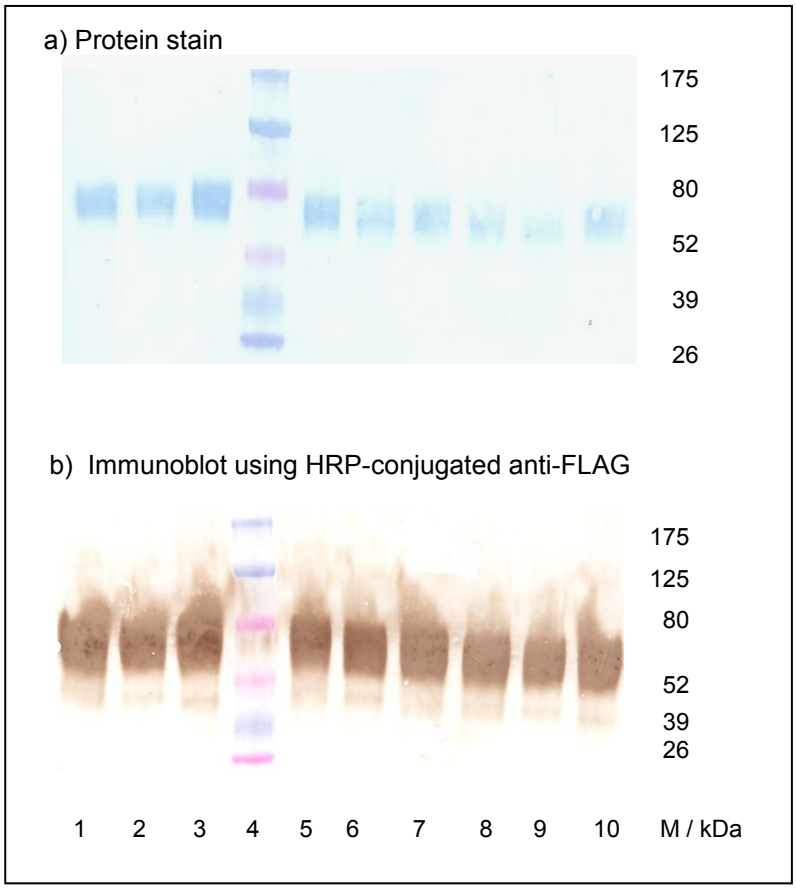

Figure 3.10 Protein stain and western blot for sr-LHR-C purified from supernatants from different culture methods. a) Protein stained western blot and b) immunoblot using HRP-conjugated anti-FLAG. Three samples from each culture system were run in sequential order of number of times the affinity gel had been used for purification. On both blots: lanes 1, 5 and 8 are samples from spinner flasks; lanes 2, 6 and 9 are samples from T-175 flasks and lanes 3, 7 and 10 are samples from the CellMax system. A marker was run in lane 4. .

The nine purified samples were tested by SDS-PAGE and the gels were western blotted and tested with anti-mouse immunoglobulin (to detect mouse anti-FLAG contamination from the affinity column) and anti-BSA (to detect bovine albumin contamination from the cell culture medium) as presence of either could affect the overall sr-protein yield. The blots in Figures 3.11 and 3.12 show that there are only trace amounts of both proteins present. BSA standards were included on the gel (Figure 3.12) at 1.0, 0.5 and 0.25 µg.

BSA was not present in all of the samples but in those that it was, it was well below 0.25 µg. The level of BSA contamination is investigated further in Protein Purification Results section 4.0.

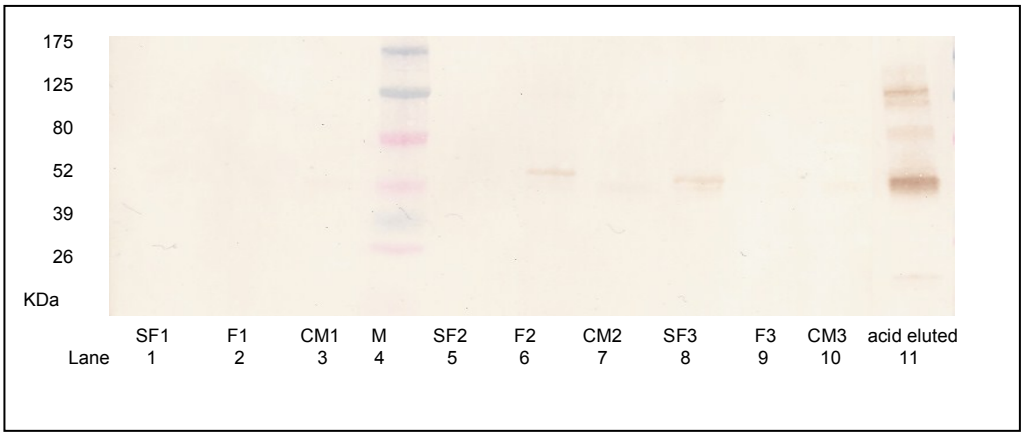

Figure 3.11 Western blot to check for contaminating mouse Ig in purified sr-LHR-C samples. Purified sr-LHR-C protein from supernatants harvested from SF = spinner flask, F = T-175 flask, CM = CellMax system were tested using HRP-conjugated anti-mouse immunoglobulin. Samples were purified on affinity gels that had been used either once, twice or three times, hence three samples from each growth system were obtained. Samples from spinner flasks were run in lanes 1, 5 and 8. Samples from T-175 flasks were run in lanes 2, 6 and 9 and samples from the CellMax system were run in lanes 3, 7 and 10. All were eluted with 3XFLAG peptide. An acid eluted sample containing mouse Ig was included for comparison (lane 11). A molecular marker (M) was run in lane 4.

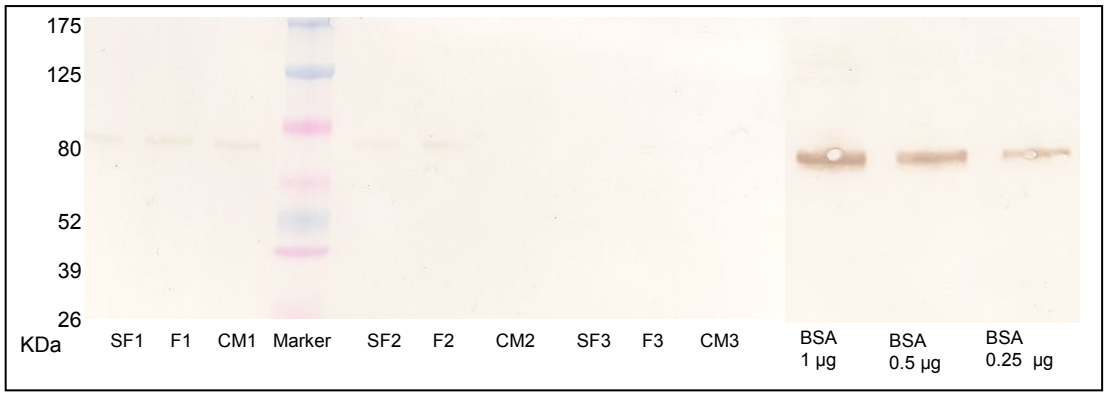

Figure 3.12 Western blot to check for contaminating BSA in purified sr-LHR-C samples. Purified sr-LHR-C protein from supernatants harvested from: SF = spinner flask, F = T-175 flask, CM = CellMax system were tested using HRP-conjugated anti-BSA. Samples were purified on affinity gels that had been used either once (1), twice (2) or three times (3), hence three samples from each growth system were obtained. All were eluted with 3XFLAG peptide. Three standards are included containing 1 µg, 0.5 µg and 0.25 µg BSA respectively. A molecular marker was run as indicated.

Sr-LHR-D was also produced in all three systems, and yield of protein was very similar to that obtained for sr-LHR-C. Although only based on purification of one 200 mL cell culture supernatant from each system, purified on one mL gels that had been used a variable number of times, yields of 610 µg, 273 µg and 209 µg were obtained for spinner flask, T-175 flask and CellMax® respectively. They showed the same order of decreasing protein yield from spinner flask to T-175 flasks to CellMax® as for sr-LHR-C in section 3.3.3 (where yields were 308 µg, 230 µg and 156 µg respectively). Figure 3.13 shows the purified sr-LHR-D compared to purified sr-LHR-C.

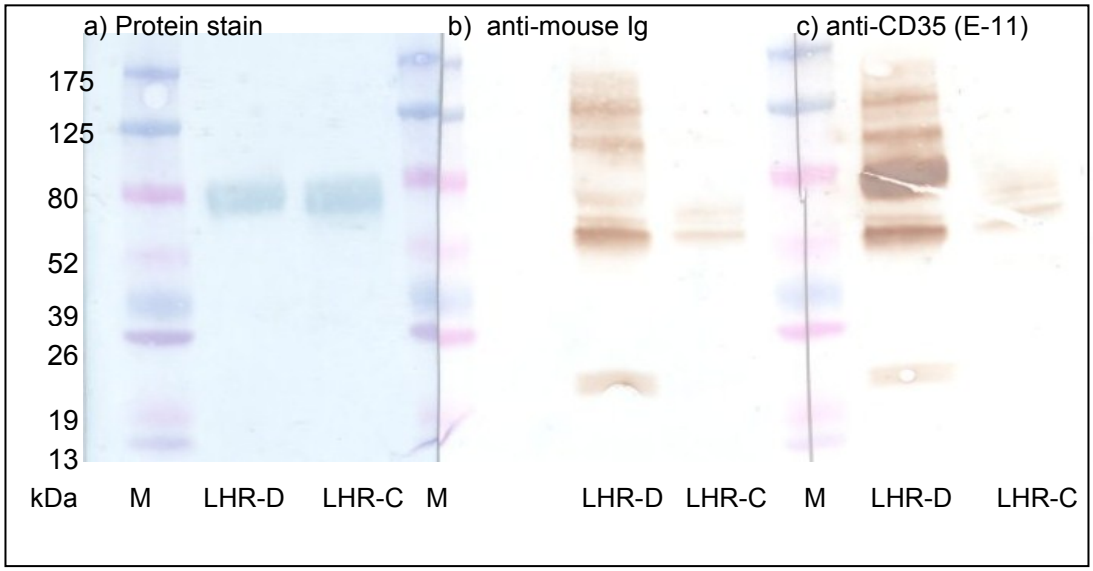

Figure 3.13 A comparison of purified sr-LHR-C and sr-LHR-D proteins. a) a protein stained western blot b) an immunoblot probed with HRP-conjugated rabbit anti-mouse immunoglobulin and c) an immunoblot probed with mouse monoclonal anti-CD35 (clone E-11) primary antibody and HRP-conjugated rabbit anti-mouse immunoglobulin secondary antibody, M=marker.

A difference is again seen between sr-LHR-C and sr-LHR-D with mouse immunoglobulins seen in the acid eluted sr-LHR-D sample but not the peptide eluted sr-LHR-C sample. Reactions with anti-CD35 (clone E-11) are much stronger with sr-LHR-D, with a band seen at approximately 65-80 kDa which corresponds with the band seen on the protein stained membrane. The sr-LHR-C protein appears to be slightly smaller than sr-LHR-D.

3.3.4 Mass spectrometry analysis of sr-LHR-C and sr-LHR-D peptides

Analysis of sr-LHR-C and sr-LHR-D peptides was performed on bands cut from a Coomassie-Blue stained SDS-PAGE gel by Dr K. Heesom at Bristol University. Pooled purified protein samples for both sr-LHR-C and sr-LHR-D were run on the gel and after tryptic digestion these were analysed by MALDI-

TOF. The protein-peptide summary results sheet returned from MALDI-TOF analysis shows the top 20 hits for each sample resulting from a search of the UniProtKB/Swiss-Prot protein sequence database (Swiss Institute of Bioinformatics, 2014). The top 20 hits for each protein are summarised in tables below. The key value for each hit is the protein score. For this search, proteins with a score >56 are classed as significant identification ($p<0.05$). The higher the score above this value, the more confidence there is in the identification (personal communication, Dr K. Heesom). The top hit for both sr-LHR-C and sr-LHR-D is Complement receptor type 1 protein, with protein scores of 129 and 170 respectively. The next best matches have much lower scores than this. The results for sr-LHR-C and sr-LHR-D are summarised in Tables 3.2 and 3.3 respectively.

Table 3.2 MALDI-TOF analysis showing the top 20 hits for sr-LHR-C.

Rank	*Protein name*	*Species*	*Protein accession number*	*Protein score*
1	Complement receptor type 1	*Homo sapiens*	CR1_HUMAN	129
2	DNA primase	*Marek's disease herpesvirus type 1 strain*	PRIM_GAHVM	59
3	Isopentenyl-diphosphate delta-isomerase	*Chlorobium limicola DSM 245*	ID12-CHLL2	54
4	Leucine-tRNA ligase	*Enterobacter sp.638*	SYL_ENT38	52
5	Acetate kinase	*Mycoplasma pneumoniae*	ACKA_MYCPN	52
6	Leucine-tRNA ligase	*Ignicoccus hospitalis*	SYL_IGNH4	50
7	Annexin A8	*Homo sapiens*	ANXA8_HUMAN	49
8	Annexin A8-like protein 2	*Homos sapiens*	AXA82_HUMAN	49
9	Uncharacterised protein KIAA0753	*Mus musculus*	K0753_MOUSE	49
10	Uncharacterised protein MJ1518	*Methanocaldococcus jannaschii DSM*	Y1518_METJA	49
11	DNA mismatch repair protein Msh2	*Bos taurus*	MSH2_BOVIN	48
12	Thiopurine S-methyltransferase	*Photobacterium profundum*	TPMT_PHOPR	48
13	Spectrin alpha chain, erythrocyte	*Mus musculus*	SPTA1_MOUSE	48
14	NADH-quinone oxireductase subunit D	*Xylella fastidosa Temecula 1*	NUOD_XYLFT	48
15	Casein kinase II subunit alpha	*Schizosaccharomyces pombe 972h-*	CSK2A_SCHPO	48
16	Ornithine carbamoyltransferase	*Campylobacter jejuni subsp. Jejuni 81116*	OTC_CAMJ8	48
17	Heparin cofactor 2	*Rattus norvegicus*	HEP2_RAT	46
18	30S ribosomal protein S3	*Sinorhizobium meliloti*	RS3_RHIME	46
19	30S ribosomal protein S3	*Sinorhizobium medicae*	RS3_SINMW	46
20	Lipoyl synthase	*Methylocella silvestris BL2*	LIPA_METSB	46

Table 3.3 MALDI-TOF analysis showing the top 20 hits for sr-LHR-D.

Rank	*Protein name*	*Species*	*Protein accession number*	*Protein score*
1	Complement receptor type 1	*Homo sapiens*	CR1_HUMAN	170
2	Serum albúmina	*Bos taurus*	ALBU_BOVIN	68
3	P53 and DNA damaged regulated protein 1	*Pongo abelii*	PDRG1_PONAB	61
4	Calcium-binding mitochondrial cancer protein	*Xenopus (Silurana) tropicalis*	SCMC2_XENTR	60
5	ATP synthase subunit alpha	*Granulobacter bethesdensis CGDNIH1*	ATPA_GRABC	60
6	Structural maintenance of chromosome protein 3	*Dictyostelium discoideum*	SMC3_DICDI	57
7	DNA polymerase catalytic subunit	*Human herpesvirus 6 strain Z29*	DPOL_HHV6Z	56
8	Cytoplasmic FMR1-interacting protein 2	*Pongo abelii*	CYFP2_PONAB	55
9	Tetratricopeptide repeat protein 12	*Homo sapiens*	TTC12_HUMAN	54
10	Exodeoxyribonuclease 7 large subunit	*Listeria monocytogenes serotype 4b*	EX7L_LISMF	53
11	ComG operon protein 1 homolog	*Bacillus halodurans*	COMGA_BACHD	52
12	Exodeoxyribonuclease 7 large subunit	*Listeria monocytogenes*	EX7L_LISMO	52
13	DNA ligase	*Thioalkalivibrio sulfidophilus HL-EbGr7*	DNLJ_THISH	52
14	NusA protein homolog	*Methanocaldococcus jannaschii DSM*	NUSA_METJA	52
15	Aspartate-tRNA ligase	*Nitrospira multiformis ATCC 25196*	SYD_MITMU	51
16	Probable GTP-binding protein EngB	*Mycoplasma pneumoniae*	ENGB_MYCPN	51
17	Ribosome biogenesis GTPase A	*Bacillus cereus ATCC 14579*	RBGA_BACCR	50
18	Uncharacterised protein AF_0844	*Archaeoglobus fulgidus DSM 4304*	Y844_ARCFU	50
19	Succinyl-CoA ligase [ADP forming] subunit beta	*Sulfurimonas denitrificans DSM 1251*	SUCC_SULDN	49
20	Putative uncharacterised protein y4kQ	*Sinorhizobium fredii NGR234*	Y4KQ_RHISN	49

In addition, in this mass spectrometry investigation, the most intense peaks in the identified protein (CR1) were selected for sequencing and these results were reported as ion scores (Dr K.Heesom, personal communication). This means that they were run in the MSMS mode of the mass spectrometer and the resulting amino acid sequence matched the identified protein which again increased confidence in the identification. Three sequences for both LHRs

were reported, those for LHR-C are shown in Figure 3.14 and sequences for LHR-D are shown in Figure 3.15.

```
                                                        QAPDHFLFAKLKTQTNA
 961 SDFPIGTSLKYECRPEYYGRPFSITCLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITD
1021 IQVGSRINYSCTTGHRLIGHSSAECILSGNTAHWSTKPPICQRIPCGLPPTIANGDFIST
1081 NRENFHYGSVVTYRCNLGSRGRKVFELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTP
1141 PNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPP
1201 PEILHGEHTPSHQDNFSPGQEVFYSCEPGYDLRGAASLHCTPQGDWSPEAPRCAVKSCDD
1261 FLGQLPHGRVLFPLNLQLGAKVSFVCDEGFRLKGSSVSH VLVGMRSLWNNSVPVCEHIF
1321 CPNPPAILNGRHTGTPSGDIPYGKEISYTCDPHPDRGMTFNLIGESTIRCTSDPHGNGVW
1381 SSPAPRCELSVRAG
```

Figure 3.14 Mass spectrometry results for sr-LHR-C. The amino acid sequence for LHR-C is shown with the three mass spectrometry sequences highlighted. These were the three most intense peaks obtained for the top hit, CR1, which were selected, and successfully sequenced. They matched sequences in the specified protein (CR1), giving further confidence that the protein is CR1.

```
                    HCKTPEQFPFASPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCL
1441 ENLVWSSVEDNCRRKSCGPPPEPFNGMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLV
1501 SGNNVTWDKKAPICEIISCEPPPTISNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFEL
1561 VGERSIYCTSKDDQVGVWSSPPPRCISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQ
1621 PGFVMVGSHTVQCQTNGRWGPKLPHCSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCE
1681 PSYDLRGAASLHCTPQGDWSPEAPRCTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCD
1741 EGFRLKGRSASHCVLAGMKALWNSSVPVCEQIFCPNPPA LNGRHTGTPFGDIPYGKEIS
1801 YACDTHPDRGMTFNLIGESSIRCTSDPQGNGVWSSPAPRCELSVPA
```

Figure 3.15 Mass spectrometry results for sr-LHR-D. The amino acid sequence for LHR-D is shown with the three mass spectrometry sequences highlighted. These were the three most intense peaks obtained for the top hit, CR1, which were selected, and successfully sequenced. They matched the sequences in the specified protein (CR1), giving further confidence that the protein is CR1.

The second hit for sr-LHR-D is BSA (Table 3.3), which could be a possible contaminant of the purified protein, from the cell culture medium. The peptides mapped to CR1 are mostly distinct from those mapped to BSA (only two, of mass 1439 and 2462, are common to both) and neither of these was

sequenced. Therefore this might imply that BSA is present at low amounts. However, given the much higher score for CR1 it is probably most likely that these peptides come from CR1 rather than BSA. If they were removed from the BSA list this may well drop the protein score for BSA below the significance threshold (K. Heesom, personal communication).

3.3.5 Antigenicity of purified proteins

The nine purified protein sr-LHR-C samples were used in the CAT inhibition test. This test is described in Materials and Methods section 2.5.3. A patient plasma sample (5635H) containing anti-KN antibodies was used. When tested with a reagent screening cell this sample gave a positive reaction indicating that KN specific antibodies in the patient's plasma had reacted with a KN specific antigen on the red cells and caused agglutination (positive control). A full investigation of this sample (performed as part of routine investigation in RCI, Filton) determined that no other antibodies directed against red blood cell antigens were present. Samples of this patient plasma were incubated with purified sr-LHR-C protein (nine samples tested individually) prior to performing the CAT test. All samples were negative by CAT, post incubation, indicating that the purified protein had inhibited the antibody so that it could not react with antigen on the reagent screening cell. A control sr-protein, specifically a type I sr-protein like the CR1 protein, could have been used in these tests instead of PBS. However, this would have added cost to the test, so a control containing the same volume of PBS substituted for purified protein was used. A positive reaction confirms that the negative reactions obtained with sr-protein were not due to a dilution of the

antibody samples. The inhibition test was only performed with one patient plasma sample due to the rarity of samples, and the quantity of sample needed for this testing, but it is does show that all the purified protein samples had KN antigen specificity (results shown in Table 3.4).

Table 3.4 CAT inhibition test using sr-LHR-C

		Neat plasma plus:									
	Neat Plasma	**PBS**	**SF1**	**SF2**	**SF3**	**F1**	**F2**	**F3**	**CM1**	**CM2**	**CM3**
5635H	2	2	0	0	0	0	0	0	0	0	0

Nine purified samples of sr-LHR-C, three each from cell culture grown in spinner flask (SF1, SF2 and SF3), T-175 flasks (F1, F2,F3) and CellMax™ (CM1, CM2 and CM3) were used in the CAT inhibition test against patient plasma (sample 5635H) known to contain a KN related antibody. Positive reactions were scored on a scale of 1 to 5 where 5 is the strongest reaction, a negative test is recorded as 0. The antibody can be seen to be reactive in the plasma sample and when diluted with an equal volume of PBS rather than sr-LHR-C. The negative reactions when sr-LHR-C is added to patient's plasma indicate that all the purified sr-LHR-C preparations inhibited the KN related antibody.

3.3.6 Comparison of costing for different culture methods

The cost of equipment and reagents used for cell culture in the three different systems, shown in Table 3.5, was compared from the point when the systems were seeded with transfected HEK293 cells. Some of the equipment is reusable: spinner flasks and the reservoir caps for the CellMax® system can be washed, sterilised and reused but for the purposes of this calculation it was

assumed that this was the first time of use to offset the handling costs. The Duo system pump for CellMax® system can also be reused many times so its cost was not included in the calculations. T-175 flasks can be used several times but more often new flasks were used so it was assumed that new flasks were used each time the flasks were reseeded.

Table 3.5 The cost of equipment and reagents needed for the three culture systems.

Equipment	***Cost (£)***	***Reagent***	***Cost (£ per litre)***
500 mL spinner flask	415.00	DMEM	5.32
T-175 flask	1.75	FCS	165.50
CM reservoir cap	206.00	PS	46.50
		LG	35.00
		Trypsin-EDTA	42.50

Culture medium was used with 10% FCS added for the spinner flask, T-175 flasks and for flushing the bioreactor in the CellMax™ system. For the reservoirs for the CM system 5% FCS was used. Costs per litre for the two types of culture medium, both containing 1% PS and 1% LG are shown in Table 3.6. Equipment and reagent costs are shown in Table 3.7.

Table 3.6 The constituents and cost of cell culture medium containing 5% or 10% FCS.

Culture medium constituent	**Cost (£ per litre cell culture medium)**
DMEM	5.32
10% FCS	16.55
5% FCS	8.28
1% PS	0.47
1% LG	0.35
Total 10% FCS	**22.69**
Total 5% FCS	**14.42**

Table 3.7 The equipment and reagent costs for pools of sr-protein produced from each system

Cell culture system	**Equipment / reagents**	**Cost (£)**
Spinner flask (600 mL pool)	500 mL spinner flask	415.00
	750 mL culture medium (10% FCS)	17.02
T-175 flask (650 mL pool)	26 x T-175 flasks	45.50
	650 mL culture medium (10% FCS)	14.75
	130 mL trypsin-EDTA	5.53
CellMax™ (720 mL pool)	Reservoir cap	206.00
	20 kDa MWCO bioreactor	355.00
	720 mL culture medium (10% FCS)	16.34
	4000 mL culture medium (5% FCS)	57.68
	Total spinner flask	**432.02**
	Total T-175 flask	**65.78**
	Total CellMax™	**635.02**

The actual volume from each pool that was purified was 300 mL. The total cost of equipment and reagents needed to produce 1 mL protein with a concentration of 1 mg mL^{-1} was calculated and is shown in Table 3.8.

Table 3.8 The equipment and reagent costs to produce 1 mg of protein from each system.

System	***Cost (£ for 300 mL)***	***Total protein yield (µg) from 300 mL***	***Cost of 1 mL protein with concentration of 1 mg mL^{-1} (£)***
Spinner flask	216.01	924	233.78
T-175 flask	30.36	691	64.40
CellMax™	264.59	467	566.57

Finally, staff costs, shown in Table 3.9, were calculated assuming a Biomedical Scientist Specialist did this work, paid at midpoint of the AFC band 6 grade with 22% added for 'on costs' i.e. National Insurance and pension payments. This would be £36,600 per annum, at an hourly rate of £18.77.

Table 3.9 Staff costs to produce 1 mg of sr-protein.

System	***Hands on time (hours)***	***Cost per pool (£)***	***Cost for 300 mL (£)***	***Cost of 1 mL protein (1 mg per mL)***
Spinner flask	2	37.54	14.08	6.35
T-175 flask	6	112.62	51.98	75.22
CellMax™	10	187.70	78.21	167.47

An assumption was made that a minimum of 9 mL of reagent (at 1 mg mL^{-1}) would be needed for NHSBT laboratories per year. This was based on approximately 30 panels using 30 µL of KNIR each time being performed by each laboratory per year (personal experience). Based on this then annual production costs would be:

Spinner flasks (233.78 + 6.35) x 9 = £2161.17

T-175 flasks (64.40 + 75.22) x 9 = £1256.58

CellMax™ (566.57 + 167.47) x 9 = £6606.36

The cost of protein purification is calculated in the next results section, Protein Purification.

3.4 Discussion

The purpose of this part of the study was to maximise production of sr-LHR protein from transfected HEK293 cells so that a reagent could be produced and supplied to eight NHSBT RCI laboratories and also IBGRL based in Filton. In addition it had to be shown that the protein produced was of the correct specificity and retained its blood group antigenicity.

The plasmids used to produce sr-proteins were validated by DNA sequencing prior to transfection into HEK293 cells. Samples of both sr-LHR-C and sr-LHR-D purified protein were sent for mass spectrometry analysis, which showed that the protein produced was CR1 related. This protein inhibited anti-KN antibody in the CAT inhibition test. It was also found that there was very little contamination with BSA or mouse immunoglobulin.

Traditionally, production of sr-proteins in mammalian cells has been based on transfection and then isolation of clonal cell lines stably expressing the required gene product (Pham *et al.*, 2006). This was the approach taken in this study, which enabled a frozen cell bank of stably transfected HEK293 cells expressing sr-LHR-C or sr-LHR-D protein, to be set up for future use. To enable the highest protein producing clones to be selected an ELISA method was developed (section 3.3.2). The first method tested was the simplest and fastest to perform relying on direct detection of sr-3xFLAG tagged protein with an HRP-conjugated anti-FLAG antibody. However, this method failed to detect sr-LHR-C protein. In this method monoclonal anti-CD35, clone E-11 was used to sensitise the microtitre plate. This antibody had been reported to

react with both LHR-C and LHR-D regions of the CR1 (CD35) molecule (Nickells *et al.*, 1998). However, it was reported by Tamasauskas *et al.*, (2001), that although LHR-D completely inhibited reactivity of anti-CD35 E-11 as measured by flow cytometry, only 50% reduction of reactivity was seen using sr-LHR-C indicating that sr-LHR-C would not react as strongly as sr-LHR-D with this anti-CD35. The second ELISA method was then used to increase sensitivity. This method, like the first, started with sensitisation of the plate with anti-CD35 followed by addition of FLAG-tagged protein but then an additional step was introduced where a primary rabbit anti-FLAG antibody was added. This was followed by addition of a secondary HRP-conjugated anti-rabbit Ig antibody. This indirect ELISA technique is generally more sensitive than a direct one due to signal amplification gained by use of the secondary antibody (Kemeny, 1991). Sr-LHR-C protein was still not detected by this technique. The third method was effectively a capture sandwich assay technique. Anti-mouse immunoglobulin was initially used to sensitise the plate so that the mouse anti-CD35 antibodies would be captured on the plate in the correct orientation to react more efficiently with the CD35 protein in the culture supernatant. Positive reactions were obtained with sr-LHR-C by this method, although reactions were not as strong as seen with sr-LHR-D-3xFLAG protein. Reactions were suitably strong enough to be able to distinguish the highest secreting clones during the cloning process.

It was hoped that this ELISA could be used to assess initial concentrations of FLAG tagged proteins in the cell culture supernatants. If estimates of the sr-protein in the starting material could be made then percentage recovery

during the purification process could be calculated. Attempts at using purified sr-LHR protein of known concentration to set up standard curves were not very successful (Figures 3.6 and 3.7). When a 'curve' was produced then generally the linear part of the curve lay within a very small range, typically between 100 to 200 ng mL^{-1} for sr-LHR-D. This meant that it was difficult to get sample OD readings within this range. The main problem was that the assay did not appear to be quantitative. Protein estimates from the starting cell culture supernatant appeared to be too low when compared to the purified protein obtained. It did not appear as though there was a linear relationship between optical densities (492 nm) or protein concentration and probably reflected deficiencies in assay optimisation which would have taken a lot of time to overcome. As it was not necessary to have a precise measure of concentration of a given sample, optimisation of the assay was not pursued as part of this study.

An alternative anti-CD35, antibody clone J3D3, was compared to anti-CD35 clone E-11 with slightly stronger reactions obtained with this reagent by ELISA. Each sr-LHR protein gave very weak reactions when blotted with either anti-CD35 reagents. A sr-LHR-D sample included as comparison seemed to react with both anti-CD35 reagents. These results appeared to contradict a study reported by Nickells *et al.*, (1998). This group reported that anti-CD35 clone J3D3 did not react with sr-LHR-D protein, whilst anti-CD35 clone E-11 did react with sr-LHR-D. However, on closer inspection of the western blots in Figure 3.8 it was clear that anti-CD35 clone J3D3 had not reacted with sr-LHR-D. A band at just below 80 kDa seen on the blot with

anti-CD35 clone E-11 was missing from the anti-CD35 clone J3D3 blot. The reactions seen were due to HRP-conjugated anti-mouse IgG reacting with mouse immunoglobulin in the sr-LHR-D preparation. There was apparently no mouse immunoglobulin in the sr-LHR-C protein. The presence of mouse immunoglobulins in the purified sr-protein preparations is investigated and discussed further in section 4.0, Protein Purification.

The cloning process was time consuming taking approximately 14 weeks to perform three rounds of cloning. Although a frozen cell bank has now been established it might be that a future development would be to use transiently transfected cells. HEK293E cells expressing the Epstein-Barr virus nuclear antigen (EBNA) have enhanced productivity of r-protein due to high levels of plasmid amplification. These cells have been used for large scale transient gene expression (Swiech *et al.*, 2012). This is a rapid technique with cells capable of producing milligram to gram amounts of recombinant protein, but it does require large numbers of cells and large amounts of purified plasmid and as a method is less easy to standardise (Pham *et al.*, 2006).

HEK293 cells were grown in three systems where they were treated in different ways and had different supernatant harvesting regimes based on standard laboratory procedures. Cells in the spinner flasks were left for up to two weeks before harvesting. This timescale was based on protocols and experience within the laboratory and fitting in with when time could be spent in the laboratory to do the harvesting work. Prior to harvesting, the cells were allowed to settle and supernatant gently removed and new cell medium

added. So with the exception of the stirring itself the cells suffered little disturbance and they tended to form aggregates so they effectively adhered to each other rather than the flask (Cartwright *et al.*, 2002). This culture method must have been conducive for cells to produce sr-protein as protein yield from this system was the highest at around 300 µg from 100 mL of supernatant. This yield compares favourably to that of another sr-blood group antigen grown in HEK293 cells. Lu^b of the Lutheran blood group system was measured at a concentration of 250 µg per litre in pre-purified cell supernatant (Seltsam *et al.*, 2007). The protein produced was V5-His-tagged Lu^b protein but the culture method used was not recorded. The V5-His tag was used in a sandwich ELISA to measure sr-Lu^b concentration, anti-V5 was used as the capture antibody and HRP-anti-His as the detection antibody. Predefined amounts of V5-His-tagged Class I protein were used as a reference to calculate quantity of V5-His-tagged protein present, but not specifically sr-Lu^b protein present.

In addition, it was found in this current study that the spinner flask system was very simple to look after with little hands on time needed, so staff costs were lower than for the other systems used. A disadvantage with the spinner flasks is that in the BITS/IBGRL laboratory Filton, there is evidence that they can become easily infected. However, this was not a problem for the 34 days the cells were grown in this study. Also, the flasks do take time to clean and sterilise and this was not included in staff costs for this system. This was because a support grade member of staff would carry this out as part of general cleaning duties within the laboratory.

The T-175 flasks once seeded with HEK293 cells were cultured without moving to allow the cells to adhere to the plastic surface. The cells were allowed to grow until they had reached approximately 80% confluency. This took about one week and at this point the flasks needed to be reseeded to prevent cells reaching 100% confluency. At this cell density it is reported that protein synthesis does decrease (McAteer and Davis, 2002). However, whether protein production from cells in this study decreased at greater than 80% confluency was not known. Optimum harvest time and the effect that cell density i.e. confluency had on protein production could have been determined using the ELISA but this was not pursued as part of this study due to time constraints. Cells were dislodged from the flasks by disruption of cell to cell and cell to plastic interactions by use of the protease enzyme trypsin with EDTA. Cells were then washed and resuspended in cell culture medium prior to reseeding the flasks. This meant that cells had to reattach to the flask surface, grow and start protein synthesis again. Despite the disruption, yields from the flasks were around 75% of those from spinner flasks. This system is the cheapest one as it does not require expensive equipment but it does require more “hands-on” time.

In the CellMax system HEK293 cells and secreted protein are held in a bioreactor which is essentially a separate compartment from which supernatant can be periodically harvested. This means that cells are exposed to a local microenvironment rather than being exposed to the bulk medium. Adequate medium flow is therefore essential to this system to prevent depletion of nutrients in the bioreactor. Supernatant was harvested twice

weekly with a syringe. Unfortunately, the bioreactor became infected so the cells may not have had sufficient time to fully colonise the bioreactor membrane, grow and produce protein. This was the most expensive system in terms of staff costs and equipment. However, the system had not been running as long as the others so protein yield was from a shorter culture time. There is evidence in the laboratory that this system can produce high yields of protein e.g. 42 mg sr-Fy^a from 190 mL cell culture supernatant from CellMax® compared to 6 mg sr-Fy^a from 200 mL cell culture supernatant from T-225 flasks, and can run for many months (Fran Green BITS/IBGRL, personal communication). If this concentration of protein could be produced either by using the CellMax® system or transiently transfected cells, then it raises the possibility of being able to use non-purified culture supernatants as reagents, which would save substantial time and money, compensating for the cost of the CellMax® system both in terms of equipment and staff time. Cell culture supernatants containing individual sr-blood group proteins of K, k, Fy^a, Fy^b or Lu^b specificities have been used in BITS/IBGRL to inhibit antibodies at 1 in 10 dilutions of cell culture supernatant to plasma (Fran Green BITS/IBGRL, personal communication). Obviously, contaminants such as BSA would be present in the supernatant and that might prove to be a problem in reagent production where consistency between each batch would need to be assessed. However, it might be worth pursuing one or both of these approaches for future reagent production.

Mammalian cells are used to express human proteins because they can perform the complex post-translational modifications widely thought to be

needed for appropriate protein folding and function. This is especially important for sr-blood group proteins as the 3-D structure seems to be important for them to retain their antigenicity. Seltsam *et al.* (2007) compared production of sr-Lu^b in eukaryotic and prokaryotic systems. HEK293 stable cell lines were transformed with the eukaryotic pcDNA3.1/V5-His-Lu^b expression construct and the prokaryotic expression plasmid pcRT7/CT-His-Lu^b was used to transfect *E. coli* cells. Sr-Lu^b protein produced from both cell lines was able to inhibit anti-Lu^b in samples tested in a haemagglutination inhibition test, indicating that the protein showed Lu^b antigenicity. However, protein produced from *E .coli* cells had to be refolded to show this reactivity. This is because r-proteins can become insoluble in inclusion bodies and can be difficult to recover as functional proteins (Villaverde and Mar Carrio, 2003). Also, most post-translational modifications are not added by bacterial systems (Assenberg *et al*., 2013).

The exact time required for the cloning process for HEK293 cells was not recorded by Seltsam *et al.,* (2007) but clones were selected to obtain stable cell lines after 10 to 16 days, whereas protein from bacterial cells was obtained in around seven working days but then required a process to refold the prokaryotic Lu^b molecules. However, Seltsam *et al*. reported that correct protein refolding occurred spontaneously after removal of the prokaryotic Lu^b protein from the denaturing environment which simplified the process. His study demonstrated that eukaryotic and re-folded prokaryotic r-Lu^b proteins were equally suited to antibody identification but whereas µg of protein was

produced from HEK293 cells, 5 to 10 mg of protein was produced from *E.coli* cells, but starting volumes of cell culture supernatant are not given.

Lutheran, like Knops, is a single pass membrane protein with an extracellular domain, which carries the blood group antigens, segmented into several discrete immunoglobulin superfamily (IgSF) domains, rather like the LHRs of CR1 (Kikkawa *et al*., 2011). This raises the possibility that functional sr-LHR proteins might be produced in bacterial cells or another non-mammalian system. Peptide fragments of the CR1 molecule have been produced from *Pischia pastoris* yeast cells (Pham *et al.,* 2010). Nuclear magnetic resonance spectroscopy was used to confirm protein folding and results of this were consistent with compactly folded protein domains. The recombinant fragments were used to study the binding of the *Plasmodium falciparum* adhesin PfTh4 to areas of the CR1 molecule, so it is not known whether these fragments displayed any blood group antigenicity (Pham *et al.*, 2010).

The aim to transfect HEK293 cells to produce sr-LHR-C and LHR-D was fully met with both proteins identified as CR1 by mass spectrometry. Stably transfected HEK293 cells were cloned and a frozen cell bank has been set up for future use. It was shown that functional sr-LHR protein could be produced relatively cheaply, with production costs of less than £2K per annum using T-175 flasks. As this culture system was also easy to use it was recommended for the production of protein for use as a reagent for NHSBT laboratories.

4.0 SR-LHR-C AND SR-LHR-D PROTEIN PURIFICATION

4.1 Introduction

The purification process aims to separate sr-LHR-C or sr-LHR-D protein, secreted by transfected HEK293 cells, from other secreted proteins and from other proteins contained in the culture medium e.g. bovine serum albumin (BSA). Affinity chromatography is the purification method of choice as the sr-LHR-C and sr-LHR-D proteins are fusion constructs with the 3xFLAG affinity tag located at their C-terminal ends. When culture medium is applied to an agarose gel with anti-FLAG M2 monoclonal antibody covalently attached, the FLAG tagged proteins will bind to the antibody. Any 3XFLAG protein bound to the gel can be eluted by competition with high concentrations of the 3XFLAG peptide or by reducing the pH. These elution conditions help to preserve the structure of protein complexes (Sigma-Aldrich, 2000).

The aim of this part of the study was to optimise a purification process based on affinity gel purification, to maximise protein yield and economy so that a routine diagnostic test could be developed for use in Red Cell Immunohaematology (RCI) departments. The purified protein must also retain its blood group antigen specificity. The immunopurification method was therefore assessed in several ways. As one of the major costs of purification is incurred through purchase of the 3XFLAG peptide, development included a comparison of yields of sr-LHR-C-FLAG and sr-LHR-D-FLAG protein obtained by competitive elution with those obtained by acid elution. Also, protein eluted by both methods was used in the inhibition test to ensure that blood group antigenicity had not been affected by the elution technique used. Another

expense is the cost of the affinity gel so the number of times the gels could be used and how this affected protein yield and antigenicity was assessed. Finally, whether yields could be increased if supernatant that had been applied to the gels was retained and reapplied to the gels again was assessed. The purity of the final product was investigated and the cost to purify enough protein (concentration 1 mg mL^{-1}) to provide as a reagent to internal NHS Blood and Transplant (NHSBT) laboratories for one year was calculated. It is recognised that other factors could affect yield such as flow rate through the gels, size of gel column used, temperature at which the purification is performed but material, time and cost meant that all these factors were not assessed but kept constant for all the purification procedures described i.e. 1 mL gels were used, supernatant was applied at a flow rate of 1 mL $minute^{-1}$, and all purification procedures were performed at room temperature (18 to 25 ºC).

4.2 Materials and Methods

4.2.1 Acid vs competitive elution of sr-proteins from anti-FLAG agarose gel

Elution using glycine-HCl pH 3.5 (acid elution) or 3XFLAG peptide (competitive elution) was compared. Both methods are recommended by the manufacturer (Sigma-Aldrich, 2010) and both are described in Materials and Methods section 2.3.2. Cell culture supernatant harvested from LHR-C transfected HEK293 cells grown in spinner flasks, T-175 flasks and in the CellMax® system were pooled giving a total volume of 1175 mL. Six 1 mL affinity gels in separate 10 mL glass columns were prepared. One 100 mL aliquot of the pooled material was run through each gel. Three gels were

eluted with 3XFLAG-peptide and three gels eluted with glycine-HCl pH 3.5. The yield of protein was assessed by BCA assay (Materials and Methods section 2.3.3) with a mean of three assays used for the final result. The approximate molecular weight (Mr) of the purified protein was assessed by SDS-PAGE (Materials and Methods section 2.3.4) followed by western blotting onto PVDF membranes (Materials and Methods section 2.3.5) and Coomassie blue protein staining (Materials and Methods section 2.3.7). Protein purity was assessed by immunoblotting with HRP-conjugated anti-mouse IgG and HRP-conjugated anti-BSA (Materials and Methods section 2.3.6). For all the blots described 2 µg of purified protein was used. Protein antigenicity i.e. whether it expressed functional KN antigens was measured by its ability to inhibit anti-KN antibodies using the CAT inhibition test (described in Materials and Methods section 2.5.3).

4.2.2 Number of uses of the affinity gel

Five further 100 mL aliquots of the pooled material from 4.2.1 were used. One affinity gel eluted by acid above was selected and the five 100 mL aliquots were applied successively to the affinity gel and eluted with acid pH 3.5 each time. The yield of protein from each elution was assessed by BCA assay (Materials and Methods section 2.3.3) with a mean of three assays used for the final result.

4.2.3 Recovery of protein

The investigations described in 4.2.1 and 4.2.2 were performed on supernatant material but the starting concentration of protein was not known.

In order to better assess recovery of protein some 'spiked' samples were prepared. Six 100 mL aliquots of cell culture medium were prepared and 50 µg of purified sr-LHR-C was added to each. Six 1 mL affinity gels were prepared (Materials and Methods section 2.3.2). Three of the 'spiked' aliquots were applied to three of the affinity gels and eluted with acid (first elution). A second acid elution was then performed to assess whether any protein might be left on the gel which could be eluted (second elution). The culture medium was retained from this first application and applied to three new gels and termed flow through (FT) material. After the flow through material had been run through the column acid elution was performed (first elution FT) and then a second acid elution was performed (second elution FT). Further as part of the procedure Vivaspin centrifuge tubes are reused, so to check that no protein was retained in the Vivaspin membrane a final membrane wash with just PBS was performed and the PBS retained. For all the above samples, protein yield was assessed by BCA assay performed in triplicate and the mean protein yield calculated.

4.2.4 Purification of sr-LHR-D from culture supernatant

When the purification method had been optimised this method was used to purify some supernatant preparations containing sr-LHR-D to ensure that yields consistent with those for sr-LHR-C were obtained and that the purified sr-LHR-D protein obtained was functionally active. This work was performed by a member of staff in BITS/IBGRL using the methods as described in this study. Supernatant containing sr-LHR-D from spinner flasks was pooled and split into three 100 mL aliquots. Three one mL affinity gels were prepared and

100 mL was applied to each gel. The flow through material was retained for each column and was put back through that column again.

4.3 Results

4.3.1 Acid vs competitive elution of sr-LHR-C from anti-FLAG agarose gel

This work was performed to assess whether there was any difference in yield or activity of eluted protein between elution of bound protein using glycine-HCl pH 3.5 or 3xFLAG peptide. Identical pooled culture supernatant was applied to six new gels and three gels were eluted by each technique and mean yield of protein was calculated. New gels were used for each purification and there was little variation between the three runs for each elution method as shown by the error bars (Figure 4.1). The mean yield (µg) from 3XFLAG eluted columns was slightly higher than from acid pH 3.5 eluted columns (148 µg vs 140 µg). Student's *t*-test was performed on the results (Kirkman, 1996) and gave a probability (P-value) of 0.004. This is low and provides statistical evidence that the null hypothesis can be rejected and that there is a significant difference in the results obtained from the two elution methods.

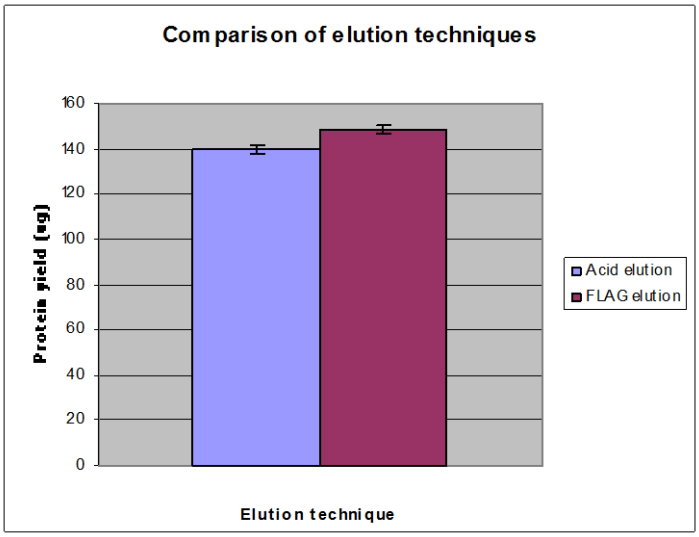

Figure 4.1 Comparison of methods for elution of sr-LHR-C protein. Yield of sr-LHR-C protein from cell culture supernatant using elution with 3XFLAG peptide and acid elution with glycine-HCl pH 3.5 is compared. Error bars represent standard deviations calculated from the data (n=3). Student's t-test P-value = 0.004.

The protein stained western blot for the six samples (Figure 4.2) shows that the eluted protein had Mr of approximately 65-80 kDa, which is comparable to that found in Protein Production section 3.3.3, for sr-LHR-C. There was no difference in gel mobility of the protein eluted by the two techniques.

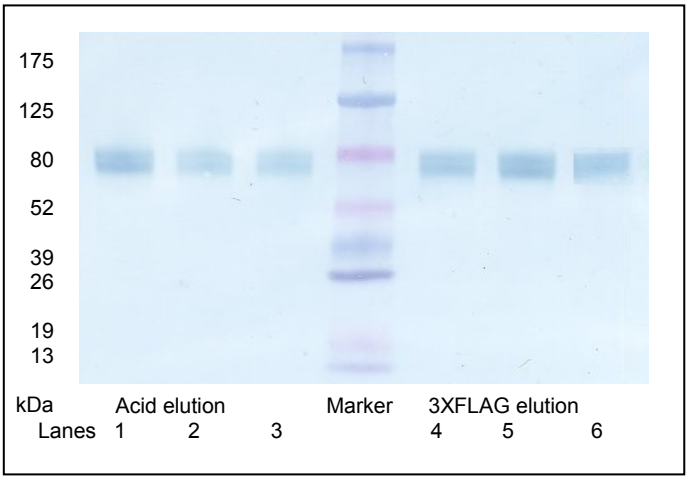

Figure 4.2 Protein stained western blot comparing elution techniques. Three acid eluted samples in lanes 1, 2 and 3 were compared to three 3XFLAG eluated samples in lanes 4, 5 and 6. The eluates were from culture supernatant containing sr-LHR-C. No difference in mobility of the eluted proteins could be seen between the two different elution techniques.

All the samples on the blot seem to show double bands, a darker band being seen at the bottom at approximately 65 kDa where BSA might appear (BSA Mr is 66.5 kDa). A western blot was performed with HRP-conjugated anti-BSA (Figure 4.3) to check for the presence of BSA in the samples (following on from work in Results section 3.3.3; Figure 3.12). Some BSA was detected on the blot in all of the samples at approximately the same level. In comparison with the standards there was significantly less than 0.25 µg of BSA present.

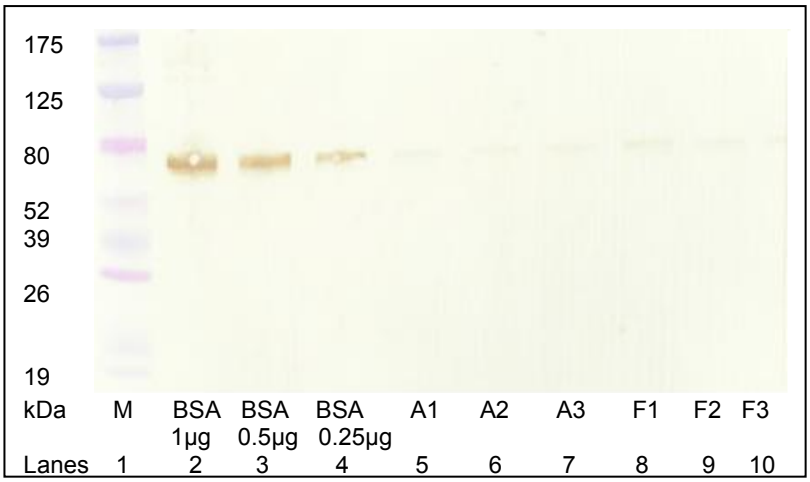

Figure 4.3 BSA contamination in acid and 3XFLAG eluted samples. Western blot with HRP-conjugated anti-BSA: three BSA standards at 1, 0.5 and 0.25 µg are shown in lanes 2, 3 and 4. Three samples eluted with glycine-HCl pH 3.5 are labelled A1, A2 and A3 in lanes 5 to 7 and three samples eluted with 3XFLAG peptide are labelled F1, F2 and F3 in lanes 8 to 10. M=marker in lane 1.

Even if 0.25 µg had been detected in the samples then this would amount to 0.25 µg in 2 µg of sample applied to the gel. This would mean that 12.5% of the sample applied was BSA and would be reported as having a purity of 87.5%. This would be an acceptable level of purity for commercial reagents. In actual fact the BSA content is much less than this. The double bands seen on the protein stain in Figure 4.2 do not appear to be due to the presence of BSA in the sample as this level of BSA would be unlikely to be detected on the protein stained membrane.

As a comparison, a purified sr-LHR-C sample was compared to a pre-purified sample of culture supernatant using HRP-conjugated anti-BSA western blot (Figure 4.4). FCS contains 1.8 g dL^{-1} BSA so the pre-purified sample on this blot contains approximately 3.6 µg BSA and is obviously overloaded at this

amount. In comparison the amount of BSA in the purified sample was very small.

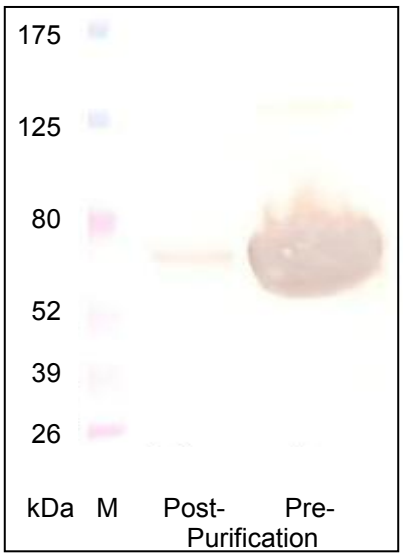

Figure 4.4 BSA contamination in a pre- and post-purified sample. Western blot with HRP-conjugated anti-BSA on post purification sr-LHR-C sample and pre-purified cell culture supernatant, M=marker.

It was suspected in Protein Production section 3.3.3 that mouse Ig from the column was present in some of the eluates. When tested with HRP-conjugated anti-mouse Ig, mouse Igs are found in the acid eluted but not the 3XFLAG eluted preparations (Figure 4.5). The acid eluted protein shows several bands, a more heavily stained band at approximately 50 kDa which corresponds to mouse IgG heavy chains. A minor band at around 20 kDa corresponds to mouse IgG light chains that can appear at 20-25 kDa (Wilson *et al*., 2002). The other bands are probably due to denatured mouse IgG fragments. No mouse IgG was detected in the 3XFLAG eluted samples.

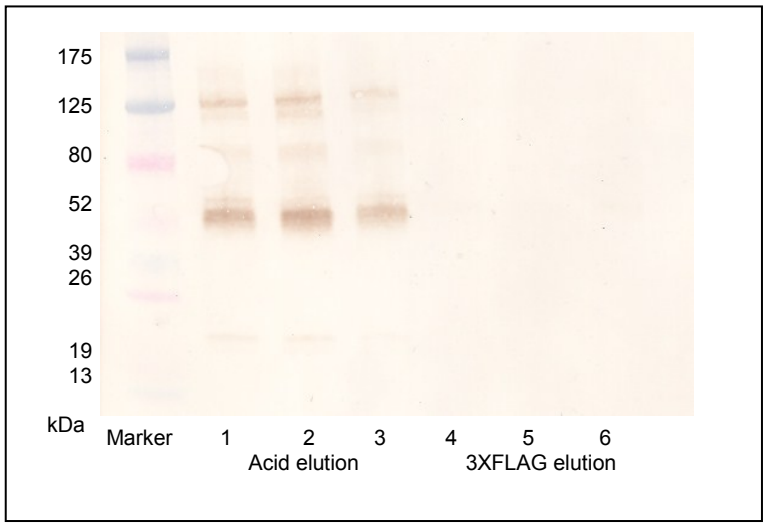

Figure 4.5 Mouse IgG contamination in sr-LHR-C samples eluted with acid and 3XFLAG. Three sr-LHR-C samples eluted with acid are in lanes 1, 2 and 3. Three sr-LHR-C samples eluted with 3XFLAG are in lanes 4, 5 and 6. All were immunoblotted using HRP-conjugated anti-mouse immunoglobulin.

To ensure that antigenicity of the eluted protein was not affected by the elution technique, CAT inhibition tests were performed. One sample known to contain anti-KN antibodies with sufficient volume (3440H) and previously shown to be inhibited by sr-LHR-C (but not sr-LHR-D) was selected and all six sr-LHR-C eluates were used in the inhibition test (Materials and Methods section 2.5.3). Results after inhibition were compared with neat plasma and a PBS dilution control i.e. PBS was used instead of sr-LHR-C in the inhibition stage (Figure 4.6). All the eluates inhibited the anti-KN antibody confirming that the antigenicity (and therefore the conformation) of the protein was retained by both elution methods.

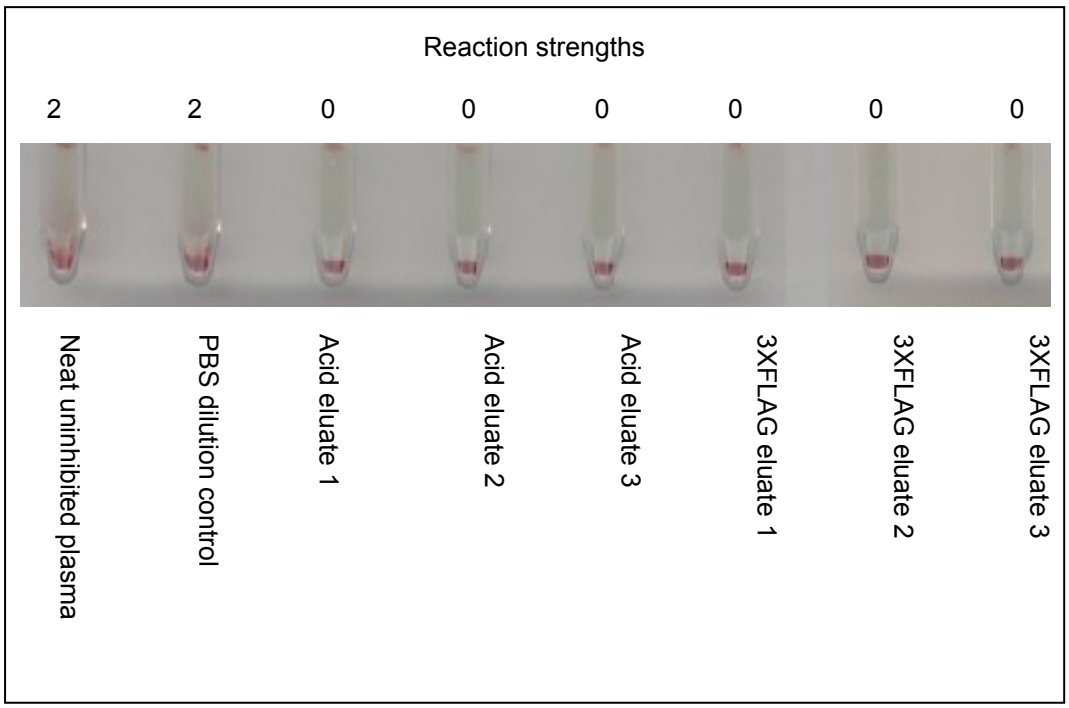

Figure 4.6 CAT inhibition tests using acid and 3XFLAG eluted sr-LHR-C preparations. Three acid eluted and three 3XFLAG eluted sr-LHR-C samples were used to inhibit patient plasma containing anti-KN antibodies. Positive tests scored on a scale of 1 to 5 where 5 is the strongest reaction, negative test = 0.

4.3.2 Number of uses of the gel

To assess how many times the gels could be used, identical 100 mL aliquots of cell culture supernatant containing sr-LHR-C were successively applied to a 1 mL gel (Figure 4.7).

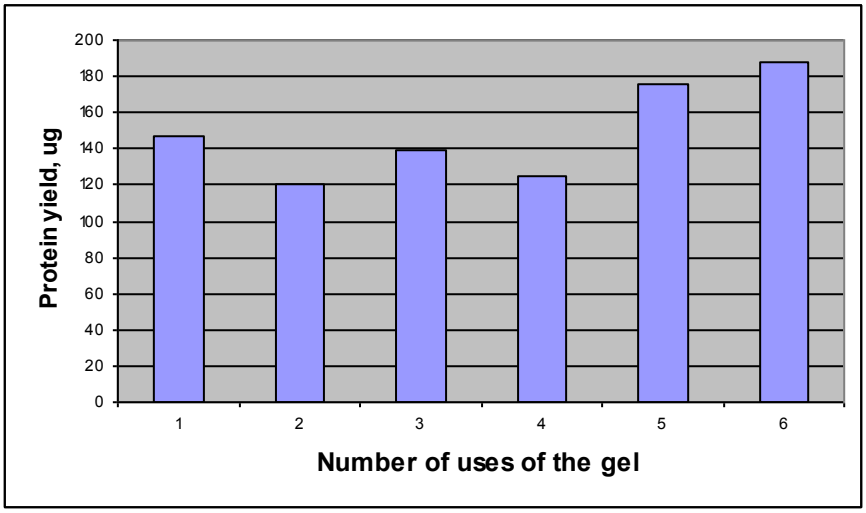

Figure 4.7 Effect of number of uses of the affinity gel columns on protein yield. Protein yield from one affinity gel column when supernatant containing sr-LHR-C was applied successively. Six aliquots of supernatant were applied and protein was eluted from the gel column with acid elution each time.

The user manual for anti-FLAG M2 affinity gel advises that if the gel is used to purify recombinant protein from periplasmic extracts from *E.coli* then the gel may be used up to 20 times without loss of binding capacity (Sigma-Aldrich, 2010) but if *E.coli* crude extracts are used then the gel should only be reused three times before loss of binding capacity is observed. Six applications was the maximum that could be achieved with the amount of cell culture supernatant available and lies between the three and twenty times recommended. The results show that there was no decrease of yield after six uses of the gel, in fact there did appear to be a slight increase in yield after five and six uses. However, this work was only performed once so no statistical analysis could be applied. From these results, six uses of the gel would be acceptable for purifying the cell culture supernatants. There was no difference in mobility of the purified protein on SDS-PAGE gels as shown in

Figure 4.8 and the size corresponded to that expected for sr-LHR-C. Double bands can again be seen in the sr-LHR-C samples.

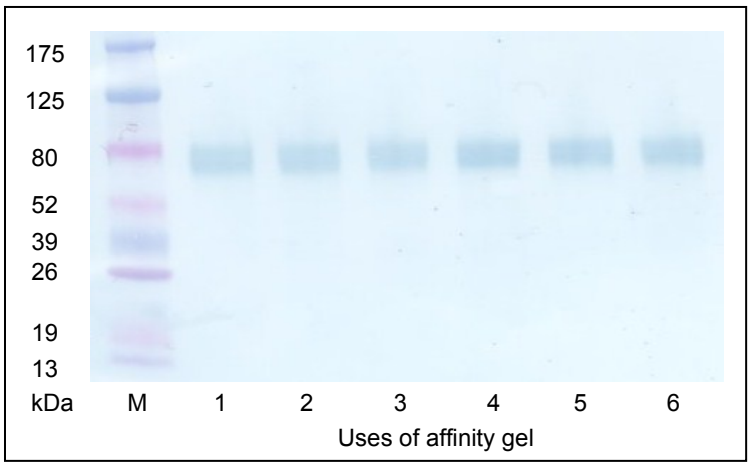

Figure 4.8 Comparison of protein in sr-LHR-C eluates from a used affinity gel column. Eluates from six successive applications of identical supernatant containing sr-LHR-C to an affinity gel column werewestern blotted and protein stained. They are shown in lanes 1 to 6 indicating 1st to 6th use of the affinity gels. M=marker.

Western blots with HRP-conjugated anti-BSA and HRP-conjugated anti-mouse IgG were performed. With anti-BSA the results were the same as for acid eluted samples seen in Figure 4.3 (i.e. very little BSA detected) and the bands were consistent for all 6 samples tested (results not shown). Low levels of mouse IgG was detected in the samples from the first four uses of the gel but by the 5th and 6th there did not appear to be much mouse IgG eluted from the gel (Figure 4.9).

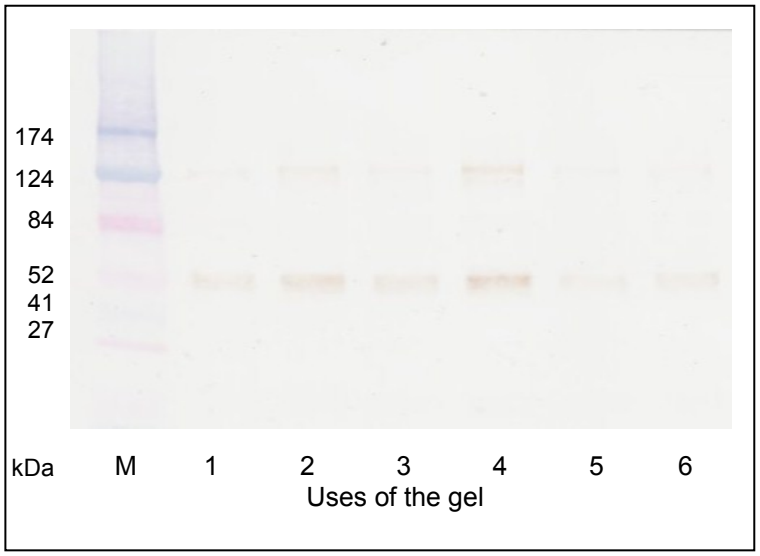

Figure 4.9 Mouse IgG contamination of sr-LHR-C eluates from a used affinity gel column. Eluates from six successive applications of identical supernatant containing sr-LHR-C to an affinity gel column were immunoblotted with HRP-conjugated anti-mouse IgG. They are shown in lanes 1 to 6 indicating 1st to 6th use of the affinity gels. M=marker.

CAT inhibition tests were performed using the six eluates to inhibit a patient sample containing anti-Kn antibodies and known to be inhibited with sr-LHR-C (Table 4.1). Results of the CAT IAT after inhibition were compared to neat uninhibited plasma and a dilution control where PBS was added to patient plasma instead of sr-LHR-C in the inhibition stage. All six sr-LHR-C eluates inhibited the KN antibody.

Table 4.1 CAT inhibition tests using eluted sr-LHR-C from used affinity gels

LHR-C eluate used for inhibition / use of gel	*Reaction vs Screening cell*
Neat uninhibited plasma	1
PBS dilution control	1
1st	0
2nd	0
3rd	0
4th	0
5th	0
6th	0

Samples are labelled from 1st to 6th use of the affinity gel. The sr-LHR-C samples were used to inhibit patient plasma containing anti-KN antibodies. Positive reactions scored on a scale of 1 to 5 where 5 is the strongest reaction, negative test = 0.

4.3.3 Recovery of sr-LHR-C protein from anti-FLAG affinity gels

Recovery of protein eluted from the affinity gels was assessed by using 100 mL aliquots of cell culture supernatant 'spiked' with 50 µg purified sr-LHR-C protein. Percentage protein recovered is shown in Figure 4.10. A mean of 56% (28 µg) of protein was retrieved on the first run through the gels (first elution). A further 34% (17 µg) was retrieved from flow through material i.e. applying the material again to a gel (first elution FT). An additional elution was performed after the first in each case (second elution, second elution FT), to assess whether any protein remained on the column after elution. From these two elutions 10% (5 µg) protein was retrieved. All the protein was retrieved when the yield from the two extra elutions were added. The gels have a binding capacity of 600 µg mL^{-1} so it would have been expected that all of the protein would have bound on the first run through, however, a significant amount of protein was recovered from the flow through material.

The best protocol to adopt appears to be to retain the flow through from the first run and apply it again to the gel.

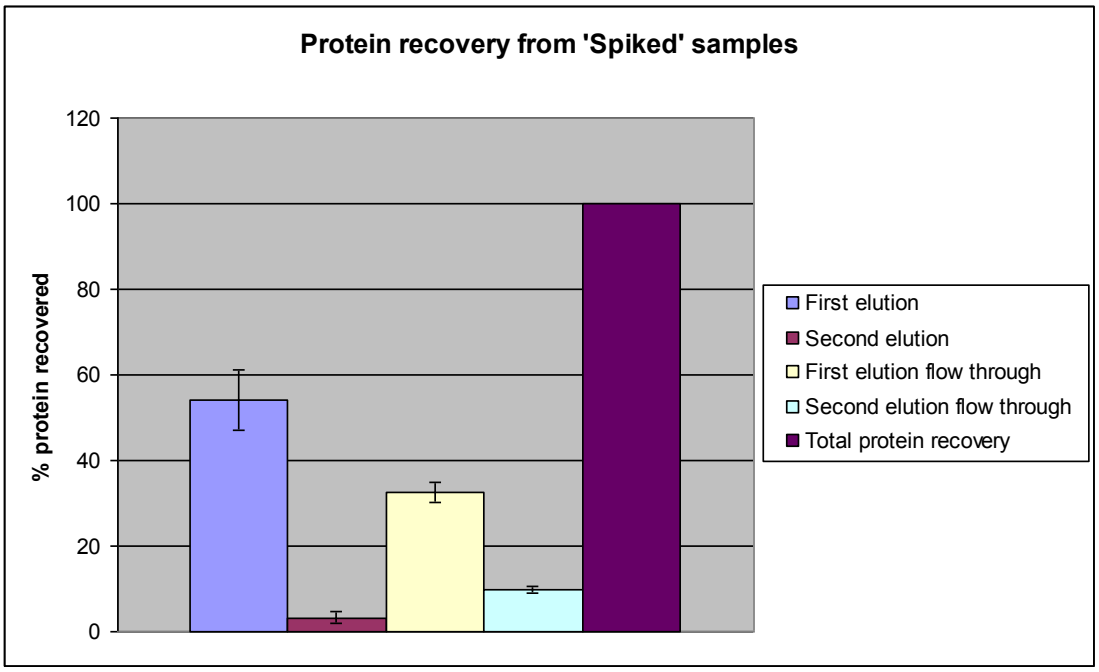

Figure 4.10 Percentage recovery of protein from three spiked samples. Error bars represent standard deviations calculated from the data (n=3).

In addition the last wash from three Vivaspin tubes was tested by BCA assay but no protein was detectable, indicating that no protein was lost through being retained in the tubes on the filtration membranes.

The three first elution samples were pooled together as were the first elution FT samples to ensure that enough sample was available for subsequent testing. These pooled samples were run on SDS-PAGE gels and blotted onto PVDF membranes. A Coomassie protein stain and western blots with HRP-conjugated anti-BSA and HRP-conjugated anti-mouse IgG were performed (Figure 4.11). The protein stain shows the same diffuse bands for sr-LHR-C

as seen before at approximately 65 – 80 kDa. There is no difference seen between the sample first applied to a new gel and then the flow through from that purification then being applied to a new gel. It is just possible to see double bands in the sr-LHR-C samples on the protein stained membrane.

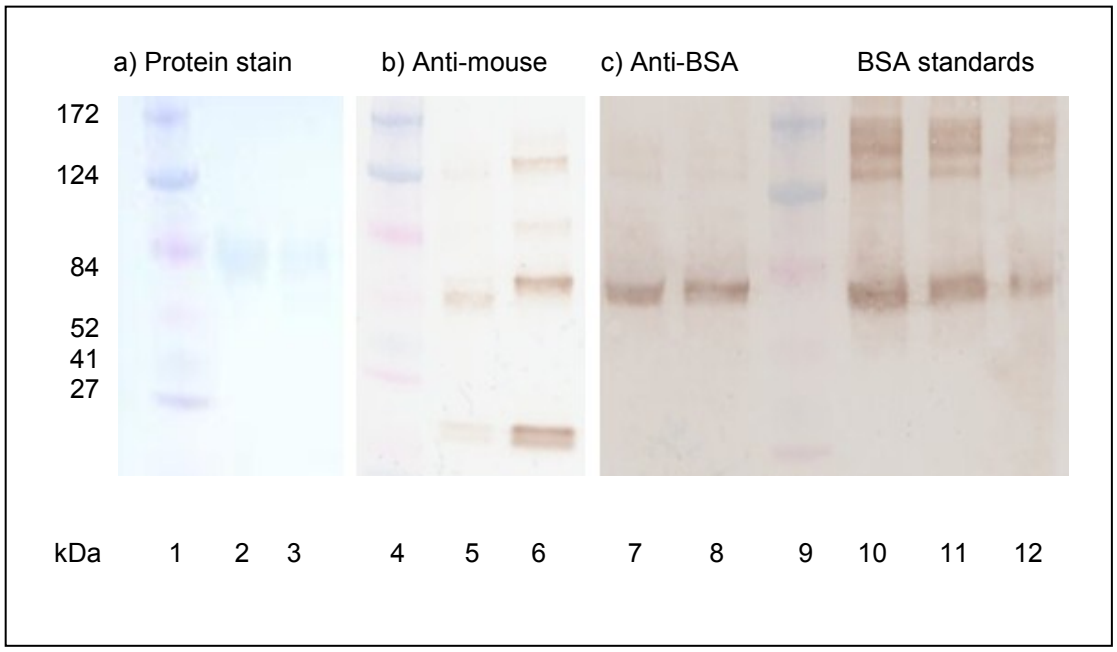

Figure 4.11 Protein content and contaminants in sr-LHR-C first elution and flow through samples. Western blots are a) protein stained, b) immunoblotted with HRP-conjugated anti-mouse Ig c) immunoblotted with HRP-conjugated anti-BSA. Lanes 2, 5 and 7 contain eluted sr-LHR-C from supernatant applied to an affinity gel column for the first time. Lanes 3, 6 and 8 contain sr-LHR-C from the supernatant collected from the first application which was applied to a new affinity gel and eluted. Lanes 10, 11 and 12 show BSA standards containing 1 µg, 0.5 µg and 0.25 µg BSA respectively. Molecular markers are shown in lanes 1, 4 and 9.

Mouse Ig is seen in both samples which would be expected as the samples were acid eluted. There appears to be more mouse Ig present in the flow through samples. BSA was also detected at around 0.5 µg which indicates

more BSA present than previously detected, but these samples were pools of three so both mouse Ig and BSA were more concentrated than previously and had had twice as many acid eluates performed as would be expected during normal purification. There were insufficient samples left to perform CAT inhibition tests but it was shown that sr-LHR-D flow through samples inhibited KN antibodies (section 4.3.5) and other sr-LHR-C flow through samples produced have inhibited KN antibodies (personal experience) so applying the cell culture supernatant to a gel again does not appear to affect the conformation of the protein.

4.3.4 Cost to purify protein for use in NHSBT laboratories

The KNIR reagent that will be distributed to NHSBT laboratories consists of equal volumes of sr-LHR-C and sr-LHR-D, both at a starting concentration of 1 mg mL^{-1}. The total cost to purify enough protein to supply 1 mL KNIR to nine NHSBT laboratories was calculated and the cost of performing acid or 3XFLAG elution was compared. The cost of equipment and reagents was calculated from the point when the cell culture supernatant was filtered prior to application onto the affinity gel columns, up to when the concentrated protein was harvested from the Vivaspin centrifuge tube. The glass 10 mL columns used to hold the affinity gel and the pumps used to run the supernatant through the affinity gels have not been included in the costs as they are reusable.

From section 4.3.1 the mean yields from elution of 100 mL cell culture supernatant using acid or 3XFLAG elution respectively were 140 µg (in 170

µL) PBS and 148 µg (in 180 µL) PBS. This means that for two applications through the one mL gel yields would be approximately 210 µg and 222 µg for acid and 3XFLAG elution respectively in approximately 340 µL PBS from 100 mL cell culture supernatant. As 1 mL KNIR will contain 500 µL of each sr-LHR (at a starting concentration of 1 mg mL^{-1}), then 300 mL cell culture supernatant applied to the columns in three 100 mL batches with the flow through material from each batch also applied to the column should be sufficient to produce 500 µL of sr-LHR at a concentration of 1 mg mL^{-1}. This will result in six applications to each affinity gel, which from section 4.3.2 is acceptable. This will require two affinity gels, one for each sr-LHR as these cannot be purified on the same gels. Twelve elutions with 3XFLAG peptide would use 600 µL peptide. Acid costs are not included as these reagents are available in the laboratory at negligible cost (Table 4.2).

Table 4.2 Comparison of equipment and reagent costs for acid and 3XFLAG eluted protein

Equipment / Reagent	***Cost***	***Quantity used***	***Total cost***
Vacuum filtration unit	£51.01 for 12	2	£8.50
Anti-FLAG M2 affinity gel	£89.60 mL^{-1}	2 mL	£179.20
3XFLAG peptide	£28 (5mg mL^{-1}) (100 µg used per elution equals 56p per elution)	600 µL	£6.72
Vivaspin centrifuge tubes	£170 for 25 tubes	6 tubes	£40.80
		Total 3XFLAG	£235.22
		Total Acid	£228.50

Costs are to produce 1 mL KNIR containing 500 µL of sr-LHR-C and sr-LHR-D.

Assuming a band 6 Biomedical Scientist (BMS) on the midpoint of their scale performed the work then with 22% added 'on costs' for National Insurance

and pension payments the hourly rate would be £18.77. Staff costs shown in Table 4.3 assumed that three purification 'sessions' were performed, running two columns at the same time.

Table 4.3 Staff costs to produce acid and 3XFLAG eluted protein

Elution technique	***Time for two gels simultaneously (hours)***	***Three procedures using two gels each time (hours)***	***Total cost***
3XFLAG	4.66	13.98	262.41
Acid	3.83	11.49	215.67

Calculations are based on two columns being run at the same time with a total of 300 mL being applied to the columns twice.

The process described above would produce 1 mL of KNIR but 9 mL is needed for NHSBT laboratories for one year. The total yearly costs are shown in Table 4.4. The saving using acid elution rather than using 3XFLAG for elution is £481 per year.

Table 4.4 Total cost to prepare a year's supply of inhibition reagent for NHSBT laboratory use

Elution technique	***Equipment / reagent costs***	***Staff costs (£)***	***Total cost (£)***
3XFLAG	2116.98	2361.69	4478.67
Acid	2056.50	1941.03	3997.53

4.3.5 Purification of culture supernatant containing sr-LHR-D

Following the findings in this study the purification method of choice is to purify 100 mL cell culture supernatant on 1 mL affinity gels and elute with glycine-HCl pH 3.5, retain the flow through from this and then apply it to the same affinity gel again and elute again with the acid. The gels can be used up to six times. This method is preferred because there is a small annual cost saving and a saving in staff time. It is also a method already in use in BITS/IBGRL for elution of other sr-proteins. It would mean a more standardised approach to affinity gel purification in the laboratory.

The work in this section has all been performed on sr-LHR-C, so this method was used to purify some sr-LHR-D from cell culture supernatants. This work was performed by a member of staff following the protocol as described from this study. The affinity gels had already been used twice so the purifications performed represented the third and fourth uses of the affinity gels. The mean yield (n=3) from the three gels was 287 µg for the first run through the gels and 181 µg for the flow through material. Inhibition tests were performed using the first run through and the flow through samples. A patient sample containing anti-KN previously shown to be inhibited by sr-LHR-D was tested with the six samples of purified sr-LHR-D and was inhibited (results not shown). Sr-LHR-C and sr-LHR-D were tested by SDS-PAGE. A Coomassie stained protein gel and a western blot using HRP-conjugated anti-FLAG are shown in Figure 4.12.

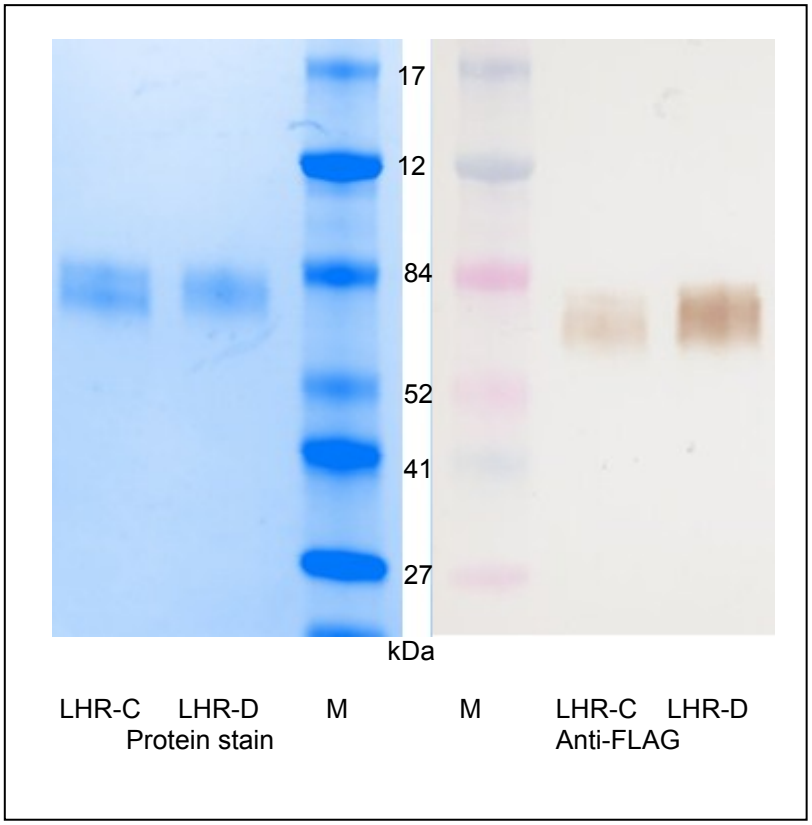

Figure 4.12 Comparison of protein and FLAG tagged protein in sr-LHR-C and sr-LHR-D preparations. Sr-LHR-C and sr-LHR-D are shown on a) a protein stained SDS-PAGE gel and b) a western blot with HRP-conjugated anti-FLAG. M=marker

The double bands previously noted for sr-LHR-C can be seen in the protein stain but not with the anti-FLAG. Sr-LHR-D appears as a diffuse band rather than a double band. Sr-LHR-C appears to be about 65-80 kDa which is consistent with other results seen in this study, with a more prominent band at the bottom of the two bands seen. In the western blot, sr-LHR-D appears to be a slightly larger molecule as the band has not migrated quite so far as sr-LHR-C. Both proteins are more uniformly stained with anti-FLAG so it appears that all of the protein is FLAG tagged which again seems to rule out BSA being present and causing the band seen in sr-LHR-C at approximately

65 kDa. The diffuse bands and double band in sr-LHR-C might be explained by N-glycosylation of the sr-LHR-C and sr-LHR-D, especially as the CR1 protein is glycosylated (Furtado *et al*., 2008) and glycosylated protein can migrate on SDS-PAGE gels as diffuse bands (Nguyen and Strong, 2007). N-glycosylation can potentially occur at any asparagine in the sequence asparagine-x-serine or threonine where x can be any amino acid except proline. The potential sites for glycosylation for sr-LHR-C and sr-LHR-D are shown in Figure 4.13.

```
                                                    GHCQAPDHFL FAKLKTQTNA
961    SDFPIGTSLK YECRPEYYGR PFSITCLDNL VWSSPKDVCK RKSCKTPPDP VNGMVHVITD
1021   IQVGSRINYS CTTGHRLIGH SSAECILSGN TAHWSTKPPI CQRIPCGLPP TIANGDFIST
1081   NRENFHYGSV VTYRCNLGSR GRKVFELVGE PSIYCTSNDD QVGIWSGPAP QCIIPNKCTP
1141   PNVENGILVS DNRSLFSLNE VVEFRCQPGF VMKGPRRVKC QALNKWEPEL PSCSRVCQPP
1201   PEILHGEHTP SHQDNFSPGQ EVFYSCEPGY DLRGAASLHC TPQGDWSPEA PRCAVKSCDD
1261   FLGQLPHGRV LFPLNLQLGA KVSFVCDEGF RLKGSSVSHC VLVGMRSLWN NSVPVCEHIF
1321   CPNPPAILNG RHTGTPSGDI PYGKEISYTC DPHPDRGMTF NLIGESTIRC TSDPHGNGVW
1381   SSPAPRCELS VRAG

                     HCKTPE QFPFASPTIP INDFEFPVGT SLNYECRPGY FGKMFSISCL
1441   ENLVWSSVED NCRRKSCGPP PEPFNGMVHI NTDTQFGSTV NYSCNEGFRL IGSPSTTCLV
1501   SGNNVTWDKK APICEIISCE PPPTISNGDF YSNNRTSFHN GTVVTYQCHT GPDGEQLFEL
1561   VGERSIYCTS KDDQVGVWSS PPPRCISTNK CTAPEVENAI RVPGNRSFFT LTEIIRFRCQ
1621   PGFVMVGSHT VQCQTNGRWG PKLPHCSRVC QPPPEILHGE HTLSHQDNFS PGQEVFYSCE
1681   PSYDLRGAAS LHCTPQGDWS PEAPRCTVKS CDDFLGQLPH GRVLLPLNLQ LGAKVSFVCD
1741   EGFRLKGRSA SHCVLAGMKA LWNSSVPVCE QIFCPNPPAI LNGRHTGTPF GDIPYGKEIS
1801   YACDTHPDRG MTFNLIGESS IRCTSDPQGN GVWSSPAPRC ELSVPA
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Figure 4.13 The potential N-glycosylation sites for sr-LHR-C and sr-LHR-D. These are marked in green on the amino acid sequence for sr-LHR-C (top) and sr-LHR-D (bottom). N-glycosylation can occur at asparagine in the sequence asparagine-x –serine where x is any amino acid except proline.

There are five potential N-glycosylation sites for sr-LHR-C and seven potential sites for sr-LHR-D, although Furtado *et al.*, (2008) when modelling the CR1 molecule speculated that there were three glycosylated sites for LHR-C and four for LHR-D for their best fit CR1 molecular model. Glycoproteins often migrate at a different rate than anticipated from a calculated molecular weight

(Kelly and Barthmaier, 2003). Both sr-LHR-C and sr-LHR-D would be approximately 50 kDa when calculated from the number of amino acids in each protein. However, glycans attached to the protein can affect the migration of glycoproteins on SDS-PAGE gels. The branched structures of glycans make the molecule more bulky and hydrophilic (Roth *et al*., 2012). The charge-to-mass ratio of the glycoprotein is also altered by the detergent used in the process. The bulky shapes and the charge-to-mass ratios are not the same as those of the protein molecular marker used for estimation of size so molecular weight estimations can vary from the predicted size. N-linked oligosaccharides may contribute 3.5 kDa or more per structure to the mass of the glycoprotein (Sigma-Aldrich, 2007). This would mean that sr-LHR-C and sr-LHR-D would be approximately 60 kDa and 64 kDa with three or four N-glycosylated sites respectively. These masses correspond more closely to those observed in this study.

4.4 Discussion

Purification using an affinity tag system has many advantages over other techniques, such as allowing a simple, one step process (Lichty *et al*., 2005). However, it is not a quick process and the affinity gels used can be very expensive. In this study the effectiveness of various aspects of the purification process were investigated with the aim of determining the fastest, most cost effective route to produce a product of the required specificity and purity. Small volumes of cell culture supernatant were purified on the affinity gels and with the gel binding capacity stated as >0.6 mg protein mL^{-1} (Sigma-Aldrich, 2010), one mL columns should have been sufficient to bind all the protein in the first run. The studies using 'spiked' samples showed that 34% protein was not recovered on the first run through the column despite there being substantially less protein present than the gel's binding capacity. The cell culture supernatant had to be applied to the column a second time to achieve retrieval of 90% of the 3XFLAG tagged protein. The affinity gel binding capacity was determined using FLAG-BAP protein (Sigma-Aldrich, 2010). This is a control protein used to determine the functional integrity of anti-FLAG M2 monoclonal antibodies. It is probable that binding capacity will be different for different proteins due to the conformation of the proteins and hence accessibility of the FLAG tag on the protein to the bound anti-FLAG antibodies in the affinity gel. Yield may be increased by passing the sample through the column again providing that the same protein is applied (Sigma-Aldrich, 2010). If more protein was produced and larger volumes of supernatant were purified then scaling up to using larger affinity gel columns and the cost effectiveness of this would need to be considered. Passing the

supernatant through the column again did not appear to affect protein structure as purified flow through material inhibited KN antibodies successfully.

Ideally, elution from affinity gels should not affect the protein tertiary structure so that biological activity is not affected (Terpe, 2003). Two elution methods recommended for the FLAG system (Sigma-Aldrich, 2010) were investigated, elution with glycine-HCl at pH 3.5 (acid elution) and competitive elution using 3XFLAG peptide. Although statistically there was significant difference between the two methods the acid elution at pH 3.5 gave comparable yields to competitive elution using 3XFLAG peptide; it is also a faster process as the final column wash with glycine-HCl recommended to regenerate the column after peptide elution by removing any residual 3XFLAG peptide is not needed. There is no difference in SDS-PAGE mobility of eluted product after acid or peptide elution, as shown on the protein stained SDS-PAGE gels.

The affinity gel has mouse IgG monoclonal Anti-FLAG M2 antibodies covalently attached to it. However, the manufacturer warns that some of the M2 antibody may not be conjugated to the resin and may appear in acid eluates as heavy and light chains at 50-60 kDa and 20-25 kDa respectively when using anti-mouse conjugated antibody in western blots (Sigma-Aldrich, 2010). It is recommended that an acid wash is performed before using the gel to remove unconjugated antibody. Although an acid wash was always performed on new gels prior to their first use, in this study it was found that acid eluates contained mouse immunoglobulin whilst 3XFLAG peptide eluates

did not. When the same column was reused up to six times in this study, it did appear from the western blots with HRP-conjugated anti-mouse antibody that the amount of mouse antibody decreased the more times the column was used. This could be explained if unconjugated antibody had gradually all been stripped off by successive uses of the gel and elution with acid. It would be useful to increase the number of acid washes at the start of the process to see if the amount of mouse antibody eluted decreased. The other explanation for the presence of mouse antibody in the eluates is that the acid process might be more likely to remove non-bound or loosely bound Ig. With the limited testing performed on reusing the affinity gel it did appear that the amount of mouse Ig in the eluates did decrease gradually and also there seemed to be no loss of protein yield when the gel was used up to six times. There was no mouse immunoglobulin found with 3XFLAG competitive elution, a much milder technique, but elution with peptide would not reduce the loss of antibody from the affinity gel as an acid wash stage is employed in the process to regenerate the gel if it is to be reused. However, competitive elution would reduce the contaminating mouse Ig in the sr-protein preparations.

At least 10% of the population have some antibodies to animal proteins. These are most often human anti-mouse antibodies (HAMA). It is not known why these develop but increased use of immune therapy with mouse derived monoclonal antibodies could partly be responsible (Klee, 2000). HAMA have been reported to cause some problems with immunological tests (Kricka, 1999) but they are not detected in serological tests like the indirect

antiglobulin test even though anti-human immunoglobulin is used. This is most likely to be because they cannot bind to antigens on the red cell surface. If the inhibition reagent produced contained mouse Ig then any anti-mouse antibodies present in patient plasma may well be inhibited but this would not matter in serological testing as anti-mouse antibodies would never be detected anyway.

Since it is unlikely that the double bands seen on the protein stained western blots (Figures 4.2, 4.8 and 4.11) are due to the presence of BSA then they are probably due to differences in glycosylation of the sr-protein. CR1 is not *O*-glycosylated but it does have *N*-glycosylation. There are 25 potential sites for *N*-glycosylation on the CR1 molecule but experimentally only 14 are known to be occupied (Furtado *et al.*, 2008). There are five potential sites in LHR-C and seven in LHR-D as shown in Figure 4.13. It is not known which sites are glycosylated but potentially LHR-D could have more glycosylation than LHR-C and this could account for the apparent larger size of LHR-D on SDS-PAGE. Both proteins probably have some glycosylation as the Mr for both would be about 50 kDa if calculated from number of amino acids, but sr-LHR-C appears to be around 65-80 kDa and sr-LHR-D to be slightly larger. The slight difference in size was seen by other workers (Tamasauskas *et al*., 2001). In their study, both sr-LHRs were seen as quite diffuse bands. Sr-LHR-C appeared to be around 80-90 kDa and sr-LHR-D about 85-95 kDa. The way the proteins have migrated in the SDS-PAGE gels in this study too could indicate that sr-LHR-D has more glycosylation than sr-LHR-C. The lower band seen in the sr-LHR-C protein on gel analysis might be due to non-

glycosylated protein that is smaller and has migrated further. It might be possible to resolve whether the proteins are glycosylated by treating them with deglycosylation reagents. If they were deglycosylated then their gel mobility should be increased and they should be seen to run as more discrete bands on SDS-PAGE gels rather than the diffuse bands seen in this study (Edge, 2003). There may also be a loss of antigen activity after deglycosylation as carbohydrate molecules may be involved in the antibody to antigen binding process (Sigma-Aldrich, 2007). As both sr-proteins have antigens that are recognised by KN specific antibodies it is probable that they are glycosylated as this important post-translational modification is involved in maintenance of protein conformation and activity (Roth *et* al., 2012). The conformation of the protein affects the accessibility of antibodies to the peptide epitopes (Lisowska, 2002).

The advantage of using mammalian system for protein production is that they can produce correctly glycosylated protein (Ariescu and Owens, 2013). BSA was found to be a very minor contaminant of the purified protein that did not cause problems for inhibition testing. When the product is produced for use in RCI laboratories though, a quality control procedure would be needed to ensure consistency between batches. As mouse Ig contamination could not be detected on SDS-PAGE gels stained for protein then this could also be a requirement for validation of each batch.

The aims of this chapter were to optimise a procedure for purification of sr-LHR-C and sr-LHR-D protein. The defined procedure is to use 1 ml affinity

gel columns, to apply the cell culture supernatant and elute bound protein. and then reapply the culture supernatant to the column and elute again to maximise yield and to reduce costs by using acid for elution of protein from the column.

5.0 DIAGNOSTIC USE OF SOLUBLE RECOMBINANT PROTEINS

5.1 Introduction

Serological techniques have been well established for many years for the detection and identification of red cell antibodies. This includes inhibition techniques where various substances have been used to neutralise antibody activity (Torres *et al.*, 2010). Soluble recombinant (sr) proteins with blood group antigen specificity are becoming more widely available and techniques using these proteins are in development (Seltsam and Blasczyk, 2009). In the future it may be possible to automate antibody identification using single sr-antigens in assays where reaction of serum, plasma or blood samples with sr-protein indicates both the presence and specificity of an antibody (Seltsam 2011). There is, however, immediate application of recombinant proteins such as sr-LHR-C and sr-LHR-D in Red Cell Immunohaematology (RCI) laboratories for the detection, identification and inhibition of specific antibodies, in this case antibodies directed against KN antigens.

The aim of this part of the study was to develop a technique, using the sr-LHR-C and sr-LHR-D proteins, to aid in investigation of KN related antibodies. Two techniques were developed using sr-LHR-C, sr-LHR-D and a mixture of both these sr-proteins now called the KN inhibition reagent (KNIR). An ELISA antigenicity test was optimised to use the individual soluble recombinant proteins to determine whether antibodies had KN specificity (Materials and Methods, section 2.4.3). A positive ELISA result indicated that KN related antibodies were present in the test plasma whereas a negative result indicated that there were no antibodies with anti-KN specificity. A new

inhibition technique was developed, based on an indirect antiglobulin test in commercial gel cards, to inhibit KN related antibodies. In the RCI laboratory, successful introduction of the inhibition test will save time and effort in investigation of patients with KN antibodies and ultimately the care of patients with these antibodies will be improved because faster provision of suitable units for transfusion will be possible.

A standard protocol for use of KNIR in an IAT test was developed and then evaluated by different members of staff for reproducibility and ease of use. The stability of the inhibition reagent developed was assessed after storage at 4ºC and -30 ºC.

5.2 Materials and Method Development

5.2.1 Antigenicity ELISA

This ELISA technique is described in Materials and Methods section 2.4.3. Sr-LHR-C and sr-LHR-D were used to coat plates at a concentration of 10 µg mL^{-1} sr-protein. Samples known to contain anti-KN antibodies when originally tested (confirmed by MAIEA technique, Materials and Methods section 2.4.4) were obtained from NHSBT IBGRL Reference Serology Laboratory, located at Filton. Seventeen samples were obtained frozen and were stored at -30 ºC until thawed for use.

5.2.2 CAT Inhibition Test

This test is detailed in Materials and Methods section 2.5.3. To assess whether sr-LHR-C and sr-LHR-D protein would inhibit Knops antibodies the

MAIEA positive samples were tested. Before performing the CAT inhibition test it was important to determine that Knops antibodies could still be detected by CAT. All samples were tested by CAT against a 3 Cell Screening Set (Materials and Methods section 2.5.2). Antibodies were still detectable by IAT in 16 out of the 17 samples. Some of these samples were used to determine the optimum dilution of sr-protein to use for effective inhibition. A series of doubling dilutions of sr-LHR-C and sr-LHR-D was prepared in PBS and then 25 µL of each dilution was incubated with 25 µL patient plasma for 30 minutes at RT. The samples were then tested by CAT with a 1 in 2 dilution of patient's plasma in PBS included as a dilution control.

Following determination of the optimum dilution for inhibition, the technique was refined to allow addition of sr-LHR reagent directly into patient plasma samples. This was to reduce the amount of sr-LHR reagent needed per test and also to reduce any dilution effect on any antibodies present.

5.2.3 Samples tested by CAT inhibition test

Once the CAT inhibition test was optimised it was used to test the IBGRL samples and a further 30 patient samples obtained from various RCI laboratories (the majority from Filton RCI). All of these samples were also tested by antigenicity ELISA.

Samples containing clinically significant antibodies but not KN related antibodies were obtained from RCI Filton and tested by CAT inhibition to ensure that clinically significant antibodies were not inhibited by this test.

Those antibody specificities considered to be clinically significant by RCI are: Anti-D, -C, -E, -c, -e, - Fy^a, -Fy^b, -Jk^a, -Jk^b, -S, -s, -M, -Kp^a and -C^w. Where it proved possible three examples of each antibody were tested by CAT inhibition.

5.2.4 Sr-reagent storage and stability testing

Several batches of sr-LHR-C and sr-LHR-D were prepared at a concentration of 1 mg mL^{-1} in PBS, with no preservatives added, and frozen at -30 ºC for future use. Individual reagents sr-LHR-C batch C157 and sr-LHR-D batch D157 were frozen on 10/5/12 and thawed and used in the CAT inhibition test on 16/4/14 nearly two years later and a KNIR reagent batch CD229 was frozen on 11/9/13 and also used on 16/4/14 after being frozen for seven months. Other sr-LHR reagents were frozen and thawed up to three times. Sr-LHR-C batch C187 and sr-LHR-D batch D216 frozen on 1/11/12 and 9/5/13 respectively were thawed, tested and refrozen on three occasions: 28/03/14, 04/04/14 and 25/04/14 and used in the CAT inhibition test. A batch of KNIR reagent was stored at 4 ºC with no added preservative and used in the CAT inhibition test 19 and 24 weeks after storage.

5.3 Results

5.3.1 Antigenicity ELISA

An antigenicity ELISA currently used in BITS/IBGRL for evaluation of other sr-blood group antigens was used to test whether the sr-proteins produced in this study showed KN specificity, prior to developing a serological inhibition assay based on the IAT (section 5.3.2). Eleven plasma samples (KN1 to KN11) confirmed as containing anti-KN antibodies by the MAIEA assay were used to validate this test. They were initially tested in one antigenicity ELISA, results shown in Table 5.1a and six further MAIEA positive samples (KN12 to KN17) were tested in a separate ELISA, results shown in Table 5.1b. Tests were performed in duplicate and a mean absorbance calculated. A negative control was included which was a plasma sample containing a blood group antibody that was not KN related. Two groups of absorbance values were obtained on both plates for both sr-proteins. In one group the absorbance values were clustered around the negative control and in the other group they were around twice the negative control value so a cut-off of twice the mean negative control value for each plate was used to define the results as positive (above x2 negative control) and negative (below x2 negative control). Using this cut-off five samples, KN9 to KN12 and KN17, gave positive results with sr-LHR-C but were negative with sr-LHR-D. Twelve samples KN1 to KN8, KN11 and KN14 to KN16 gave opposite reactions i.e. they were positive with sr-LHR-D but negative with sr-LHR-C. The reactions obtained with samples containing known KN related antibodies confirmed that the sr-proteins produced had KN antigenicity. The 30 patient samples were also tested by this technique (section 5.3.3).

Table 5.1 Antigenicity ELISA results for samples containing KN related antibodies

Table 5.1a						
Sample	LHR-C1 OD492	LHR-C2 OD492	LHR-C Mean	LHR-D1 OD492	LHR-D2 OD492	LHR-D Mean
KN1	1.348	1.353	1.351	3.832	3.48	3.656
KN2	0.827	0.809	0.818	3.245	3.030	3.138
KN3	0.964	0.864	0.914	2.922	3.027	2.975
KN4	0.587	0.593	0.590	2.728	2.793	2.761
KN5	1.056	0.957	1.007	2.645	2.568	2.607
KN6	1.363	1.267	1.315	3.571	3.395	3.483
KN7	1.415	1.566	1.491	3.210	3.268	3.239
KN8	1.650	1.711	1.681	3.555	3.504	3.530
KN9	3.355	3.434	3.395	1.538	1.486	1.512
KN10	3.127	3.335	3.231	0.985	1.074	1.030
KN11	0.866	0.813	0.840	3.238	3.212	3.225
Control	1.194	1.194	1.194	1.066	0.856	0.961

Table 5.1b						
Sample	LHR-C1 OD492	LHR-C2 OD492	LHR-C Mean	LHR-D1 OD492	LHR-D2 OD492	LHR-D Mean
KN12	2.928	2.928	2.928	1.273	1.072	1.173
KN13	2.939	2.888	2.914	1.233	1.222	1.228
KN14	1.394	1.631	1.513	2.938	3.010	2.974
KN15	1.538	1.469	1.504	2.987	2.987	2.987
KN16	1.170	1.579	1.375	3.001	2.983	2.992
KN17	2.913	3.007	2.995	2.267	2.110	2.189
Control	1.267	1.384	1.326	1.779	1.882	1.831

Tables 5.1a and 5.1b show results for two antigenicity ELISAa. Negative control sample was patient plasma lacking KN antibodies. Tests were performed in duplicate (C1 and C2, D1 and D2) and a mean calculated. A ratio of greater than 2:1 of mean test absorbance value to negative control absorbance values indicates a positive result i.e. presence of KN related antibody. In Table 5.1a absorbance > 2.388 for LHR-C and > 1.862 for LHR-D indicates a positive result (shaded orange). In Table 5.1b absorbance > 2.652 for LHR-C and > 2.662 for LHR-D indicates a positive result (shaded orange).

5.3.2 CAT Inhibition test

Three antibody samples KN12, KN13 and KN17, which gave positive reactions with sr-LHR-C but were negative with sr-LHR-D by ELISA, and three antibody samples KN14, KN15 and KN16, which gave positive results with sr-LHR-D but not sr-LHR-C, were incubated with decreasing concentrations of stock sr-LHR-C and sr-LHR-D respectively. The starting stock material i.e. purified sr-LHR-C and sr-LHR-D contained 1 mg mL^{-1} protein. Doubling dilutions of sr-LHR from 1/2 to 1/2048 (with plasma sample KN15 dilutions of sr-protein were extended to 1/8192) were prepared in PBS, 25 µL of these dilutions were incubated with 25 µL plasma for 30 minutes at RT. After incubation a CAT test was performed using reagent cells shown to react with anti-KN antibodies. A 1 in 2 dilution of plasma sample in PBS (25 µL plasma + 25 µL PBS) was also included as a dilution control. The results of these tests are shown in Table 5.2.

Table 5.2 Determination of dilution of sr-LHR to use for the CAT inhibition test

Sample	Inhibited with LHR-	Plasma + PBS	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192
KN12	LHR-C	3	0	0	0	0	0	1	1	2	2	3	3	NT	NT
KN13	LHR-C	2	0	0	0	0	0	0	0	0	0	0	1	NT	NT
KN17	LHR-C	2	0	0	0	0	0	1	1	2	2	2	2	NT	NT
KN14	LHR-D	2	0	0	0	0	0	0	0	0	1	1	2	NT	NT
KN15	LHR-D	3	0	0	0	0	0	0	0	0	0	0	0	1	1
KN16	LHR-D	2	0	0	0	0	0	1	2	2	2	2	2	NT	NT

Results of CAT inhibition test using doubling dilutions of sr-LHR reagents. Plasma + PBS indicates plasma diluted 1 in 2 in PBS. Dilutions are expressed as reciprocals e.g. 2 = 1 in 2 dilution. Grading of reactions on gel IAT card: 0=negative. Positive reactions are graded 1 to 5 in order of increasing strength of reaction. NT = not tested.

There was wide variation in the concentration of sr-LHR protein that was needed to inhibit the KN antibodies. The lowest concentration needed was seen for sample KN15 where a 1 in 2000 dilution, 0.5 µg mL^{-1}, brought about inhibition. Three samples KN12, KN17 and KN16 were inhibited up to a 1 in 32 dilution or 30 µg mL^{-1} sr-LHR reagent. Similar variability was seen when using sr-LHR-C or sr-LHR-D. From these results a dilution of 1 in 20 (50 µg mL^{-1}) was chosen for the inhibition test as at this dilution, for both sr-LHR reagents, inhibition was effected with all plasma samples tested and choice of this dilution erred on the side of caution rather than picking a 1 in 40 dilution. A gel card CAT inhibition test showing reactions of a representative sample, KN12, is shown in Figure 5.1.

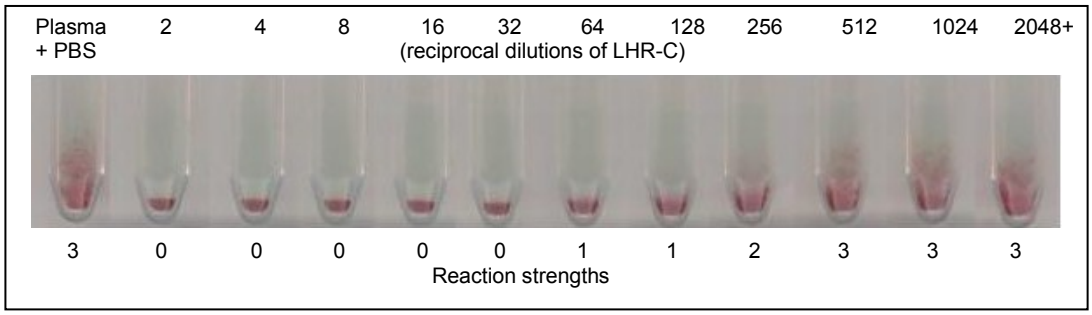

Figure 5.1 Representative sample showing inhibition with dilutions of LHR-C. Doubling dilutions of LHR-C were prepared and incubated with patient plasma (KN12). A score of 0 = negative. Positive reactions are graded 1 to 5 in order of increasing strength. A negative result indicating inhibition is seen at 1 in 32 but from 1 in 64 onwards positive reactions can be seen indicating that the anti-KN antibody has not been inhibited at these dilutions. Plasma diluted 1 in 2 in PBS was included as a dilution control.

5.3.3 Patient samples tested by CAT inhibition test

When the optimum dilution of sr-LHR reagent had been decided then the technique for patient plasma testing was modified to make the 1 in 20 dilution directly into patient's plasma. This meant that a stock dilution could be made in one tube, for example to test inhibited patient plasma against a 10 cell ID panel 14 µL sr-LHR was added to 266 µL plasma for a 1 in 20 dilution. After the inhibition incubation stage, 25 µL of this mixture was then added to each well of a gel card containing 50 µL test cells. The amount of sr-LHR per test was 1.25 µg.

A total of 47 samples were tested (including the 17 IBGRL samples tested in sections 5.3.1 and 5.3.2) by CAT inhibition test. Prior to performing the inhibition test they were tested by IAT to confirm that antibody could still be detected as KN antibodies can become undetectable on storage. In the CAT

inhibition test they were tested with individual sr-LHR reagents and also a mixture of the two reagents, the KNIR reagent. This contained equal volumes of stock sr-LHR-C and sr-LHR-D, so contained 500 μg mL^{-1} of each protein and was therefore diluted 1 in 10 for use. The peptide ELISA was also performed on all the samples using individual sr-LHR reagents. The pattern of reactions fell into four groups. Those inhibited by sr-LHR-C alone (group one – 15 samples), those inhibited by LHR-D alone (group two – 25 samples) and those not inhibited at all (group three –five samples). In these groups there was agreement between the results obtained by ELISA and CAT inhibition test, where CAT inhibition was performed, as in one sample (KN9) antibody was no longer detectable by CAT (it was detectable by ELISA). The last group (group four – two samples) reacted with both sr-proteins by ELISA.

5.3.3.1 Group one samples

These were inhibited by sr-LHR-C and KNIR and reactive with sr-LHR-C only by peptide ELISA. Five samples (KN9, KN10, KN12, KN13 and KN17) were positive by MAIEA and therefore known to contain KN related antibodies. However, antibodies were no longer detectable in sample KN9 by CAT. Two patient samples were reported to contain KN related antibodies: sample 2904A contained anti-McC^a and sample 4337G contained anti-Sc2 (although the amount of sample 4337G was limited so it was only tested with KNIR by CAT inhibition). The other samples contained antibodies that had reacted like HTLA antibodies in serological testing, results are shown in Table 5.3.

Table 5.3 Results of CAT, antigenicity ELISA and CAT inhibition for group one samples.

	CAT Screening cells			ELISA Reactions with		CAT inhibition test Inhibition with				Notes
	1	2	3	LHR-C	LHR-D	LHR-C	LHR-D	KNIR	PBS	
KN9	0	0	0	+	0	NT	NT	NT	NT	MAIEA positive
KN10	1	2	1	+	0	Y	N	Y	N	MAIEA positive
KN12	1	3	1	+	0	Y	N	Y	N	MAIEA positive
KN13	0	2	1	+	0	Y	N	Y	N	MAIEA positive
KN17	0	0	2	+	0	Y	N	Y	N	MAIEA positive
4337G	1	3	0	+	0	NT	NT	Y	N	Limited sample anti-Sc2/K
8204R	1	3	3	+	0	Y	N	Y	N	
2904A	2	3	3	+	0	Y	N	Y	N	Anti-McC^a
5635H	3	2	3	+	0	Y	N	Y	N	
5839I	0	1	2	+	0	Y	N	Y	N	
0598L	2	0	0	+	0	Y	N	Y	N	
92241	3	1	0	+	0	Y	N	Y	N	
05452	0	1	2	+	0	Y	N	Y	N	
1587B	2	3	2	+	0	Y	N	Y	N	
5948K	2	1	2	+	0	Y	N	Y	N	

Group one samples tested by CAT using a 3 Cell Screening Set. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction. For the antigenicity ELISA, + = positive reaction 0 = negative reaction. In the CAT inhibition test PBS is a dilution control, Y = inhibition, N = no inhibition, NT = not tested, KN9 because antibody activity was no longer detectable by CAT and sample 4337G due to being a limited sample. Previously defined antibody specificities or if positive by MAIEA test is indicated in the final column.

The results of CAT inhibition testing performed on sample 2904A as a representative sample are shown in Figure 5.2. Sample 2904A was known to contain a KN antibody, anti-McC^a. A 3 Cell Screening Set was used to perform the test, rather than a full ten cell ID panel. Plasma was diluted with the same volume of PBS as sr-LHR reagents as a dilution control. Negative reactions were seen after incubation of patient's plasma with sr-LHR-C and

the KNIR reagent (containing sr-LHR-C) for this sample indicating that the antibody had been inhibited. Positive reactions are still seen after incubation with sr-LHR-D, indicating that this reagent did not inhibit the anti-McC^{a}. There are no underlying antibodies as shown in the negative reactions obtained with all three screening cells after incubation with sr-LHR-C. The PBS dilution control gave positive reactions as expected.

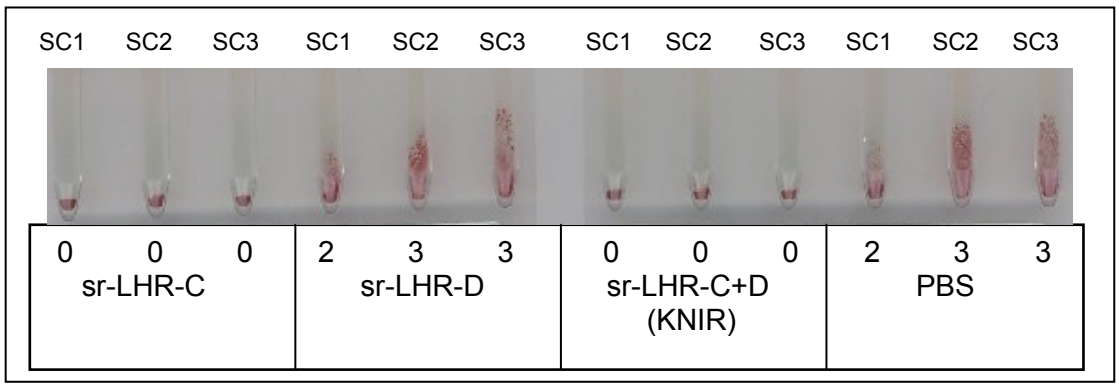

Figure 5.2 Gel card CAT inhibition test for a representative group one sample. Results of testing sample 2904A by CAT inhibition test using a 3 Cell Screening Set, labelled screening cell SC1, SC2 and SC3. The PBS dilution control gave positive reactions with all three screening cells. This activity was inhibited on incubation with sr-LHR-C and KNIR reagent but positive reactions were still obtained after incubation with sr-LHR-D. A score of 0 = negative. Positive reactions are graded 1 to 5 in order of increasing strength.

For the four MAIEA positive samples (KN10, KN12, KN13 and KN17) the CAT inhibition test was repeated with sr-LHR-D but with 25 µL patient plasma inhibited with 25 µL sr-LHR-D (starting concentration 1 mg mL^{-1}). The antibodies were still not inhibited with sr-LHR-D even though this represented a ten fold increase of sr-LHR-D i.e. 12.5 µg sr-LHR-D per test (results not shown).

5.3.3.2 Group two samples

These were inhibited by sr-LHR-D and KNIR reagent but not by sr-LHR-C. They were positive in the ELISA with sr-LHR-D but not sr-LHR-C. Twelve of these samples had anti-KN antibodies as determined by MAIEA test. Two others had been reported as having anti-Kn^a and McC^a specificity; one contained anti-McC^a alone. One sample contained anti-Yk^a and two others contained a mixture of anti-Kn^a and anti-K. The rest had been referred for investigation because they had reacted like HTLA antibodies during investigation in RCI. The results are shown in Table 5.4. For one sample, 4033B, the reactions obtained when tested against a standard 10 cell ID panel of reagent red cells, used for antibody identification, are shown in Table 5.5. An antibody with probable anti-K specificity could be seen as reactions with K+ cells (cells 2, 6 and 7) were stronger than other reactions seen with the panel. However, other reactions were obtained against panel cells 1, 3, 5, 8 and 10. Following RCI policy at least two positive and two negative reactions are required to determine antibody specificity. It would not be possible from the results of the CAT test to decide whether these reactions were due to the presence of other antibodies. RCI has access to another two antibody identification and these would both need to have been set up to exclude the presence of anti-S, -s, -Fy^a, -Fy^b, -Jk^a and -Jk^b i.e. clinically significant antibodies. The inhibition test has inhibited these reactions against the first panel cells so they are likely to have been due to an anti-KN antibody present together with the anti-K. The anti-K can be clearly seen in the inhibited plasma and it can be confirmed that no more clinically significant antibodies are detectable in the plasma. In routine use this would

considerably simplify testing samples with mixtures of antibodies like sample 4033B.

Table 5.4 Results of CAT, antigenicity ELISA and CAT inhibition for group two samples.

	CAT Screening cells			ELISA Reactions with		CAT inhibition test Inhibition with				Notes
	1	2	3	LHR-C	LHR-D	LHR-C	LHR-D	KNIR	PBS	
KN1	1	2	1	0	+	N	Y	Y	N	MAIEA positive
KN2	1	1	1	0	+	N	Y	Y	N	MAIEA positive
KN3	1	2	1	0	+	N	Y	Y	N	MAIEA positive
KN4	1	2	0	0	+	N	Y	Y	N	MAIEA positive
KN5	0	1	0	0	+	N	Y	Y	N	MAIEA positive
KN6	1	2	0	0	+	N	Y	Y	N	MAIEA positive
KN7	1	1	0	0	+	N	Y	Y	N	MAIEA positive
KN8	2	2	1	0	+	N	Y	Y	N	MAIEA positive
KN11	2	3	1	0	+	N	Y	Y	N	MAIEA positive
KN14	0	2	0	0	+	N	Y	Y	N	MAIEA positive
KN15	1	0	3	0	+	N	Y	Y	N	MAIEA positive
KN16	1	0	2	0	+	N	Y	Y	N	MAIEA positive
47581	3	0	3	0	+	N	Y	Y	N	Anti-Kn^a/McC^a
93683	4	5	3	0	+	N	Y	Y	N	Ant-E/C^w
5786E	3	3	3	0	+	N	Y	Y	N	Anti-Yt^a/E/Fy^a/Yk^a
65986	0	0	3	0	+	N	Y	Y	N	
5804P	2	3	2	0	+	N	Y	Y	N	Anti-Kn^a/K
4033B	2	1	3	0	+	N	Y	Y	N	Anti-Kn^a/K
2555K	3	3	3	0	+	NT	NT	Y	N	Limited sample Anti-Kn^a/McC^a
2929T	1	2	2	0	+	N	Y	Y	N	Anti-McC^a
54967	3	3	3	0	+	N	Y	Y	N	
5532V	2	3	2	0	+	N	Y	Y	N	
5619H	3	3	3	0	+	N	Y	Y	N	
57359	1	0	2	0	+	NT	NT	Y	N	Limited sample
5948K	0	3	0	0	+	N	Y	Y	N	

Group two samples tested by CAT using a 3 Cell Screening Set. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction. For the antigenicity ELISA, + = positive reaction 0 = negative reaction In the CAT inhibition test PBS is a dilution control, Y = inhibition, N = no inhibition, NT = not tested, 2555K and 57359 because they were limited samples. Previously defined antibody specificities or if positive by MAIEA test is indicated in the final column.

Table 5.5 Sample 4033B tested with a standard 10 cell ID panel used in RCI for antibody identification.

NHSBT Reagents **Panel 1**

Antibody Investigation Worksheet

Sample name				**Requestor**				**Database ref No.**				**Tested by**										
Date of Birth				**Hosp no**				**Sample No.**				**4033B**				**Date Tested**						
Product		**Lot No.**		**Product**		**Lot No.**		**Product**		**Lot No.**		**Product**		**Lot No.**								
Panel in Alsevers		**R144 3254**		**Panel in CellStab**		**R154 3254**		**Panel in CellMedia**		**NA**		**Panel in LISP**		**R146 3254**								
Panel Papainised in Alsevers		**R143 3254**		**Panel Papainised in CellStab**		**R153 3254**		**Panel Papainised in CellMedia**		**NA**		**EXPIRY DATE: 2012.09.20**										

	Rh	M	N	S	s	P1	Lu^a	K	k	Kp^a	Le^a	Le^b	Fy^a	Fy^b	Jk^a	Jk^b	Other	CAT	Patient's plasma plus:				
																			LHR -C	LHR -D	KNIR	PBS	
1	$R_1^wR_1$	**0**	**+**	**0**	**+**	**3**	**0**	**0**	**+**	**0**	**0**	**+**	**0**	**+**	**0**	**+**	**weak Lub**	2	2	0	0	2	
2	R_1R_1	**+**	**0**	**+**	**0**	**0**	**0**	**+**	**+**	**0**	**0**	**0**	**+**	**0**	**+**	**0**		3	3	3	3	3	
3	R_2R_2	**0**	**+**	**0**	**+**	**2**	**0**	**0**	**+**	**0**	**+**	**0**	**+**	**0**	**0**	**+**		1	1	0	0	1	
4	**r'r**	**0**	**+**	**0**	**+**	**0**	**0**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**+**	**+**		0	0	0	0	0	
5	**r''r**	**+**	**0**	**+**	**0**	**0**	**0**	**0**	**+**	**0**	**0**	**+**	**0**	**+**	**+**	**0**		2	2	0	0	2	
6	**rr**	**+**	**+**	**0**	**+**	**2**	**0**	**+**	**0**	**0**	**+**	**0**	**0**	**+**	**+**	**0**		3	3	3	3	3	
7	**rr**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**+**	**0**	**0**	**+**	**+**	**0**	**0**	**+**	**Ch-**	3	3	3	3	3	
8	**rr**	**+**	**0**	**+**	**0**	**3**	**0**	**0**	**+**	**+**	**+**	**0**	**0**	**+**	**0**	**+**		1	1	0	0	1	
9	**rr**	**0**	**+**	**+**	**0**	**4**	**0**	**0**	**+**	**0**	**0**	**+**	**+**	**0**	**+**	**0**		0	0	0	0	0	
10	**rr**	**+**	**0**	**0**	**+**	**4**	**+**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**0**	**+**		1	1	0	0	1	
Auto																							

Neat plasma from sample 4033B reacted with all panel cells tested by CAT except cells 4 and 9. Incubation prior to testing with sr-LHR-D on its own or when contained in cocktail KNIR (sr-LHR-C and sr-LHR-D) shows that an antibody with KN specificity is present in the plasma (reacting with cells: 1, 3, 5, 8 and 10 and when this is removed it is easy to identify anti-K also present reacting with K+ cells numbers: 2, 6 and 7 (shaded in grey)). No other antibodies were present. A score of 0 = negative. Positive reactions are graded 1 to 5 in order of increasing strength. PBS indicates a dilution control.

Another sample in group two contained two clinically significant antibodies, anti-E and anti-C^w, as well as an antibody with KN specificity. Testing the plasma by CAT, using the 3 Cell Screening Set, shows reactions with all three screening cells but after inhibition only positive reactions with cells 1 (C^w positive) and cell 3 (E positive) are seen. The gel card results for this sample, 93683, are shown in Figure 5.3.

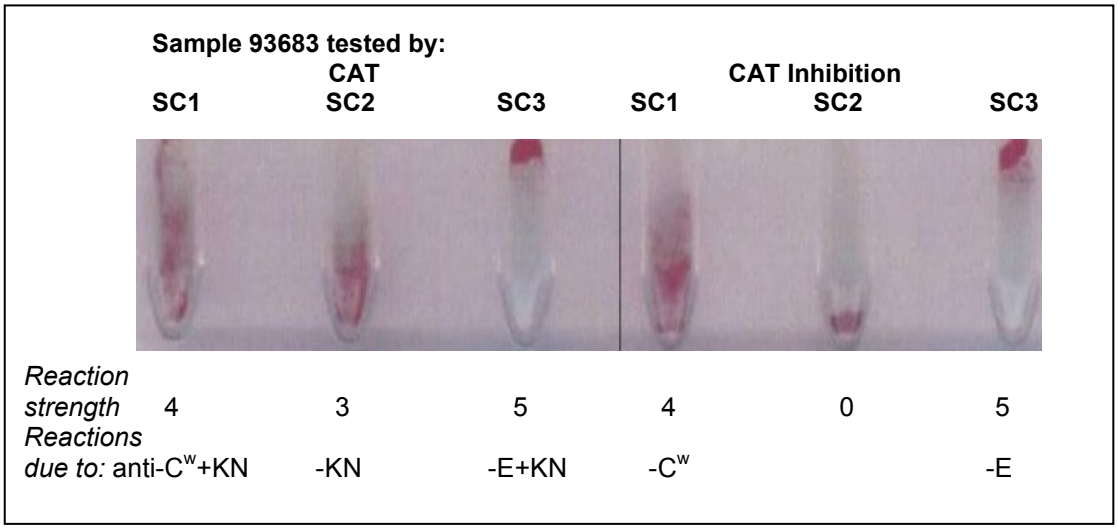

Figure 5.3 Gel card results for a sample containing multiple antibodies. A column agglutination gel card showing reactions of CAT and CAT inhibition testing for sample 93683. CAT shows reactions with all three screening cells. After inhibition with KNIR reagent the reactions with the screening cell 2 disappear. Reactions with screening cells 1 and 3 remain as they are due to anti-C^w reacting with C^w positive screening cell 1 and anti-E reacting with E positive screening cell 3. Reactions with screening cell 2 were due to anti-KN antibody that was inhibited by KNIR reagent. A score of 0 = negative. Positive reactions are graded 1 to 5 in order of increasing strength.

For the twelve MAIEA positive samples (KN1 to 8, KN11, KN14 to KN16) the CAT inhibition test was repeated with sr-LHR-C but with 25 µL patient plasma inhibited with 25 µL sr-LHR-C (starting concentration 1 mg mL^{-1}). The

antibodies were still not inhibited with sr-LHR-C even though this represented a ten fold increase of sr-LHR-C i.e. 12.5 µg sr-LHR-C per test.

5.3.3.3 Group three samples

The antibodies in these samples were not inhibited in the CAT inhibition test and were negative by ELISA. None of these antibodies had been defined as KN antibodies but their reactions by IAT were non-specific and they were referred for testing as possible KN antibodies. Results are shown in Table 5.6. During investigation in RCI, sample 3387C had been tested against reagent ID panel cells that had been treated with 2-aminoethylisothiouronium (AET), because anti-KN specificity was suspected. KN antigens are destroyed by AET and this technique can be used in the laboratory to help identify KN antibodies. In this case, however, the antibody did not appear to be KN related from the results of the ELISA and CAT inhibition.

Table 5.6 Results of CAT, antigenicity ELISA and CAT inhibition for group three samples.

	CAT Screening cells			*ELISA Reactions with*		*CAT inhibition test Inhibition with*				*Notes*
	1	2	3	LHR-C	LHR-D	LHR-C	LHR-D	KNIR	PBS	
3387C	3	3	3	0	0	N	N	N	N	
42106	0	2	1	0	0	N	N	N	N	
8385M	1	0	0	0	0	N	N	N	N	Anti-C
4984S	2	2	2	0	0	N	N	N	N	
59215	3	3	2	0	0	N	N	N	N	

Group three samples, results of antibody screening by CAT using a 3 Cell Screening Set. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction. For the ELISA, + = positive reaction 0 = negative reaction. In the CAT inhibition test PBS is a dilution control, Y = inhibition, N = no inhibition. Previously defined antibody specificities or if positive by MAIEA test is indicated in the final column.

Further investigation was performed on sample 3387C; full ID panels were tested against inhibited plasma (Table 5.7). Referral to IBGRL at Filton for more specialist investigation revealed that this antibody had anti-AnWj specificity. These antibodies are clinically significant as examples have caused severe haemolytic transfusion reactions (Xu *et al*., 2012). AnWj is a high frequency antigen occurring at >99% in the Caucasian population (Xu *et al*., 2012). The antigen is also destroyed by AET treatment. Prior to inhibition testing this antibody had been reported by RCI as a KN related antibody based on the serological pattern of reactions by CAT i.e. reacting with all cells and the fact that it did not react with AET treated panel cells. The specificity was amended in RCI and a new report sent to inform the hospital that anti-AnWj had been confirmed. Fortunately, the patient had not required transfusion.

Of the other samples in group three, sample 42106 was found to contain anti-Cs^a. The Cs^a antigen is another high frequency antigen and the antibody reacts in a very similar way to KN antibodies and it is often included in the HTLA category. The antibody is not clinically significant (Moulds, 2010). One sample was found not to have any detectable antibodies, and a further sample contained anti-C but no other antibodies. The anti-C could be seen in the screening results as only SC1 is C positive and this is the only cell this sample reacted with. It did not react typically like an HTLA antibody. The final two samples in this group were determined to contain non-specific 'cold reacting' antibodies. Pre-warming cells and plasma before performing the IAT can stop these samples from giving positive reactions by IAT. These are normally due

to the antibodies fixing complement and then this being detected due to anti-complement contained in the AHG reagent. They are not clinically significant antibodies.

Table 5.7 Sample 3387C tested with a standard 10 cell ID panel used in RCI for antibody identification.

NHSBT Reagents **Panel 1**

Antibody Investigation Worksheet

Sample Name				**Requestor**				**Database ref No.**				**Tested by**											
Date of Birth				**Hosp no**				**Sample No.**		**4033B**		**Date Tested**											
Product		**Lot No.**		**Product**		**Lot No.**		**Product**		**Lot No.**		**Product**		**Lot No.**									
Panel in Alsevers		**R144 3254**		**Panel in CellStab**		**R154 3254**		**Panel in CellMedia**		**NA**		**Panel in LISP**		**R146 3254**									
Panel Papainised in Alsevers		**R143 3254**		**Panel Papainised in CellStab**		**R153 3254**		**Panel Papainised in CellMedia**		**NA**		**EXPIRY DATE: 2012.09.20**											
	Rh	**M**	**N**	**S**	**s**	**P1**	**Lu^a**	**K**	**k**	**Kp^a**	**Le^a**	**Le^b**	**Fy^a**	**Fy^b**	**Jk^a**	**Jk^b**	Other	**CAT**	**Patient's plasma plus:**				**AET**
																			LHR -C	**LHR -D**	**KNIR**	**PBS**	
1	**$R_1^wR_1$**	**0**	**+**	**0**	**+**	**3**	**0**	**0**	**+**	**0**	**0**	**+**	**0**	**+**	**0**	**+**	**weak Lub**	2	2	2	2	2	0
2	**R_1R_1**	**+**	**0**	**+**	**0**	**0**	**0**	**+**	**+**	**0**	**0**	**0**	**+**	**0**	**+**	**0**		2	2	2	2	2	0
3	**R_2R_2**	**0**	**+**	**0**	**+**	**2**	**0**	**0**	**+**	**0**	**+**	**0**	**+**	**0**	**0**	**+**		2	2	2	2	2	0
4	**r'r**	**0**	**+**	**0**	**+**	**0**	**0**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**+**	**+**		2	2	2	2	2	0
5	**r''r**	**+**	**0**	**+**	**0**	**0**	**0**	**0**	**+**	**0**	**0**	**+**	**0**	**+**	**+**	**0**		2	2	2	2	2	0
6	**rr**	**+**	**+**	**0**	**+**	**2**	**0**	**+**	**0**	**0**	**+**	**0**	**0**	**+**	**+**	**0**		2	2	2	2	2	0
7	**rr**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**+**	**0**	**0**	**+**	**+**	**0**	**0**	**+**	**Ch-**	2	2	2	2	2	0
8	**rr**	**+**	**0**	**+**	**0**	**3**	**0**	**0**	**+**	**+**	**+**	**0**	**0**	**+**	**0**	**+**		2	2	2	2	2	0
9	**rr**	**0**	**+**	**+**	**0**	**4**	**0**	**0**	**+**	**0**	**0**	**+**	**+**	**0**	**+**	**0**		2	2	2	2	2	0
10	**rr**	**+**	**0**	**0**	**+**	**4**	**+**	**0**	**+**	**0**	**+**	**0**	**0**	**+**	**0**	**+**		1	0	1	1	1	0
Auto																		0					

Neat plasma from sample 3387C reacted with all panel cells tested by CAT except patient's own cells, designated as 'Auto' in the panels (antibody is therefore an alloantibody). Incubation of patient's plasma with sr-LHR-C, sr-LHR-D, KNIR (sr-LHR-C and sr-LHR-D) or PBS prior to testing by CAT shows no antibody inhibition. Treatment of panel cells with AET and then testing by CAT shows no positive reactions i.e. the antigen on the panel cells must have been destroyed by the AET treatment.

5.3.3.4 Group four samples

Only two samples reacted with both sr-LHR reagents. Neither sample reacted by CAT so neither were tested by CAT inhibition. Results are shown in Table 5.8.

Table 5.8 Results of CAT, antigenicity ELISA and CAT inhibition for group four samples.

	CAT Screening cells			*ELISA Reactions with*		*CAT inhibition test Inhibition with*				*Notes*
	1	2	3	LHR-C	LHR-D	LHR-C	LHR-D	KNIR	PBS	
42874	1	0	0	+	+	NT	NT	NT	NT	Ant-C+E+M+S+ Co^b+Kn^a/McC^a
57375	0	0	0	+	+	NT	NT	NT	NT	KN related

Table 5.8 Group four samples, results of antibody screening by CAT using a 3 Cell Screening Set. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction. For the ELISA, + = positive reaction 0 = negative reaction. In the CAT inhibition test PBS is a dilution control, NT = not tested. Previously defined antibody specificities or if positive by MAIEA test is indicated in the final column.

The ELISA was repeated and these results were confirmed. Sample 42874 was tested by IAT in RCI and only the anti-C was detected as seen here with the weak positive result by CAT against SC1, the only C positive cell. Sample 57375 was reported as an HTLA antibody possibly KN related. It appears that the ELISA detected KN related antibodies when they were at a level that was undetectable by IAT as seen in these two samples and sample KN9.

5.3.4 Testing clinically significant antibodies by CAT inhibition test

As well as proving that the KNIR reagent inhibited KN antibodies, it was important to show that no clinically significant antibodies were inhibited. Patient samples containing clinically significant antibodies were tested by CAT

inhibition test. Three examples of each antibody were tested, where possible. Reactivity for all antibody specificities tested was not affected by incubation with the inhibition reagent. Representative results are shown in Figure 5.3, where it can be seen the clinically significant antibodies, anti-E and anti-C^w are not inhibited by KNIR reagent. Numbers of each antibody tested by CAT Inhibition are listed below in parentheses: Anti-D (3), anti-C (3), anti-E (3), anti-c (3), anti-e (3), anti-K (3), anti-Fy^a (3), anti-Fy^b (2), anti-Jk^a (3), anti-Jk^b (1), anti-S (3), anti-s (3), anti-M (3) anti-C^w (3) and anti-Kp^a (3).

Some of the above antibodies were also tested by antigenicity ELISA. All gave negative results: anti-D (2), anti-C (1), anti-E (2), anti-c (1), anti-e (2), anti-K (1), anti-Fy^a (2), anti-Jk^a (2) anti-Jk^b (1), anti-s (1), anti-M (1) and anti-C^w (1).

5.3.5 Inhibition test validation

Prior to distribution of KNIR reagent to RCI laboratories a validation process will be followed for each batch to ensure that the reagent is fit for purpose and to reduce inter assay variability. This will include ensuring that: the protein concentration is 1 mg mL^{-1}; BSA and mouse IgG contamination is assessed; protein stained SDS-PAGE gels show a protein band at the correct molecular mass and an unrelated sr-blood group antigen does not inhibit KN related antibodies. Inhibition will be determined using known examples of KN related antibodies and clinically significant antibodies. Each time KNIR is used it will be a requirement to use a PBS diluted plasma sample as a dilution control.

A standard operating procedure (SOP) has been developed (Appendix 1) to describe the CAT inhibition test and use of KNIR reagent. This was tested by staff in RCI and IBGRL. RCI staff performed the CAT inhibition test in gel cards. IBGRL use glass tubes routinely for IAT testing rather than commercial gel cards. They performed their testing following the SOP but used tubes instead of gel cards. Whether using gel cards or tubes the results obtained by other members of staff were in 100% concordance with those expected.

5.3.6 Storage of sr-reagents and KNIR

Results of testing sr-LHR-C and sr-LHR-D reagents and a KNIR reagent which had all been frozen and thawed are shown in Table 5.9. Sr-LHR-C batch C157 and sr-LHR-D batch D157 were frozen for nearly two years and KNIR reagent batch CD229 was frozen for seven months. These reagents were thawed and used in CAT inhibition tests to inhibit KN antibodies in two samples: group one sample 5635H and group two sample 5786E. Inhibition results were as expected in that the KN related antibody in sample 5635H was inhibited with sr-LHR-C but not sr-LHR-D and opposite results were seen for sample 5786E where the antibody was inhibited with sr-LHR-D but not sr-LHR-C. The KNIR reagent mixture inhibited both samples as expected.

Table 5.9 Effect on inhibition using frozen / thawed batches of reagent

Sample number	PBS dilution control	sr-LHR-C C157	sr-LHR-D D157	KNIR CD229
5635H	1	0	1	0
5786E	3	3	0	0

Batches C157 and D157 had been frozen for nearly two years, KNIR reagent batch CD229 had been frozen for seven months. There was no deterioration in their ability to inhibit KN antibodies. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction.

Samples frozen and thawed up to three times were still able to inhibit KN antibodies. Sr-LHR-C batch C187 and sr-LHR-D batch D216 were frozen, thawed and tested on three occasions. All testing results were as expected compared to previous testing. Unfortunately, it was not possible to test the same patient samples again on each occasion due to limited sample sizes but at least one sample from group one (inhibited with sr-LHR-C but not sr-LHR-D) and one sample from group two (inhibited with sr-LHR-D but not sr-LHR-C) were tested on each date. Results of CAT inhibition testing on samples containing KN antibodies are shown in Table 5.10.

Table 5.10 Effect of freeze-thawing reagents used to inhibit KN antibodies tested by CAT inhibition

Sample number	25/02/14			28/03/14			04/04/14		
	Plasma	C187	D216	Plasma	C187	D216	Plasma	C187	D216
5893I	2	0	2						
2929T	3	3	0						
8204R				1	0	1			
KN3				2	2	0			
KN10							1	0	1
5619H							3	2	0

The same sample batches C187 (sr-LHR-C) and D216 (sr-LHR-D) was thawed, used and frozen again on the dates shown. There was no deterioration in ability of the sr-LHR reagent samples frozen and thawed up to three times to inhibit KN antibodies. Samples 58931, 8204R and KN10 are group one samples. Samples 2929T, KN3 and 5619H are group two samples. Positive reactions for the CAT test are graded 1 to 5 in order of increasing strength or 0 for no reaction.

A batch of the KNIR inhibition reagent was stored refrigerated at 4ºC with no added preservative. It was used at 19 weeks with no decrease in reactivity. However, after 24 weeks storage it failed to inhibit known KN antibodies.

5.3.7 Time savings if the CAT inhibition was used to investigate patient samples in NHSBT laboratories

No cost savings could be determined for RCI if the KNIR reagent was used. This is because reducing the complexity of investigation with use of the KNIR reagent would currently result in passing on a reduced charge for work to our customers. However, RCI has committed to an Operational Improvement Programme to become more efficient and save “hands-on” testing time. Time savings are estimated to be two hours per sample investigated. The KNIR reagent supplied would allow up to 30 ID panels to be set up which is based

on the current number of cases investigated in RCI that would benefit from use of the KNIR reagent (personal experience). Assuming 30 cases with a reduction in two hours testing time, this would be 60 hours per RCI laboratory saved per year, 480 hours for RCI per year for the eight RCI laboratories. This time saving would count towards RCI achieving its planned time savings and would be for BMS band 6 time.

Use of the KNIR reagent would mean that Filton RCI laboratory would no longer prepare an AET treated panel which would save approximately one hour per week. There would also be less reliance on the use of rare cells which in some instances and in some laboratories may not be available for use.

5.4 Discussion

It had been shown that a complete sr-CR1 protein could be used to inhibit KN system antibodies (Moulds and Rowe, 1996). Later workers speculated that using smaller parts of the CR1 protein for inhibition may be more efficient and cost effective. They showed that individual sr-LHRs of CR1 could be produced and that sr-LHR-D and to a lesser extent sr-LHR-C could inhibit most examples of antibodies with KN specificity (Tamasauskas *et al.*, 2001). In this study sr-LHR-C and sr-LHR-D proteins were produced, purified and then used in two techniques to identify and inhibit KN system antibodies. Individual sr-LHR-C and sr-LHR-D were used in both the antigenicity ELISA and CAT inhibition test and also a mixture of the two LHRs, KNIR, was used in the inhibition test.

The inhibition test using sr-CR1 has previously been performed in a tube IAT test using 6 µg sr-CR1 per test (Moulds and Rowe, 1996). Inhibition using individual sr-LHRs was also based on a tube IAT test but the concentration of individual sr-LHRs used for inhibition was not known. Inhibition was brought about by incubation of equal volumes of sr-LHR and patient plasma containing KN antibodies (Tamasauskas *et al.*, 2001). In this current study, a commercial gel card based IAT, was used for the CAT inhibition test using 1.25 µg sr-LHR to effect inhibition. During the course of this current study another group also investigated inhibition of KN antibodies, although they only produced and used sr-LHR-D (Seltsam *et al.*, 2014). They distributed their reagent to several laboratories to evaluate and after the inhibition incubation stage some of the laboratories used a tube IAT test, whilst others used a gel

card IAT test as in this study. Their LHR-D reagent was supplied either on its own or as part of a mixture containing other sr high frequency blood group antigens. Inhibition was performed using 0.5 to 1.5 µg sr-LHR per test. Independently, both this current study and Seltsam's work showed that a similar concentration of LHR protein could be used to bring about inhibition when based on a 30 minute incubation time of sr-reagent with patient's plasma. A smaller amount of sr-LHR reagent than complete sr-CR1 could be used for inhibition of KN antibodies.

In their study, Seltsam *et al.* (2014) found that when using the single sr-LHR-D reagent 12 out of 14 samples were inhibited. On increasing the amount of inhibition reagent used from 0.5 µg to 1.5 µg they found that the other two antibodies were also inhibited. Using their mixture of other sr-proteins which included sr-LHR-D, 21 examples of KN related antibodies were successfully inhibited, three samples needed 2 µg or more to effect inhibition, whilst six KN related antibodies were not inhibited even with 10 µg of protein. In this current study there were no examples found where increasing the inhibition reagent concentration brought about inhibition, even when ten times as much inhibition reagent was used (12.5 µg). In Seltsam's study therefore six out of 21 samples were not inhibited by sr-LHR-D (22%). Tamasauskas *et al.*, (2011), tested six examples of anti-Kn^a and found that three were inhibited by sr-LHR-C alone and three with LHR-D alone (50%). In this study 38% KN related samples were not inhibited by sr-LHR-D but were inhibited by sr-LHR-C either on its own or as part of the KNIR reagent. This illustrates the

effectiveness of using both sr-LHR-C and sr-LHR-D together in the inhibition test to detect KN system antibodies.

The antigenicity ELISA is a useful test to confirm KN specificity of an antibody. Using the separate sr-LHR reagents in both ELISA and CAT inhibition tests has given some interesting findings. All the amino acid polymorphisms responsible for KN blood group system antigens have now been localised to the LHR-D region (Veldhuisen, 2011). However, this study and two others have found that not all CR1 related antibodies are inhibited by sr-LHR-D. (Tamasauskas *et al.*, 2001 and Seltsam *et al.*, 2014). Seltsam speculates that these antibodies may be directed to so far undefined epitopes not expressed on LHR-D. In this study it was shown that most KN related antibodies react with one sr-LHR or the other but not usually both. It does not appear that this is a quantitative effect as increasing the concentration of sr-protein used to attempt to inhibit these antibodies did not effect inhibition. Of those antibodies with known KN specificity, two out of 11 anti-Kn^{a} antibodies and one out of two anti-McC^{a} antibodies reacted only with sr-LHR-C. One other study (Tamasauskas *et al.*, 2001) only found anti-Kn^{a} antibodies reacting with sr-LHR-C alone. The two LHR regions show high homology (Moulds *et al*., 2004). This can be seen in Figure 5.4 where the proteins are aligned, particularly from approximately 252 amino acids from the start of both proteins. The Kn^{a} antigen has valine at position 1561. Around this amino acid there is the sequence FEL**V**GERSIYCTS starting at 164 amino acids from the beginning of LHR-D. On LHR-C at 165 amino acids from the start the sequence is: FELVGEPSIYCTS with the exception of proline (P) instead of

arginine (R) as the seventh amino acid, this sequence is identical. The closely matching sequences around the Kn^a / Kn^b polymorphism are highlighted in Figure 5.4. It could be that the conformations of the proteins are very similar around this point and that some anti-Kn^a antibodies are directed against this epitope and not that on LHR-D. If models of the two molecules are compared and matched around the Kn^a / Kn^b SNP, the predicted conformation can be seen to be very similar (Figure 5.5).

```
LHR-C  HCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSITCLDNLVWSSPKDVCKR
       ::..:..: ::.     :  .::.::::.::::: :.:. :::.::.:::::: .: :.:
LHR-D  HCKTPEQFPFASPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCLENLVWSSVEDNCRR

LHR-C  KSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAECILSGNTAHWSTKPPIC
       :::  ::.: :::::. :: : :: .::::. : ::::  :. :..:::.. :. : :::
LHR-D  KSCGPPPEPFNGMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLVSGNNVTWDKKAPIC

LHR-C  QRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNLGSRGRKVFELVGEPSIYCTSNDDQ
       . : :  ::::.:::: :.:: .:: :.::::.:. :  :...:::::: :::::.:::
LHR-D  EIISCEPPPTISNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFELVGERSIYCTSKDDQ

LHR-C  VGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQ
       ::.::.: :.::  :::: :.::::.: :  :::.:.:.:...:::::::::: : . :.::
LHR-D  VGVWSSPPPRCISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQPGFVMVGSHTVQCQ

LHR-C  ALNKWEPELPSCSRVCQPPPEILHGEHTPSHQDNFSPGQEVFYSCEPGYDLRGAASLHCT
       . ..: :.:: :::::::::::::::: :::::::::::::::::.::::::::::::
LHR-D  TNGRWGPKLPHCSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCEPSYDLRGAASLHCT

LHR-C  PQGDWSPEAPRCAVKSCDDFLGQLPHGRVLFPLNLQLGAKVSFVCDEGFRLKGSSVSHCV
       ::::::::::::.::::::::::::::.:::::::::::::::::::::: :.::::
LHR-D  PQGDWSPEAPRCTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCDEGFRLKGRSASHCV

LHR-C  LVGMRSLWNNSVPVCEHIFCPNPPAILNGRHTGTPSGDIPYGKEISYTCDPHPDRGMTFN
       :.:::.:::.:::::::.:::::::::::::::::: ::::::::::::: :::::::::
LHR-D  LAGMKALWNSSVPVCEQIFCPNPPAILNGRHTGTPFGDIPYGKEISYACDTHPDRGMTFN

LHR-C  LIGESTIRCTSDPHGNGVWSSPAPRCELSVRA
       :::::.:::::::.:::::::::::::::: :
LHR-D  LIGESSIRCTSDPQGNGVWSSPAPRCELSVPA
```

Figure 5.4 Comparison of LHR-C and LHR-D protein sequences. The two sequences of LHR-C and LHR-D were aligned using LALIGN software. The yellow highlight shows the similarity between LHR-C 165 amino acids from the start of the sequence and LHR-D 164 amino acids from the start. The greyed out area shows the highly homologous parts of the two molecules. The dots indicate degree of homology with two dots indicating complete homology and one dot indicating partial homology, whilst no dots indicates no homology.

Conformation of the molecules could also be affected by glycosylation as discussed in Protein Purification section 4.3.5. Figure 5.5 shows that around the yellow highlighted area (shown in Figure 5.4) the molecular conformation is very similar for LHR-C and LHR-D.

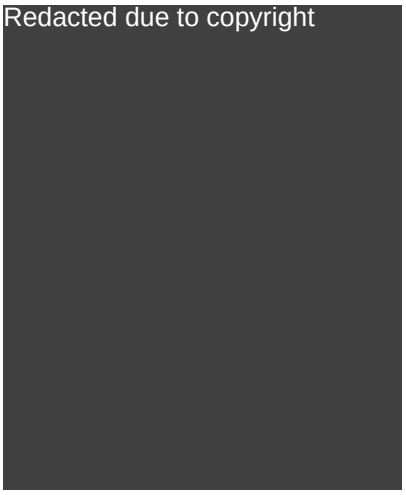

Figure 5.5 Comparison of the structure of LHR-C and LHR-D molecules. A model of LHR-C (blue) and LHR-D (red) matched around the Kn^a / Kn^b polymorphism on LHR-D and the corresponding part of the molecule on LHR-C is shown. The green line indicates a matching area of the molecules. Models from Furtado et al., (2008) aligned using the Swiss-Prot database at www.ebi.ac.uk/swissprot.

In practice the mixed inhibition reagent will be used for inhibition in RCI laboratories so the differences seen when using individual sr-LHRs would not be seen. However, a strategy for RCI might be for RCI laboratories to refer any inhibited plasma samples to RCI, Filton. Samples could be batched and then tested by an antigenicity ELISA using the individual sr-LHRs. This would enable data to be gathered on the reactivity of KN antibodies and so would add to knowledge of the KN blood group system.

KN antibodies are difficult to investigate in the laboratory. The current RCI protocol for investigation of antibodies includes an algorithm which specifies that if an antibody reacts with all or most reagent panel cells by IAT and enzyme techniques with variable or weak reaction strengths then the possibility of the presence of a KN related antibody should be considered. The next step is to test with cells that lack antigens such as Kn^a, Yk^a and McC^a. If negative with one or more of these cells then the antibody may have KN specificity. However, these cells are rare and may not be available to the laboratory. If they are available they are usually frozen and need to be recovered for use. It might be necessary to refer the sample to IBGRL who have access to plasma and red cells from previously investigated cases or who can perform the monoclonal antibody-specific immobilization of erythrocyte antigens assay (MAIEA) test. However, if blood is required for the patient, the antibody may react with all panel and donor cells that it is tested with and the presence of underlying clinically significant antibodies needs to be confirmed or excluded. At this point it becomes difficult to resolve the investigation and if transfusion is urgently required, donor units may have to be selected that give the weakest reactions by IAT, which is not ideal for the patient. Some RCI laboratories will use RBCs treated with AET to denature KN antigens for antibody investigations but AET also denatures other antigens, for instance AnWj. This is particularly dangerous as seen in this study where retrospective testing of a patient sample indicated that an anti-AnWj was wrongly reported as a KN related antibody. KN antibodies are not clinically significant but anti-AnWj is and has caused severe haemolytic transfusion reactions (Xu *et al.*, 2012).

In other countries e.g. USA (Judd, 2006) and Argentina (Torres *et al*., 2010) it seems that greater emphasis is placed on trying to identify KN and other HTLA antibodies. RBCs treated with the enzyme ficin, or the reducing agents DTT or AET may be used or serum inhibition and titration studies may be performed. These workers will also try to use RBCs negative for high frequency HTLA antigens. The rationale is that Ch/Rg and JMH antibodies will not react with ficin treated cells; anti-Ch/Rg antibodies are inhibited by pooled plasma; anti-JMH does not react with DTT treated cells. KN antibodies react variably with ficin treated RBCs and usually do not react with DTT or AET treated RBCs. Incubation of patient's plasma with pooled plasma will inhibit Ch/Rg. However, this extra testing takes time and may not, ultimately, reveal the identity of the antibody. If an antibody is inhibited by the KNIR then it is immediately known that the antibody is KN related and even more importantly, the presence of underlying antibodies can be seen. It was shown that no clinically significant antibodies are inhibited by either sr-LHR-C or sr-LHR-D. Inhibition with the new KNIR reagent is a simple and fast technique that identifies the presence of a KN antibody and provides information on any underlying clinically significant antibodies that may be present in one test that takes one hour to complete, including setting up and reading gel cards; 30 minutes for the inhibition stage, 15 minutes for incubation on the gel card and 10 minute centrifugation step.

Crossmatching is the process where a patient's plasma is tested against donor red cells to ensure that the donations are compatible and safe to issue for transfusion. The compatibility test is a CAT test (Materials and methods

section 2.5.2) where patient's plasma is tested with red cell samples from the units selected for transfusion. CAT inhibition test could easily be used for crossmatching. As the plasma would have been modified by inhibition, RCI protocol dictates that the crossmatch should be performed in parallel with normal, uninhibited patient's plasma. It would give reassurance that incompatibility was due to the presence of clinically insignificant antibodies if the KN antibody was inhibited and all the crossmatched units were compatible with the inhibited plasma. The units issued would be suitable for issue, although not termed compatible, a term reserved for negative reactions obtained with unmodified patient plasma, but there would be reassurance that the units would benefit the patient. The process from identification of KN antibody to issue of crossmatch suitable blood would be very rapid at less than two hours in comparison to current practice which would take approximately four hours (personal experience).

Both techniques proved successful and will be valuable additions to the repertoire of RCI and IBGRL laboratories, where time taken for investigation and provision of suitable blood would be significantly reduced. KNIR reagent stored for up to four months at 4ºC showed no deterioration in its ability to inhibit KN related antibodies. However, after five months some deterioration was noted. Samples stored for over a year once thawed did not show any difference in ability to inhibit, nor did samples that had been thawed and frozen up to three times. In practice it would be useful to prepare frozen reagent in ready to use aliquots so that they could be stored in the individual laboratories, thawed for use and the appropriate volume of plasma added for

inhibition. Currently, the Medicines and Healthcare Products Regulatory Agency (MHRA) indicate that NHSBT is assigned the status of a single Health Institution, so that supplying the KNIR reagent to RCI laboratories would be exempt from CE marking (personal communication from M. James, National Reagents Strategy Manager). However, to sell the reagent to laboratories outside NHSBT would require it to be CE marked and this is discussed in the next section.

The aim to use sr-LHR-C and sr-LHR-D proteins in a test to aid in antibody investigation was fully met. The ELISA which was developed will not be used routinely due to a lack of equipment and expertise in this technique in RCI laboratories. The inhibition technique utilises a primary antibody investigation technique, a gel card IAT test, in regular use in RCI. Addition of an inhibition stage to this technique will provide an efficient and standardised testing method for samples containing KN related antibodies in RCI laboratories. Ultimately, patient care will be improved by faster identification of KN related antibodies and by simplifying the identification of clinically significant antibodies that may be masked by KN related antibodies..

6.0 FINAL DISCUSSION

6.1 Investigation of Knops antibodies

This study aimed to produce a reagent to simplify investigation of Knops related antibodies in RCI laboratories. Despite reports in the literature on use of whole sr-CR1 protein to inhibit anti-KN antibodies, no commercial reagent is available. Also, the use of a single part of the CR1 protein, sr-LHR-D, has been advocated for use as an inhibition reagent but a commercial reagent based on just sr-LHR-D is not available either. This study has shown that a cocktail containing both sr-LHR-D and sr-LHR-C is more effective at inhibiting Knops related antibodies, than sr-LHR-D on its own. Sr-LHR-C and sr-LHR-D proteins were produced in HEK293 cells. The proteins produced proved to have KN antigenicity so use of a mammalian system produced protein in the correct conformation to retain its blood group specificity. The sr-proteins were produced as 3XFLAG tagged proteins. The presence of the tag allowed a one-step purification of the desired proteins from other protein contained in the cell culture supernatant. The proteins produced were antigenic with the FLAG tag attached so cleavage of the tag and further purification was not required. There was little contamination with BSA after the purification process. Although acid elution did elute a small amount of the mouse anti-FLAG antibody from the affinity gel it was chosen as the elution method of choice due to slightly reduced cost over peptide elution, it being a quicker process than peptide elution and acid elution is already in use in BITS/IBGRL for elution of other sr-blood group proteins. The presence of mouse immunoglobulin does not affect the serological IAT test or the antigenicity ELISA. Also, the affinity gels could still be used up to six times after acid

elution. The novel inhibition test in commercial IAT gel cards was developed for use in NHSBT laboratories. When distributed to these laboratories it will be an invaluable reagent, providing a faster way of identifying KN related antibodies in patient's plasma identifying whether there are any underlying antibodies in the plasma that might be clinically significant and ultimately ensure faster provision of compatible blood for patients.

The cheapest cost to produce 9 mL reagent to distribute to NHSBT laboratories was calculated as £5,255 (production cost using T-175 flasks was £1,257 and purification using acid elution was £3,998). If aliquoted into ready to use amounts and stored frozen until use, reagent activity would last for at least one year. The 1 mL amount would easily allow 30, ten cell ID panels to be set up which should be enough for one year for each laboratory based on current activity and frequency of anti-Kn antibodies (personal knowledge). There would be a saving on use of rare KN negative cells and if a sample does not react with e.g. Kn^a negative cells it is only an indication towards presence of anti-Kn^a and so further investigation would be required. The inhibition test confirms, in one test, that anti-KN antibody is present by the fact that it is inhibited. In the Red Cell Reference laboratory, IBGRL, use of this reagent would replace use of the MAIEA test. The Head of RCR (Nicole Thornton, personal communication) has said that performing the MAIEA would not provide any advantage to them if the KNIR reagent was available to use. The MAIEA is a time consuming test and can only indicate that an antibody has KN specificity and this could be achieved more rapidly by use of KNIR reagent. For RCI, time savings of approximately 560 hours for band 6

BMS staff would be achieved. A further advantage of using KNIR reagent would be standardisation of investigation of KN related antibodies across RCI laboratories with a national standard operating procedure in place. Currently, there is no consistent approach with some RCI laboratories having access to rare cells, others using AET treated panels.

6.2 Future Developments

6.2.1 CE marking of reagents

Under current European Union (EU) guidelines the *in vitro* diagnostic device (IVD) includes reagents (European Union, 1998) and states that they should be CE marked (see below). However, reagents produced within Health Institution laboratories for use in that environment are not covered by this directive as they are not subject to commercial transactions. The current viewpoint from the Medicines and Healthcare Products Regulatory Agency (MHRA) under guidance issued in August 2013 is that NHSBT is regarded as a Health Institution so supplying reagent to NHSBT laboratories would be exempt from CE marking (personal communication from Malcolm James, NHSBT National Reagents Strategy Manager). However, following a poster presentation about the findings of this study at the British Blood Transfusion Society (BBTS) annual meeting in October 2013 (Appendix 2), an enquiry has been received from a national testing laboratory in Finland asking if they could be supplied with the KN inhibition reagent. To do this CE marking of the reagent would be required.

CE marking is verification by the manufacturer that the product meets EU safety, health or environmental requirements (European Union, 2010). As there already is demand from abroad and there may be further demand and in case CE marking becomes necessary even for provision within NHSBT, it is the intention to CE mark the KN inhibition reagent cocktail. This will require carrying out a conformity assessment, setting up a technical file, issue of the EC declaration of conformity and affixing a CE mark to the product. If the reagent is supplied to a foreign country then a further complexity is the requirement to issue instructions and labels in the country's specified language e.g. in the case of Finland labels would need to be in Finnish and Swedish. As the intention would be to supply KNIR frozen then there would be a requirement to check that the product meets its intended purpose up to the date of expiry and at least one month beyond this for three separate batches. Also validation that the product meets its intended purpose once thawed for three separate batches. This would involve sending the reagent to the furthest RCI laboratory at Newcastle via NHSBT transport in dry ice for them to test and also for them to send back a vial that could then be tested in Filton. For Finland this would involve sending by air transport. An EU declaration of conformity could be issued by RCI Reagents under self-certification or through BITS/IBGRL who issue monoclonal antibodies and a CE mark could then be affixed to the reagent.

6.2.2 Use of sr-blood group proteins in RCI

Use of KNIR reagent will represent the first use of sr-blood group proteins in RCI laboratories. The CAT inhibition test utilises a test system already

familiar to RCI staff. Feedback on use of the reagent has been positive from both RCI and RCR laboratories. A recent study showed that several sr-blood group antibodies could be incorporated into inhibition techniques (Seltsam *et al*., 2014). Sr-blood group proteins expressing the most relevant and high prevalence antigens of the respective blood groups Scianna, Rodgers, Chido, Knops and JMH were produced and mixed together in a cocktail for use. Antibodies of these systems are all clinically insignificant so use of the cocktail would inhibit any of these antibodies if they were present in a single test. This would eliminate the need to identify the exact antibody specificity.

Sr-blood group antigens including sr-K, sr-k, sr-Fy^a, sr-Fy^b and sr-Lu^b have been produced in BITS/IBGRL under a contract with a commercial company BioRad. The cell lines and license to use them are owned by BioRad but they can be used internally within NHSBT (personal communication from K Ridgwell). During the course of this study an antibody under investigation in RCI was found to react with all panel cells and was proving difficult to identify. Before examples of rare cells that were negative for high frequency antigens were thawed, the patient's plasma was tested by CAT inhibition test using sr-Lu^b kindly provided by Fran Green from BITS/IBGRL. The antibody was inhibited and thus identified as anti-Lu^b, a clinically significant antibody.

6.2.3 Collaboration between research and diagnostics laboratories within NHSBT

The above use of sr-Lu^b to resolve a patient antibody investigation in RCI highlights one of the most satisfying aspects of this project and that is the

collaboration that has grown between RCI and BITS/IBGRL. The need for a reagent to inhibit KN antibodies was identified by RCI and working together a reagent has been produced. Ultimately the reagent will be produced in BITS/IBGRL according to the recommendations made in this study. Frozen HEK293 cells prepared in this study have been retrieved and grown by Amelia White in BITS/IBGRL. She has grown them successfully in T-175 flasks and purified the protein on affinity columns according to the protocol described here. She has found growth in T-175 or the bigger T-225 flasks to be the best method for growing the cells and is currently trying to produce enough protein for distribution to the nine NHSBT laboratories.

PUBLICATIONS AND PRESENTATIONS

An abstract for the British Blood Transfusion Society annual meeting in October 2013 was accepted for a poster presentation, Appendix 2.

An oral presentation was given at a BITS/IBGRL Work in Progress meeting held in March 2014.

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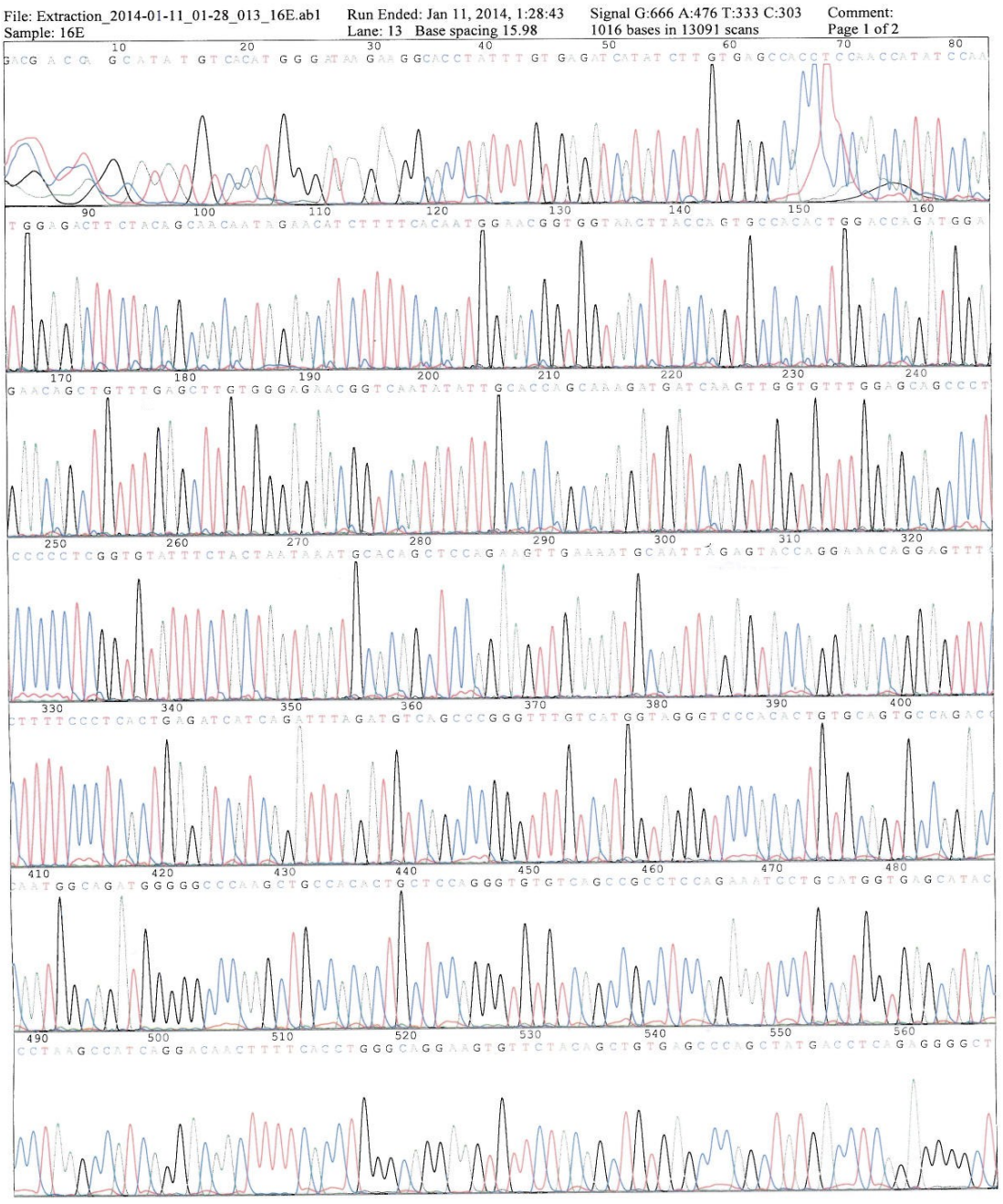
APPENDIX 1

Sequence analysis for the primer designed to sequence around the Kn^{a} / Kn^{b} polymorphism. The fragment shown in figure 3.5 lies between bases 178 and 192.



APPENDIX 2 Standard operating procedure KN (Knops) antibody inhibition test

STANDARD OPERATING PROCEDURE *This SOP replaces* *NEW*	**Copy Number**
	Effective **DRAFT FOR EVALUATION**

Summary of Significant Changes

N/A

Purpose

To inhibit KN antibodies to aid in their identification and allow detection of any underlying clinically significant alloantibodies.

Responsibilities

Restrictions

Definitions

Column agglutination test (CAT) Indirect antiglobulin test (IAT) performed in gel cards

KN Inhibition Reagent (KNIR) – a mixture of soluble recombinant proteins carrying KN antigens. Store frozen at -30°C.

KN system (Knops) – contains 9 antigens: KN1 (Kn^a), KN2 (Kn^b), KN3 (McC^a), KN4 (Sl1), KN5 (Yk^a), KN6 (McC^b), KN7 (Vil), KN8 (Sl3), KN9 (KCAM)

CAT Inhibition test – CAT test performed with plasma inhibited with KNIR

RT – Room temperature

Items Required

Automatic pipette
Pipette tips
Indelible pen
BIO-RAD AHG cards
Reagent cells in CellStab

INF335 – Grading of Reactions
SOP1180 – Compatibility testing
KNIR reagent – supplied as aliquots of 30 µL

STEP	DETAILS	INFORMATION
1. Perform inhibition	1.1 Thaw tube of KNIR inhibition reagent	KNIR is provided frozen in 30 µL amounts
	1.2 Label tube with test identity	
	1.3 Add 270 µL of patient's plasma to tube and mix well	This represents a 1 in10 dilution
	1.4 In a separate tube add 30 µL PBS to 270 µL patient's plasma and mix well	Dilution control
	1.4 Incubate both tubes at RT for 30 minutes	
2. Perform CAT Inhibition test	2.1 Label card(s) with test identity	
	2.2 Place 50µL of 0.8% CellStab ID-panel cells in the reaction chambers of AHG cards	
	2.3 Add 25µL of inhibited plasma or patient's plasma with PBS to each well	Incubate for 15 minutes
	2.4 Place tests in a 37°C air incubator	
	2.5 Centrifuge in an appropriate centrifuge	
	2.6 Read results	
	2.7 Write results on worksheet	
3. Result interpretation	3.1 Compare results between the initial CAT, CAT using the dilution control and CAT using inhibited plasma	If antibodies with KN specificity are present positive results obtained by CAT and with the dilution control will be negative by CAT Inhibition test
4. Crossmatching	4.1 Use inhibited plasma for crossmatching in parallel with untreated plasma	SOP1180

APPENDIX 3 - Poster presented at British Blood Transfusion Society meeting October 2013

Production of soluble recombinant proteins for investigation of antibodies directed against the KN blood group system

NHS Blood and Transplant

Wendy Etheridge[1], Kay Ridgwell[2], Ruth Morse[3]

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Introduction

Knowledge of gene sequence has allowed the amino acid sequence of molecules carrying blood group antigens to be determined and their structures within the red blood cell membrane to be predicted.

Soluble recombinant (sr) blood group antigens have been developed that retain their antigenic activity but are free of the red blood cell membrane. Antigens of the KN blood group system are carried on Complement Receptor 1 (CR1) protein. CR1 is a single pass protein with a large extracellular domain.

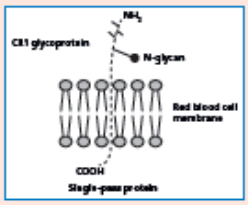

Figure 1: CR1 glycoprotein shown within red blood cell membrane (adapted from Reid ME and Lomas-Francis C. (2004) The Blood Group Antigens Facts Book (2nd edn). Elsevier Ltd, London.

A complete srCR1 molecule and smaller fragments, termed long homologous repeats (LHRs) have been shown to inhibit antibodies directed against antigens of the KN system. These antibodies are not clinically significant ie they do not cause Haemolytic Transfusion Reactions (HTRs) or Haemolytic Disease of the Foetus and Newborn (HDFN) but their presence can mask underlying clinically significant antibodies. This can make investigation and provision of blood for patients with these antibodies more difficult and time consuming.

Methods

Soluble recombinant (sr) LHR-C and LHR-D FLAG-tagged proteins were produced from stably transfected HEK293 cells, followed by affinity purification using FLAG-agarose gel column chromatography. PBS solutions containing approximately 1mg mL^{-1} srLHR-C or srLHR-D were prepared.

An inhibition test was optimised to use 50 µg mL^{-1} protein to inhibit KN antibodies prior to testing the inhibited samples by gel column agglutination technology (CAT).

CAT is an indirect antiglobulin test (IAT) performed in gel cards for antibody detection. Inhibition was achieved by incubation of patient plasma with srLHR protein(s) for 30 minutes at RT (Figure 2).

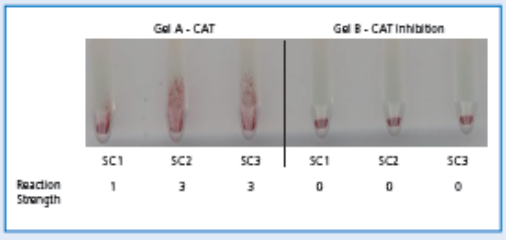

Figure 2: CAT (gel A) and CAT inhibition (gel B) cards for a sample containing anti-KN (SC = reagent screening cells).

Results

Patient samples (n=29) that were either known or suspected through serological testing to contain antibodies to KN antigens were tested by CAT inhibition. Results are shown in Figure 3. The two samples that were not inhibited did not have anti-KN specificity. One proved to be anti-Csa and the other anti-AnWj.

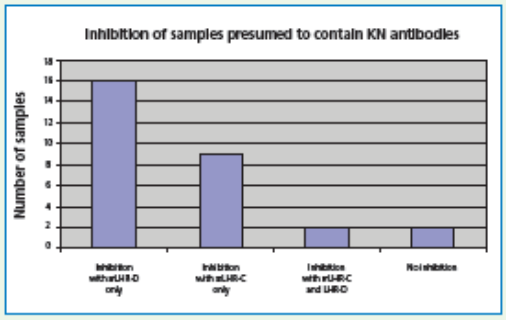

Figure 3: Graph showing results of inhibition testing of samples using individual LHRs.

Other antibodies were present in some samples eg anti-E and anti-C^w. Although the KN specific antibodies also present were inhibited these other antibodies were not and could still be clearly detected in serological tests (Figure 4).

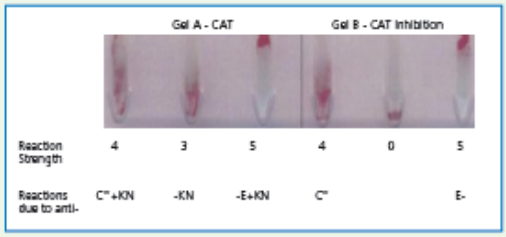

Figure 4: CAT (gel A) and CAT inhibition (gel B) cards for samples with anti-KN and other antibodies.

Discussion

KN antibodies are notoriously difficult to work with as they give variable reactions and react with most reagent red cells that they are tested against. About 50% of samples with KN antibodies also contain other red cell antibodies. Inhibiting the nuisance anti-KN antibodies allows detection of any important clinically significant antibodies that may also be present.

The inhibition technique using a cocktail containing srLHR-C and srLHR-D will greatly improve investigation of these antibodies in RCI laboratories, reducing the time taken to investigate and then find compatible donor units for the patient. In addition it has been shown here that antibodies appearing to have KN specificity, but that are not inhibited, should be tested further as they have the potential to be antibodies that are clinically significant like the anti-AnWj found here, an antibody incorrectly reported as a KN antibody but that actually has caused severe HTR.