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Summary

The autologous red cell agglutination assay reagent consists of an antibody or antibody fragment of a human erythrocyte-specific monoclonal antibody (mAb) conjugated to an antigen of interest. This bi-functional reagent causes the agglutination of the patient's erythrocytes in the presence of the antigen-specific antibodies in the patient's serum. Previously, such reagents have been produced either by chemical conjugation or recombinant expression in bacteria. These protocols required laborious processes for purification and refolding. The aim of the work reported in this article was to explore the production of the agglutination assay reagent as both a single chain Fv (scFv) antibody fragment and recombinant full-length mAb, expressed in a secreted form in commonly used mammalian cell lines. The DNA encoding the anti-erythrocyte antibodies was linked to that of a diagnostic peptide from West Nile virus, which requires glycosylation for recognition by antibodies present in the sera of infected horses. The expression vectors were designed to allow the rapid directional insertion of DNA encoding other immunogenic peptides to mediate the secretion of agglutinating scFv and full-length mAb reagents from transfected mammalian cells. Stable cell lines were produced for the expression of most, but not all of the constructs. The recombinant reagents could be used directly from the cell culture media after a simple concentration step. The results indicate that further modifications to increase the yield of recombinant protein will enable the direct use of culture supernatant in diagnostic assays without further processing.

Keywords

red cell agglutination, mammalian expression, scFv, recombinant antibody, diagnostic, West Nile virus

Abbreviations

mAb - monoclonal antibody; scFv – single chain Fv antibody fragment; WNV – West Nile virus; E – envelope protein; HRP – horseradish peroxidase; hyg – hygromycin; gpt – guanine phosphoribosyl transferase; Ig – immunoglobulin, OD – optical density

1. Introduction

The autologous red cell agglutination technology was developed and commercialised by AGEN Biomedical Ltd. for the rapid detection of circulating antibodies of clinical interest. The technology is based on a monoclonal antibody (mAb) which binds to but does not agglutinate erythrocytes. If a diagnostic peptide is conjugated to the mAb, peptide-specific antibodies in patient sera crosslink erythrocytes through the mAb and mediate easily observable agglutination within two minutes (Kemp *et al*., 1988; Rylatt *et al*., 1990; Hillyard *et al*., 1991). The technology has been applied in a commercial assay to detect antibodies in Human Immunodeficiency virus (HIV)-infected patients, with sensitivities and specificities of greater than 99% (Hillyard *et al*., 1991; Wilson *et al*., 1991).

The autologous red cell agglutination technology represents a simple and robust rapid diagnostic assay platform based on a single reagent. Because the read-out system is the patient's own erythrocytes, there is no need for secondary reagents such as fluorophore- or gold-labelled antibodies, reagent-coated latex beads or fixed heterologous erythrocytes. The assay is performed on a simple plastic tray, which avoids the need for more complicated devices such as those required for immunochromatographic technologies. However, broader exploitation of this technology has been hindered by the conjugation protocols required to produce the diagnostic reagents (John *et al*., 1990; Wilson *et al*., 1991). The mAb must be reduced to an Fab fragment and chemically conjugated to the synthetic peptide in a process requiring several purification steps. A separate chemical protocol must be developed for each diagnostic analyte. The presence of heterophile (anti-mouse immunoglobulin (Ig)) antibodies also complicates the interpretation of the assay results and has led to the inclusion of mouse immunoglobulin in the reaction mix.

To overcome these barriers to greater use of the technology, several groups have explored the use of recombinant reagents. The feasibility of this approach was established initially in a bacterial expression system that used a single chain Fv fragment (scFv) derived from the original anti-erythrocyte mAb. The DNA encoding the scFv was linked to that encoding the HIV gp41 peptide to produce a functional reagent (Lilley *et al.*, 1994; Coia *et al*., 1996). Although the performance of the recombinant molecule was equivalent to that of the

chemically conjugated reagent, the yield of recombinant was considered unacceptable for commercial purposes.

Anti-erythrocyte Fab fusion proteins have also been described. In one approach, an Fab with HIV-1 and HIV-2 terminal fusion peptides was expressed using a system which induced secretion of the recombinant proteins into the bacterial periplasmic space, where proper chain folding and disulphide bond formation could take place (Dolezal *et al*., 1995). In another approach, an anti-erythrocyte Fab-HIV peptide fusion protein was produced through cytosolic expression as inclusion bodies in *Escherischia coli* (Gupta *et al*., 2001; Gupta and Chaudhary, 2002, 2003, 2006). Although functional reagents were produced with both methods, the recovery of the reagent involved extensive extraction and purification procedures, as is common with other bacterial systems (for example, Choi *et al*., 2004). Similarly, in the approach used by Gupta *et al*., (2001) the recombinant molecules were extracted from the inclusion bodies by solubilisation and denaturation, followed by renaturation to assemble the Fab. The published protocol resulted in a pure reagent, but it involves several purification steps that appeared to span many days.

In the decade since the original publications describing the recombinant scFv-HIV reagent, there have been significant advances in protein expression systems. scFvs have been produced in bacterial, mammalian, yeast and insect cell systems with varying success in terms of yield and function (Ridder *et al*., 1995; Kretzschmar *et al*., 1996; Sanchez *et al*., 1999; Fernandez *et al*., 2000; Freyre *et al*., 2000; Dai *et al*., 2003). Increasing numbers of recombinant therapeutics are being manufactured using mammalian expression systems (Wurm, 2004). These systems ensure that there is authentic post-translational modification of the recombinant versions of proteins expressed naturally in mammalian cells, such as antibodies, hormones, cytokines and viral proteins. The secretion of the protein into the cell culture medium, especially synthetic serum-free medium, facilitates simple protein purification (Dorai *et al.*, 1994). Although the reported protein yields vary, fed-batch fermentation can yield 0.5 to 2 g/L protein (Bebbington *et al*. 1992; Zhou *et al*. 1997).

The studies reported in this article were undertaken to assess the feasibility of mammalian expression systems for the production of recombinant autologous agglutination reagents for

use in antibody-detection assays. In particular, the aim was to develop a system based on generic vectors into which antigenic peptides could be incorporated rapidly and one in which the culture supernatant could be used for agglutination reactions without further processing. Recombinant expression of these agglutination reagents would enable rapid, on-demand production for minimal cost. This is particularly important for developing countries where outbreaks of new pathogens are frequent and there is a requirement for inexpensive, 'point-of-care' assays. Such a system will be very useful in response to the emergence of new pathogens.

2. Materials and Methods

2.1. Antibodies

The anti-erythrocyte and anti-D-dimer mAb and fragments were supplied as purified proteins by AGEN Biomedical Ltd (Brisbane, Australia). The anti-erythrocyte (specifically, anti-human glycophorin A, Rylatt *et al*., 1990) mAb 1C3/86 (mAb 1C3) is the basis for the commercial human autologous agglutination assay reagents. The D-dimer-specific mAb 3B6/22 (mAb 3B6) is used in the commercial D-dimer assays manufactured under the Dimertest™, SimpliRED™ or Simplify™ trademarks, and was provided in both the hybridoma-derived and recombinant chimeric (human-mouse) forms. The mAb 1C3 Fab (blocked) fragment was prepared by proprietary protocols adapted from Wilson *et al*. (1991). MAb 17D7, which recognizes a 19-residue peptide from the envelope (E) protein of West Nile virus (Sanchez *et al*., 2005; Hobson-Peters *et al*., 2008) was supplied in ascitic fluid by Melissa Sanchez and Robert Doms (University of Pennsylvania). Anti-HIV-1 mAb 1B1/114 (mAb 1B1) was provided by AGEN Biomedical Ltd. (Brisbane, Australia) in purified form. The West Nile virus (WNV)-positive horse serum was obtained about three weeks following experimental infection with WNV_{NY99} (Richard Bowen, Colorado State University, personal communication), and its reactivity to the 19-mer E protein peptide has been described previously (serum 3, Hobson-Peters *et al*., 2008). A pool of sera from WNV-naïve horses was used as an additional negative control.

2.2. Polymerase chain reaction (PCR) protocol

PCR was performed in a final volume of 25 µL. The reaction mix comprised 10 mM Tris-Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 – 300 µM each dNTP (Sigma-Aldrich, Saint Louis, MO, USA), 1.25 U mAb-bound *Taq* DNA polymerase (Jumpstart™, Sigma-Aldrich, Saint Louis, USA) and 500 nM each of a sense and anti-sense primer (Sigma-Genosys, Castle Hill, Australia, Table 1). The reactions contained 0.5 – 200 ng of plasmid as template. PCR products were visualised on an UV transilluminator, following separation through an agarose gel (generally 2% agarose in Sybrsafe™ (Invitrogen (Molecular Probes Inc.) Eugene, OR, USA)).

2.3 Construction of vectors expressing recombinant mAb 1C3 antibodies

The construction of plasmids expressing the mAb 1C3-based scFv vectors has been described previously (Hobson-Peters *et al*., 2008). Construction of vectors for expression of a

recombinant version of the full-length mAb 1C3 was based on plasmids which encode the D-dimer-specifc mAb 3B6. These plasmids, pSVhygHuC_K and pSVgptHuIgG_1, separately encode the mAb 3B6 light and heavy variable region genes linked to the human kappa light chain and IgG_1 heavy chain genes, respectively (Macfarlane *et al*., 2006). The cloning strategy, as outlined in Fig. 1, was to substitute the mAb 3B6 variable regions in the two plasmids with those of mAb 1C3. In brief, the original pSV vectors were digested with *Hin*DIII and *Bam*HI to release the immunoglobulin signal peptide and 3B6 variable regions. These were replaced with the 1C3 variable regions which had been generated by using overlap PCR to ligate three PCR products: DNA encoding the immunoglobulin signal peptide with *Hin*DIII site amplified from the original pSV vectors, either the mAb 1C3 V_L or V_H region genes amplified from the mAb 1C3 scFv bacterial plasmid pGC038C_L (Coia *et al*., 1996) and the 3' region of the parental pSV vector containing splice sequences and the *Bam*HI site. The plasmids were named pSV1C3V_L and pSV1C3V_H.

The construction of a plasmid to express chimeric mAb 1C3 with the WNV epitope WN19 attached to the C-terminus of the heavy chain was done in two steps (Fig. 2). An intermediate construct (pSV1C3VHI) was produced, in which the stop codon at the end of heavy chain was removed and two unique, non-complementary restriction enzyme sites, *Xho*I and *Eag*I, were inserted. The sequence encoding WN19 was amplified from pCBWN (Davis *et al*., 2001) and inserted into pSV1C3VHI using these two sites. An additional restriction enzyme site (*Bsp*E1) was incorporated at the 3' end of the peptide WN19 sequence to facilitate future insertions via the *Xho*I and *Bsp*EI sites. This design also enables replacement of peptide WN19 through *Xho*I/*Eag*I or *Bsp*EI/*Eag*I digestion. The primers used in the cloning reactions are indicated in Figs. 1 and 2 and listed in Table 1. To confirm that the expected constructs had been prepared, the plasmids were sequenced by the Australian Genome Research Facility (AGRF, Brisbane) by ABI Prism BigDye Terminator version 3.1. At least one forward and one reverse reaction were submitted for each plasmid.

2.4 Glycophorin ELISA

The wells of an ELISA plate (Greiner Bio-one GnbH, Neuburg, Germany) were coated with glycophorin A (Sigma-Aldrich, Saint Louis, MO, USA) in coating buffer (50 mM $NaHCO_3$, 50 mM Na_2CO_3 pH9.6) for either 1 h at room temperature with gentle agitation, or overnight at 4 °C. The wells were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and blocked with 5% skim milk in PBS (100 μL) for 1 h. After

washing, diluted mAbs, Fabs or recombinant antibodies as culture supernatant were added (50 μL) and serially diluted in PBS/T. Following a 1 h incubation, the wells were washed four times and glycophorin-bound antibody detected with goat anti-mouse Ig (Dako, Glostrup, Denmark) diluted 1:1000 in PBS/T (50 μL/well). After a 1 h incubation, the plates were washed four times and 50 μL/well horseradish peroxidase (HRP)-conjugated rabbit anti-goat Ig (Dako, Glostrup, Denmark) diluted 1:1000 with PBS/T was added and incubated for an additional 1 h. After a final six washes, 100 μL/well ABTS substrate solution [2 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 0.003 % H_2O_2 (v/v), 25 mM citric acid, 25 mM tri-sodium citrate (pH4.4)] was added and incubated for 1 h. The reaction was stopped with 50 μL/well 3.9 % oxalic acid and the plate was read on an automated plate-reader at 405 nm. The optical density (OD) readings represent the average of two wells.

The ELISA was optimised using a checkerboard titration of human glycophorin A (200 – 6.25 ng/well) and mAb 1C3 (anti-glycophorin A) or 3A1/48 (negative control mAb, Bundesen *et al*., 1985) Fab (800 – 0.0018 ng/well), using the conditions described below. The optimal concentration of glycophorin A was 50 ng/well and was used for all subsequent assays.

The recombinant proteins were added as cell lysates (COS cells) or culture supernatants (NS0 cells, undiluted or diluted 1:2 or 1:6). The cell lysates were prepared by harvesting with a cell scraper into PBS. The scFv proteins were detected with goat anti-mouse Ig, followed by HRP-conjugated rabbit anti-goat Ig, as described above. The recombinant mAb 1C3 was detected using a 1:1000 dilution of rabbit anti-human IgG (Dako, Glostrup, Denmark), followed by HRP-conjugated goat anti-rabbit Ig (Dako, Glostrup, Denmark, 1:1000). For identification of stable cell lines secreting recombinant 1C3, culture supernatant was added undiluted.

To determine the relative concentration of the scFv and recombinant 1C3 antibodies, absorbance values for the recombinant protein (serially diluted in PBS/T in duplicate) were compared to a standard curve for a known concentration of purified, hybridoma-derived 1C3 Fab or intact antibody.

For all ELISAs where the recombinant reagents were present in cell culture supernatant, supernatant from untransfected wild type cells was used as a negative control.

2.5 Cell culture, transfections and stable cell-line selection

COS cells (COS-7L, Invitrogen (Gibco), Carlsbad, CA, USA) were grown in RPMI 1640 medium containing 10% foetal bovine serum (FBS), 50 U/mL penicillin, 50 µg/mL streptomycin and 2 mM glutamax-1 (Invitrogen (Gibco), Carlsbad, CA, USA). NS0 mouse myeloma cells (European Collection of Cell Cultures, Salisbury, UK) were grown in either RPMI 1640 medium containing 10% FBS or HyQ CDM4NS0 (Hyclone, Perbio Science, Logan, UT, USA) medium supplemented with 10 µg/ml insulin and 6 mM L-glutamine. The cells were incubated at 37 °C with 5% CO_2. COS cells were treated with trypsin/EDTA (Invitrogen (Gibco), Carlsbad, CA, USA) to detach from the flask surface, while NS0 cell lines were passaged by gentle aspiration.

Transient transfections of COS and NS0 cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. NS0 cells transfected with the pCDscFv constructs, which contain the *neo*^{r} gene, were selected in 0.5 mg/mL Geneticin (Invitrogen (Gibco), Carlsbad, CA, USA). The plasmids expressing the recombinant mAb 1C3 heavy and light chains were co-transfected into NS0 cells and selected with 10 µg/mL mycophenolic acid, 0.25 mg/mL xanthine, 13.5 µg/mL hypoxanthine, 5 mM Hepes in RPMI supplemented with 10% FBS. Once selected, the cells were maintained in non-selective medium. The cells were cloned by limiting dilution. The mAb-secreting clones were identified by glycophorin ELISA and converted to growth in CDM4NS0 in roller bottles, except that the yield analysis experiment with the recombinant mAb 1C3-secreting cells was performed in CDM4NS0 supplemented with 10% FBS.

2.6 N-glycosidase F (PNGase F) digestion

The recombinant proteins, as COS cell lysates, were digested with PNGase F (NEB, Ipswich, MA, USA) according to the manufacturer's instructions, as in Hobson-Peters *et al*. (2008).

2.6 Western blot

Antigens were mixed with 1 x NuPage LDS sample buffer (Invitrogen, Carlsbad, CA, USA). For reduced antigens, dithiothreitol (97 mM) was added and the samples heated at 70 °C for 10 min. The samples were gently sheared by repeated aspiration through a 29 gauge insulin needle to fragment COS cell genomic DNA. The antigens were separated by SDS-PAGE (4-12% gradient polyacrylamide gels, Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose (0.2 µm pore size, Invitrogen, Carlsbad, CA, USA). Membranes were blocked with 5% skim milk powder in PBS for 1 h at room temperature. Primary antibody was diluted

in 1% skim milk powder in PBS (mAb 17D7 in ascitic fluid (1:500); goat anti-human kappa light chain, free and bound (1:250, Sigma); goat anti-human IgG (gamma chain-specific, Dako, Glostrup, Denmark, 1:250) and incubated for 1 h at room temperature with gentle rocking. The blot was washed three times with PBS/T and the relevant HRP-conjugated secondary antibody added for 1 h (HRP-conjugated goat anti-mouse IgG + IgM (H+L) (minimal cross reaction to human serum proteins, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, 1:2000); HRP-conjugated rabbit anti-goat Ig (Dako,Glostrup, Denmark, 1:1000)). After a final three washes, the blots were developed with substrate solution (1.5 mM diaminobenzidine, 0.06% (v/v) H_2O_2 in PBS, pH 7.2).

2.7 Ammonium Sulphate precipitation of recombinant scFv fusion proteins

Ammonium sulphate precipitation of the recombinant scFv proteins from cell culture supernatant was performed as described by Wang *et al*. (2001). Briefly, the cell culture supernatant of stable NS0 cell lines growing in CDM4NS0 medium was harvested and the cells removed by centrifugation, followed by filtration through a 0.45 µm filter. Solid ammonium sulphate was added slowly to a final concentration of 60% (w/v) while stirring at 4 °C. Stirring was continued for a further 30 min, followed by centrifugation of the mixture at 6,500 g for 30 min at 4 °C. The pellet was dissolved in 1 mM EDTA in PBS and the ammonium sulphate removed via dialysis against 1 L of 1 mM EDTA/PBS (changed once), overnight at 4 °C using a 3.5 kDa dialysis cassette (Slide-a-Lyzer, Thermo Scientific (Pierce), Rockford, IL, USA) or dialysis cup (Thermo Scientific (Pierce), Rockford, IL, USA). The dialysed sample was concentrated further using a 10,000 molecular weight cut-off centrifugal concentrator (Vivaspin, Sartorius, Aubagne Cedex, France).

2.8 Yield Analysis

NS0 stable cell lines scFv-C_L clone 2H4 and scFv-C_L-WN19 clone AB4/23-25 (AB4) were cultured in CDM4NS0 medium in roller bottles. On day 0, cells were seeded at 0.3 x 10^6 cells/mL (100 mL total). Daily from day 3 onwards, 3 mL was removed from each bottle, the cells were assessed for viability using trypan blue exclusion and the supernatant was recovered (1,000 r.p.m., 5 min, Clements Orbital 310, Clements Medical Equipment, Pty. Ltd., Somersby, Australia). Harvesting ceased once the viability reached ≤ 20%. The concentration of glycophorin-reactive scFv in each sample was estimated by glycophorin

ELISA and a standard curve, which was generated using a known concentration of 1C3 Fab diluted in CDM4NS0 medium.

A similar procedure was used for recombinant mAb 1C3 stable cell line 3H4/221 (3H4), except that the cells were cultured in CDM4NS0 medium supplemented with 10% FBS and the standard curve was generated using hybridoma-derived, intact 1C3 in the glycophorin ELISA.

2.9 Detection of light and heavy chain gene integration into NS0 genome by PCR

Genomic DNA was extracted from NS0 cells using the GenElute kit (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions. PCR was performed as in 2.2 using 5 μL of the purified NS0 DNA, or approximately 10 ng plasmid DNA and the primer pairs VL3/VL4 (light chain-specific); VH3/VH4 (heavy chain-specific) or tg2/WN11 (amplifies heavy chain sequence plus peptide WN19 sequence, Table 1).

2.10 Red cell agglutination assays

Red cell agglutination assays were performed using protocols based on those described by Lilley *et al*. (1994). For direct assays, 10 μL human blood (collected into citrate) was mixed on a plastic slide with dialysed, ammonium sulphate-concentrated scFv-C_L and scFv-C_L-WN19 reagents (40 μL). The amount of scFv-C_L and scFv-C_L-WN19 added per assay was estimated as 40 and 20 ng respectively, using the glycophorin ELISA. MAb 17D7 as ascitic fluid (1 μL as a 1:4 dilution in PBS), mAb 1B1 (0.5 μg) or 25 μL WNV-positive horse serum was added and the reagents were mixed for 2-3 seconds with a plastic rod, and then rocked manually for 5 min.

For indirect assays, 25 μL human blood (collected into citrate) was incubated with 1 μg scFv-C_L-WN19 as 5 mL cell culture supernatant (CDM4NS0) and mixed gently for 30 min. The erythrocytes were pelleted at 1,000 r.p.m. (Jouan CR312, Jouan Ltd., Ilkeston, UK) and the supernatant was removed. MAbs 17D7 or 1B1 were added to 10 μL of the pre-absorbed erythrocytes as for the direct assays and made up to 50 μL final assay volume using the culture supernatant. In a modified format, the erythrocytes were pre-incubated with scFv-C_L or scFv-C_L-WN19 in cell culture medium containing 10% FBS. In this instance, the coated

erythrocytes were mixed with 25 µL WNV-positive horse serum and the total reaction volume made up to 50 µL with PBS.

For agglutination assay using the recombinant mAb 1C3, 5 mL of cell culture supernatant, recovered from transfected NS0 cells and with an approximate concentration of 70 ng/mL, was incubated with 25 µL human blood for 30 min with gentle mixing. The treated erythrocytes were recovered by low-speed centrifugation (as described above) and 10 µL of the cells was mixed with 8 µL rabbit anti-human Ig (Dako, Glostrup, Denmark), or control rabbit antiserum (Dako, Glostrup, Denmark). The total reaction volume was made up to 50 µL using r1C3 cell culture supernatant.

3. Results

3.1. Glycophorin ELISA

To detect and quantify the recombinant autologous agglutination reagents, a glycophorin-binding ELISA, described originally by Lilley *et al*. (1994), was optimized using checkerboard titration of glycophorin A and the Fab product of the hybridoma-derived mAb 1C3. The results (not shown) indicated that maximal binding of the antibody occurred when wells were coated with 50 ng glycophorin. These conditions were used in all subsequent ELISAs. Standard curves were established with the hybridoma-derived 1C3 Fab or intact mAb to quantitate the scFv and full-length recombinant antibodies, respectively.

3.2. scFv-based agglutination reagents

The initial expression system examined was based on the scFv construct previously used for bacterial expression (Lilley *et al*., 1994; Coia *et al*., 1996). This construct contains the variable region genes from mAb 1C3 linked to a constant light chain gene (scFv-C_L). The generation of mammalian expression vectors derived from the scFv-C_L construct has been described previously (Hobson-Peters *et al*., 2008). Two constructs were used in the studies reported here: one expressing the scFv-C_L alone (pCDscFv) and another, pCDscFvWN19, which expresses a 19-residue West Nile virus (WNV) immunogenic peptide fused to the C-terminus of the scFv-C_L protein (scFv-C_L-WN19). Restriction enzyme recognition sites generating non-complementary overhangs were included either side of the insertion site for the WNV peptide coding sequence, to enable directional insertion of DNA encoding other diagnostic peptides. In addition, the bacterial signal peptide was replaced with an immunoglobulin leader sequence to facilitate secretion from transfected cells, and a Kozak consensus sequence was inserted to enhance protein expression.

Sequencing of the mammalian vectors revealed five nucleotide differences to the scFv-C_L sequence (Lilley *et al*., 1994, Gregory Coia, CSIRO, Personal communication). Three of the nucleotide differences were synonymous substitutions. One difference is predicted to lead to the substitution of an alanine with a threonine residue in the V_L region. Since threonine residues are present at this position in other immunoglobulin sequences listed on the NCBI database (for example gi10834748), this substitution is unlikely to cause a functional change. There is also a change from lysine to methionine in the C_L region. As the function of the C_L

region is as a spacer to extend the attached peptide away from the erythrocyte surface, it is anticipated that this change is of little functional significance.

It has been shown previously through Western blot analysis of lysates of transiently transfected COS cells that the vectors (pCDscFv and pCDscFvWN19) direct the expression of proteins of the predicted mass and expected immunoreactivity with anti-(mouse Ig) antibody, WN19 specific-mAb and sera from WNV-infected horses (Hobson-Peters *et al*., 2008). The sequence of additional experiments performed using these reagents is provided in figure 3A. To determine whether a functional antigen-binding molecule was expressed, lysates of transfected COS cells were analyzed in the glycophorin ELISA. The results (Fig. 4A) confirmed that an antibody fragment capable of binding to glycophorin was expressed from both the pCDscFv and pCDscFvWN19 vectors.

To obtain sufficient scFv reagents for use in agglutination assays, NS0 cells stably transfected with pCDscFv and pCDscFvWN19 were generated. NS0 cells are used for the commercial production of recombinant antibodies (Barnes *et al*., 2000; Chu and Robinson, 2001), as these cells are capable of secreting up to 2.7 mg/mL of recombinant protein (Zhou *et al*., 1997). After transfection, the cells were selected in medium containing Geneticin (G_{418}), and cells which survived selection were cloned by limiting dilution. Several clones secreting the recombinant proteins were identified by analyzing the culture supernatants in the glycophorin ELISA. The estimated concentration of the glycophorin-binding reagent in the supernatants varied between 50 and 200 ng/mL. Two clones, 2H4 and AB4, which secreted scFv-C_L and scFv-C_L-WN19, respectively, at 200 ng/ml were expanded for further analysis in agglutination reactions. Glycosylation of the NS0-secreted scFv-C_L-WN19 molecule was confirmed by digestion with the glycosidase PNGase F (data not shown).

The utility of the scFv-C_L-WN19 and scFv-C_L reagents recombinant molecules in the direct red cell agglutination assay was assessed following concentration by ammonium sulphate precipitation. The anti-WNV mAb 17D7 (approximately 0.5 µg) effectively cross-linked the erythrocytes upon addition of approximately 20 ng of the concentrated scFv-C_L-WN19 reagent (Fig. 5A), thus demonstrating the functionality of the eukaryotic-expressed scFv-C_L-WN19 protein in the agglutination assay. As expected, there was no agglutination in a similar assay upon the addition of the negative control mAb 1B1, nor with scFv-C_L and mAb 17D7. No agglutination was observed with WNV-infected horse serum, perhaps due to the lower

concentration of WN19-specific antibodies in the infected serum compared to ascitic fluid (data not shown).

To determine whether the strength of agglutination could be enhanced and to investigate the use of the recombinant scFvs secreted directly into the cell culture medium, without any further processing, indirect agglutination assays were performed. In these assays, human erythrocytes were incubated in harvested supernatant to allow adsorption of the reagents to the erythrocyte surface. The coated erythrocytes were washed and used in agglutination assays. Erythrocytes pre-coated with scFv-C_L-WN19 reagent were cross-linked with the anti-WN19 mAb 17D7, to a greater extent than in the direct assay (Fig. 5B). There was no agglutination in a similar assay upon addition of mAb 1B1, nor with another anti-WNV E protein mAb which binds a different epitope to mAb 17D7 and was also supplied in ascitic fluid (data not shown).

Pre-coated erythrocytes were agglutinated following addition of serum from a WNV-infected horse, but not with normal horse serum (Fig. 5C). No agglutination was observed with erythrocytes coated with the scFv-C_L reagent, although it should be noted that only an estimated 3.5 ng of the reagent was available per assay compared with 35 ng of scFv-C_L-WN19. Slight background agglutination of the human erythrocytes was observed after addition of WNV-positive horse serum, in the absence of the scFv-C_L or scFv-C_L-WN19 reagents (Fig. 5C). The WNV-positive horse serum caused slight agglutination of erythrocytes from four different people, indicating that this reactivity was not specific to individual patients (data not shown).

In all, the results demonstrate that a functional autoagglutination reagent was produced by transfected mammalian cells. Although the concentration was too low for use of the supernatant in a direct assay without concentration, no further purification of the reagent was necessary.

3.3. Recombinant 1C3 antibody fusion proteins

A second set of experiments (summarized in Fig. 3B) examined an alternative mammalian expression system that is used in our laboratory for the production of recombinant D-dimer-specific mAb 3B6 (Morris *et al*., 2004). Transfected NS0 cells yield about 20 µg of the recombinant protein per ml of culture supernatant under unoptimised culture conditions and

about 0.2 mg/mL under optimized conditions (M. Gerometta, AGEN Biomedical Ltd., Personal communication). The system uses two vectors to produce a human IgG_1 molecule – one expressing the antibody light chain ($pSVhygHuC_k$) and the second expressing the antibody heavy chain ($pSVgptHuIgG_1$). These vectors contain the hygromycin (hyg) or *E.coli* guanine phosphoribosyl transferase (gpt) resistance genes, respectively, for selection of stable cell lines. Each vector encodes the murine heavy chain immunoglobulin enhancer region, the murine heavy chain promoter, and a signal peptide sequence (including the signal sequence intron). The DNA encoding the mAb 3B6 V_L and V_H regions in the expression plasmids was replaced with the corresponding sequences from mAb 1C3 (Fig. 1). The resultant vectors were named $pSV1C3V_L$ and $pSV1C3V_H$. A third vector, $pSV1C3V_HWN19$, was created by inserting DNA encoding the WN19 peptide at the 3' end of the heavy chain gene (Fig. 2). As for the scFv-C_L-WN19 construct, restriction enzyme recognition sites generating non-complementary overhangs were included either side of the insertion site to enable directional insertion of sequences encoding other diagnostic peptides. Sequencing of the plasmids indicated that the DNA encoding the mAb 1C3 variable regions and the WN19 epitope had been correctly inserted.

The $pSV1C3V_L$ and $pSV1C3V_H$ vectors were transfected separately into COS cells to confirm the expression of the expected antibody molecules prior to the preparation of stable cell lines. Lysates of transfected cells were examined by Western blot using antibodies specific for the human kappa and gamma chains, respectively. The light chain molecule had an apparent molecular mass of about 25 kDa (reduced, Fig. 6A). The heavy chain had an apparent molecular mass of about 50 kDa (in reduced form) and migrated slightly more quickly than both its murine (hybridoma-derived) counterpart and the recombinant 3B6 heavy chain (Fig. 6B). A distinct doublet was observed for the $1C3_H$ molecule, which could not be attributed to differences in glycosylation (Fig. 6C) and was not apparent for the other heavy chain molecules. The 3B6 heavy chain expressed in both COS and NS0 cells (Fig. 6B; $3B6_H$ and r3B6 respectively) migrated a little more slowly and in COS cells was expressed to a similar level as the recombinant mAb 1C3 heavy chain molecule. Upon expression of plasmid $pSV1C3V_HWN19$ (r1C3-WN19), it was noted that the increase in size of the heavy chain molecule was proportional to the size difference between scFv-C_L-WN19 and scFv-C_L (data not shown). Incubation of the r1C3-WN19 and r1C3 heavy chain molecules with PNGase F revealed that there was a greater difference in size between the treated and untreated r1C3-WN19 heavy chain molecule than that of r1C3 (Fig. 6C). Together, these data suggested that

a glycosylated peptide WN19 molecule was expressed on the end of the antibody Fc region. However, this molecule was not recognised by mAb 17D7 (Fig. 6D). The reason for this is not apparent as the presence of the correct DNA sequence for peptide WN19 in the r1C3-WN19 heavy chain plasmid was confirmed by sequencing.

To determine if a glycophorin-binding antibody could be expressed by pSV1C3V_L and pSV1C3V_H, COS cells were co-transfected with the vectors and cell lysates were examined in the glycophorin ELISA. The results (Fig. 4B) showed that glycophorin-binding antibodies were present in the lysate from the co-transfected cells. Surprisingly, reactivity was also observed in the lysate from cells transfected with pSV1C3V_H alone, albeit less than that observed for the lysates from cells transfected with both pSV1C3V_L and pSV1C3V_H.

Together, these data demonstrate that the variable region sequences of mAb 1C3 were subcloned correctly into the original mAb 3B6 vectors to mediate the expression of a full-length, chimeric (mouse-human) antibody capable of binding to glycophorin. Furthermore, it appeared that the WNV-specific peptide WN19 was fused to the C-terminus of the antibody heavy chain, although it was not recognized by mAb 17D7.

NS0 cells were co-transfected with pSV1C3V_L and pSV1C3V_H and selected for gpt expression with mycophenolic acid to obtain stable cell lines expressing the full-length antibody. Surviving cells were cloned by limiting dilution and several clones secreting glycophorin-reactive antibody were identified by ELISA. One clone (3H4) was expanded in serum-free CDM4NS0 medium in roller bottles. To demonstrate that the recombinant mAb 1C3 was functional in agglutination assays, erythrocytes were coated by incubation in culture supernatant from clone 3H4. Agglutination was clearly observed when anti-human Ig was added to the erythrocytes (Fig. 5D). No agglutination was observed in the absence of cross-linking anti-human Ig, as is the case for the original hybridoma-derived mAb 1C3 (data not shown).

In light of the inability of mAb 17D7 to recognise the r1C3-WN19 heavy chain molecule produced in COS cells, it was considered unlikely that a similar molecule secreted by NS0 cells could be used in an agglutination assay and cross-linked with mAb 17D7. However, it was hoped that a stably expression r1C3-WN19 molecule may provide valuable insight into the effect of glycosylated fusion peptides at the 1C3 antibody Fc terminus on glycophorin binding, both in ELISA and erythrocyte agglutination assays. NS0 cells were stably co-transfected with plasmids pSV1C3V_HWN19 and pSV1C3V_L and the transfected cells were

selected with mycophenolic acid. Surviving cells were cloned by limiting dilution and cloned cell supernatants were analyzed by the glycophorin ELISA. Despite several transfections, all of which yielded mycophenolic acid-resistant cloned cells, only one clone (3B7) was identified which secreted glycophorin-reactive antibody, albeit at barely detectable levels. To try to identify the reason for the lack of productive clones, PCR analysis was used to assess the presence of the heavy and light chain genes in the various stable clones. Three clones were examined: clone 3H4 (as described above – produces glycophorin-reactive recombinant mAb 1C3), clone 3B7 (r1C3-WN19) and clone 1A6. The last clone was produced following transfection with pSV1C3V_HWN19 and pSV1C3V_L, and was resistant to mycophenolic acid, but did not secrete detectable glycophorin-reactive antibody.

PCR amplification of genomic DNA from the clones with primers specific for the V_H or V_L region indicated stable integration of the heavy chain genes in all three clones, but the light chain gene was not apparent in clone 1A6. (Fig. 7A and B). The latter result explains the lack of secretion of a functional antibody from 1A6 despite its resistance to mycophenolic acid, as the gpt gene is part of pSV1C3V_HWN19. When primers specific for the C-terminal heavy chain domain (IgG_{1-3}) and the WN19 peptide sequence were used, PCR product was observed for clones 3B7 and 1A6 (Fig. 7C) as expected.

A further attempt to isolate a stable cell line secreting recombinant mAb 1C3 with the WN19 fusion protein was undertaken by re-transfecting clone 1A6 with pSV1C3V_L and selecting for the presence of the hygromycin-resistance gene present on this plasmid. Despite the generation of several hygromycin-resistant cells, none secreted detectable glycophorin-reactive antibody. PCR analysis of these clones demonstrated that the light chain gene was not present (data not shown).

3.4. Yield analysis

As a better understanding of the dynamics of expression of the recombinant fusion protein in culture is expected to be useful to optimise the yield of the protein, the concentration of glycophorin-reactive scFv protein and the viable cell density of three permanently transfected, cloned cell lines during *in vitro* culture was determined.. The analysis with the scFv-C_L-secreting clone 2H4 indicated that the maximum concentration of recombinant fusion protein and maximum cell viability count were detected after about six days of culture in serum-free

CDM4NS0 medium (Fig. 8A). Thereafter, both the viable cell count and the concentration of glycophorin-reactive scFv decreased. The results with the scFv-C_L-WN19-secreting clone AB4 (Fig. 8B) showed that both parameters peaked at days 3 to 4 after culture initiation, and declined thereafter.

The third clone analysed was 3H4, which secretes recombinant mAb 1C3. In an attempt to increase yield, the clone was grown in CDM4NS0 medium supplemented with 10% FBS. Although the yield of glycophorin-reactive antibody was greater than that from the scFv cell lines, with a peak concentration of about 700 ng/mL (Fig. 8C), the antibody production curve was similar to those of the scFv stable cell lines, in that the maximum viable cell count and antibody concentration were detected on day 8.

These data differ from those obtained with other cell lines, where the recovery of antibody increases linearly after the maximal cell count has been reached (Bebbington *et al*., 1992; Brown *et al*., 1992; Bibila *et al*., 1994; Robinson *et al*., 1994; Keen and Hale, 1996). To ensure that the antibody yields were not affected by an overgrowing population of non-secreting cells from which integrated DNA had been removed, Geneticin selection was re-administered at 200 µg/mL to the scFv-secreting cell lines. Very little cell death was observed in the stable cell lines at this concentration of Geneticin, while there was complete death of the wild type cells cultured under the same conditions (data not shown). There was a negligible increase (<10%) in secreted glycophorin-reactive scFv produced by the cells growing in the medium containing Geneticin.

Similarly, to ensure that the antibody yields from clone 3H4 which secretes recombinant mAb 1C3 were not affected by the presence of a mixed population of antibody-secreting and non-secreting cells, the cell line was passaged 34 times and then re-cloned by limiting dilution. Assessment of the supernatant from each clone in ELISA revealed that 97.3% (36/37) of the clones were expressing glycophorin-reactive antibody (data not shown). Therefore, it was concluded that the low yield was not due to instability of the integrated genes, which may have arisen due to premature removal of the mycophenolic acid selection.

4. Discussion

The autologous red cell agglutination assay is simple, rapid and has the unique advantage in that the sample itself contains the read-out system. The only external requirements are the agglutination reagent, a simple plastic well and a mixing rod to perform the agglutination reaction. These characteristics make the technology ideal for rapid response to outbreaks of emerging diseases due to the potential speed and ease with which new reagents could be manufactured for newly arising variants of current pathogens. For diseases that arise intermittently, the reagents could be stored for extended periods and deployed rapidly following outbreaks. The key to the widespread use of this technology is to develop a method for rapid, easy and economical production of the agglutination reagents with various fusion peptides. The aim of the current experiments was to develop and assess a mammalian expression system to fulfill these requirements. In particular, it was envisaged that the agglutination reagents, if secreted into culture supernatant, may be used without further purification to facilitate the rapid and economic deployment of the diagnostic assays. To that end, various vectors based on an anti-erythrocyte mAb were constructed to mediate secretion into culture medium of agglutination reagents. The vectors were developed to allow for the directional insertion of DNA encoding a variety of antigenic epitopes. The experiments show that culture supernatant may be used to mediate agglutination. However, they also suggest that greater yields are required for direct assays, and that further insight is required to ensure stable expression of some constructs.

Various expression systems have been used for the production of recombinant versions of molecules of biomedical interest, including bacterial, yeast, insect and mammalian cells. Mammalian systems offer three distinct advantages. First, glycosylation of peptide WN19 is required for recognition by sera from infected animals (Hobson-Peters *et al*., 2008). Although bacteria can glycosylate some antigenic moieties (McBride *et al*., 2007), it is not certain whether this applies to all mammalian and viral epitopes requiring glycosylation and other post-translational modifications. It is likely that mammalian cells will provide more authentic modifications for antigens expressed naturally in mammalian cells, including those of viral origin. This is important as many newly emerging and re-emerging diseases are caused by viruses (Cleaveland *et al*. 2001). Second, mammalian cells lines, in particular the mouse myeloma cell line NS0, have been used extensively in industry for the production of proteins

including recombinant antibodies (Barnes *et al*., 2000; Chu and Robinson, 2001) and have been used in the manufacture of several commercial products such as as Zenapax (Dacliximab) (http://www.emea.auropa.eu/humansdocs/PDFs/EPAR/Zenapax/017599en6.pdf) and Synagis (Palivizumab) http://www.emea.europa.eu/humandocs/PDFs/EPAR/Synagis/190499en6.pdf). AGEN Biomedical Ltd. has used this cell line for large-scale production of an anti-D-dimer antibody (Macfarlane *et al*., 2006). As these cells grow in suspension, it is possible to cultivate them to high densities. Indeed, these cells when stably transfected, are capable of producing up to 2.7 g/L of recombinant protein (Zhou *et al*., 1997). Other groups achieved as much as 1-2 mg/L (Ridder *et al*., 1995) or 400 mg/L (Howard *et al*., 2002) using large-scale transient transfection of COS cells. Third, the secretory apparatus of mammalian cells has been well-studied, and this understanding aids the design of expression vectors and the secretion of intact protein molecules into cell culture medium.

The current work examined two forms of recombinant antibody, the scFv and the full-length molecule. The scFv construct is a small antigen-reactive molecule. As a single chain is required, there is no need for disulphide bridges to link different proteins and this form of antibody is well suited to bacterial expression. The scFv previously used to produce autologous agglutination reagents in bacteria was adapted for expression in mammalian cells by inserting the relevant coding regions, together with mammalian leader and Kozak sequences, into a commercially available mammalian expression vector (Hobson-Peters *et al*., 2008). Glycophorin-reactive scFv molecules (with and without the peptide WN19 fusion protein) were secreted by stably transfected NS0 cells at levels sufficient for the cell culture medium to be used without further processing in an indirect red cell agglutination assay. In two independent transfection events, six scFv-C_L and eleven scFv-C_L-WN19 stable transfectants were identified. Disappointingly, most of the clones secreted between 50 and 200 ng/mL glycophorin-reactive scFv, which was not sufficient for use in direct agglutination assays. Direct agglutination was achieved after concentration of the proteins in the cell culture medium.

The concentration of the secreted fusion protein produced by the stable NS0 cell lines did not increase by delaying harvest. Transfectants secreting different amounts of recombinant protein were expected (Barnes *et al*., 2001, 2007), due to the chromosomal integration site of

the foreign gene (Wilson *et al*., 1990). The low expression of the stable cells was unlikely to be due to poor cellular growth, as during the harvesting trial, the number of viable cells was within the range of growth of similar NS0 stable cell lines (Bebbington *et al*., 1992; Brown *et al*., 1992; Bibila *et al*., 1994; Robinson *et al*., 1994; Keen and Hale, 1996; Barnes *et al*., 2001, 2006, 2007). Unexpectedly, the harvesting trial indicated that once the cell viability dropped below 50%, there was a decrease in the net glycophorin-reactive scFv protein in the cell culture medium. This contrasts with other published antibody production cycles of NS0 recombinants which show that the majority of recombinant antibody production occurs usually when the cell viability starts to decrease (Bebbington *et al*., 1992; Brown *et al*., 1992; Bibila *et al*., 1994; Robinson *et al*., 1994; Keen and Hale, 1996). A possible explanation for the decrease in scFv accumulation seen in the current experiments is proteolytic degradation. However, no specific degradation products were visible during Western blot analysis of the secreted NS0-produced scFv molecule (data not shown). In addition, analysis of cellular lysates did not indicate the presence of large amounts of protein retained within the cells (data not shown).

Production of a full-length recombinant antibody was undertaken to study of the effect of: a) extending the peptide away from the erythrocyte to make it more accessible to the crosslinking antibodies, b) using a bivalent antibody to increase avidity for the erythrocytes and c) having two peptide WN19 tails (due to the presence of one peptide per Fc of the intact antibody). In the commercial assays marketed by AGEN Biomedical, an Fab fragment was used to facilitate chemical coupling of the synthetic peptide on the hinge thiols, and to remove the Fc region which can cause false positive reactions due to the presence of anti-mouse antibodies or other interferents such as rheumatoid factor, present in human sera (M. Gerometta, Personal communication). The expression of the mAb 1C3 variable regions with human IgG constant regions will minimise these reactions when using human blood. Although the affinity of the full-length antibody with the scFv version was not compared, Coia *et al*. (1996) showed that the scFv expressed in bacteria showed lower affinity for glycophorin than the hybridoma-derived Fab, while Dolezal *et al*. (1995) noted that there was little difference in the affinity shown by Fab expressed in hybridoma cells or bacteria. These results suggest that the full-length recombinant mAb 1C3 will have superior erythrocyte binding capacity compared to the scFv molecule.

The plasmids chosen for expression of the full-length molecule have been used in our laboratory to generate large quantities of a recombinant anti-D-dimer antibody in NS0 cells (Macfarlane *et al*., 2006). This expression system uses two vectors which encode variable regions fused to human kappa light and IgG_1 constant regions respectively. Using a series of overlap PCRs, the mAb 1C3 variable region genes were engineered into the heavy and light chain expression vectors. A second heavy chain vector was also prepared which incorporated peptide WN19 after the carboxy terminus of the antibody heavy chain molecule. Culture medium from NS0 cloned cell lines stably transfected with the light and heavy chain plasmids (without WN19) mediated agglutination in an indirect assay without purification of the recombinant antibody. However, the concentration of antibody was shown to be about 700 ng/mL, which was too low to effect agglutination in a direct assay. This level is considerably below that observed in optimised cell lines, which suggests that greater levels of secreted protein can be achieved.

An interesting observation was that the harvesting profile of recombinant mAb 1C3 clone 3H4 was similar to that of the scFv stable clones, in that there was no increase in antibody accumulation post log-phase growth of the cells. Analysis of mRNA levels would assist in determining if the plateau in antibody accumulation is due to cessation of antibody synthesis, or if the antibody is being degraded by cellular proteases. It should be noted that the lower (and most intensely stained) band of the doublet observed for both the $1C3_H$ and $1C3_H$WN19 molecules produced as COS lysates could not be attributed to glycosylation (Fig. 6C) and is possibly the result of N-terminal cleavage within the antibody variable region.

Despite the inability of mAb 17D7 to bind the r1C3-WN19 heavy chain molecule produced transiently in COS cells, several attempts to create a NS0 stable cell line expressing this molecule were made. It was envisaged that production of this molecule would enable investigation into the impact of the peptide on the antibody molecule's affinity and expression. Despite the ability of transiently transfected COS cells to express the heavy chain–WN19 fusion peptide, and the presence of heavy and light chain genes in one r1C3-WN19 NS0 clone (clone 3B7), very little secreted, glycophorin-reactive antibody was detected from the stable cell line. It can be argued that the visibly lower quantity of light and heavy chain PCR products, resulting from the amplification of the r1C3-WN19 transfected

cell DNA, in comparison to the amount of product obtained when using the recombinant mAb 1C3 clone (3H4/221) DNA as a template, could be the result of fewer copies of the light and heavy chain genes. Previous studies have shown that an excess of light chain polypeptide is required for efficient mAb folding and assembly (Schlatter *et al*., 2005). Furthermore, the heavy chain : light chain gene ratio can greatly affect the abundance of secreted mAb (Schlatter *et al*., 2005). Southern blot (Bebbington *et al*., 1992), or quantitative PCR analysis of the genomes of the stable cell lines produced in this study is required to determine the copy number of each gene. The secretion of functional r1C3-WN19 protein may have also been hindered by the peptide WN19 tails at the antibody terminus, preventing the correct folding of the antibody. However, expression of much larger and multi-glycosylated proteins at the end of other $F(ab')_2$ and intact antibodies have been reported without deleterious effect on antibody affinity and production (Gillies *et al.*, 2002; Sharifi *et al*., 2002; Biela *et al*., 2003). In addition, the recombinant COS-produced $1C3_H$ and $1C3_H$WN19 (data not shown) molecules were capable of binding glycophorin in the absence of the light chain molecule, indicating that the requirement of the light chain for target recognition of 1C3 is negligible. Thus, it seems unlikely that the poor expression of glycophorin reactive r1C3-WN19 was caused by the presence of the peptide tails, although this is the only difference between the recombinant mAb 1C3 and r1C3-WN19 constructs.

To adapt the production of the agglutination reagents described herein to commercial scale, several issues must be addressed. First, the expression levels by the mammalian cells must be increased significantly to achieve inexpensive production of reagents for use in direct agglutination assays. Greater yields were expected from the NS0 stable cell lines, particularly since NS0 cells are used frequently in the commercial production of recombinant antibodies. However, the fact that both the scFv and full-length (recombinant 1C3) stable NS0 cell lines displayed similar secretion curves, which differ from those in other systems, suggests that there is site-specific proteolytic degradation occurring within the variable region of the 1C3 antibody. Second, these data highlight the importance of peptide choice in producing a successful agglutination reagent. While the scFv-C_L-WN19 reagents were rapidly cross-linked by mAb 17D7, strong agglutination with the strongest WNV-positive horse serum was elusive. This indicates that peptide WN19 is not sufficiently immunogenic during WNV infection to induce an antibody response detectable by rapid immunoassays. The final issue to be addressed is the problem associated with the stable integration of both the heavy and light

chain plasmids (for the production of the intact antibody) in a single cell. Enlisting a vector that encodes the light and heavy chain genes in a single plasmid may solve this problem. However, the fact that the 1C3 heavy chain molecule appears to bind glycophorin in the absence of the light chain molecule suggests that co-expression of the two molecules may not be necessary.

In summary, recombinant scFv-C_L-WN19 and recombinant mAb 1C3 molecules were functional in red cell agglutination assays when cross-linked with mAb 17D7 or WNV-positive horse anti-serum and anti-human immunoglobulin respectively. However, the mammalian expression systems did not yield recombinant protein at concentrations sufficient for use in direct agglutination assays without concentration or purification. Nevertheless, these studies have generated valuable plasmid constructs which will facilitate the efficient incorporation of alternative peptides and greater yields of these bi-functional agglutination reagents may be achieved by optimizing cell culture conditions or different vector systems to produce greater quantities of recombinant proteins.

Acknowledgements

The authors wish to thank Agen Biomedical Ltd. for supply of the mAb used in this study; Michael Gerometta, AGEN Biomedical Ltd for useful discussion and advice; Kym Hoger and Kirstie Breslin, AGEN Biomedical Ltd for invaluable advice on NS0 cell culture; Richard Bowen, Colorado State University, Fort Collins, CO, USA for generous supply of horse serum; Melissa Sanchez and Robert Doms, University of Pennsylvania, Philadelphia, PA, USA for supply of mAb 17D7 and Gwong-Jen J. Chang, Centers for Disease Control and Prevention, Fort Collins, Colorado, CO, USA for the pCBWN plasmid. This research was supported by AGEN Biomedical Ltd. and the Australian Biosecurity CRC for Emerging Infectious Disease.

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Fig. 1. Cloning strategy for the construction of plasmids for expression of the light and heavy chains of mAb 1C3 (Fig.1 A and B). The generation of fragments for the V_H construct only is shown. For the V_L construct, the strategy was similar except that primers VL2 – VL6 and pSVhygHuC_k were used instead of VH2 – VH6 and pSVhygHuIgG_1, respectively. The complete plasmids are depicted in Fig. 1C.

Fig. 2. Cloning strategy for the construction of a plasmid for expression of the heavy chain of mAb 1C3 plus the peptide WN19.

Fig. 3. Study design flowchart

Fig. 4. Reactivity of COS cell-expressed 1C3 scFv and full-length antibody molecules to glycophorin in ELISA. Lysates of COS cells transiently expressing the 1C3 scFv-C_L or scFv-C_L-WN19 molecules (A) or 1C3 heavy and light chains alone, or co-expression of the 1C3 or 3B6 heavy and light chains (B), were assessed for reactivity to glycophorin in ELISA. The absorbance (OD 405 nm) of two replicates was plotted and the standard deviation shown.

Fig. 5. Agglutination assays using recombinant scFv-C_L, scFv-C_L or r1C3 and human erythrocytes. The reagents were incubated with the WN19-specific mAb 17D7 or negative control, HIV-specific mAb 1B1 in direct (A) or indirect (B) agglutination assays, and with WNV positive or negative horse sera in indirect assays (C). Recombinant 1C3 was incubated with rabbit anti-human IgG or rabbit control serum in an indirect assay with human erythrocytes (D).

Fig. 6. Recombinant 1C3 heavy and light chain analysis. Lysates of COS cell-expressed 1C3 heavy chain ($1C3_H$), 3B6 heavy chain ($3B6_H$), 1C3 light chain ($1C3_L$), 3B6 light chain ($3B6_L$),

1C3-WN19 heavy chain ($1C3_H$-WN19) or scFv-C_L-WN19, along with hybridoma-derived mAbs 1C3 (^{Hyb}1C3) and 3B6 (^{Hyb}3B6) and recombinant 3B6 (r3B6) produced in NS0 cells, were reduced prior to SDS-PAGE and Western blotting. The blots were probed with anti-human kappa chain (A), or gamma chain (B, C) or mAb 17D7 (D). Cell lysates of $1C3_H$ and $1C3_H$-WN19 were treated with PNGase F to remove N-linked glycans prior to Western blot (Panel C, +). Lysates of untransfected cells (mock) were included as controls.

Fig. 7. PCR analysis of stable cell lines. PCR analysis was performed using purified NS0 DNA from the stable cells and light chain- and heavy chain-specific primers (A,B). Purified plasmids were used as controls (V_L and V_H – pSV1C3V_L and pSV1C3V_H respectively). 3H4: r1C3 clone 3H4/221; 3B7: glycophorin-reactive molecule secreting r1C3-WN19 clone 3B7; 1A6: r1C3-WN19 clone 1A6 (does not secrete glycophorin-reactive mAb); WT – wild type untransfected cells. (C) Presence of peptide WN19 gene was confirmed for two clones using one peptide sequence-specific and one heavy chain-specific primer.

Fig. 8. Recombinant protein accumulation (■) and viable cell density (o) plotted against time in culture. Stable NS0 cell lines expressing scFv-C_L (a) and scFv-C_L-WN19 (b) were cultured in chemically defined serum-free medium, while NS0 cell line secreting recombinant mAb 1C3 (c) was cultured in the same medium with 10% FBS.

Table 1. PCR primers used for construction of expression plasmids

Name	Sequence [a, b, c]	Restriction enzymes or stop codon [b]
Overlap PCR primers for pSV1C3V_L and pSV1C3V_H (Fig.1)		
VL1	CCATACACATACTTCTGTGTTCC	
VL2	**ACTGTGACATGACGATGTC**GGAGTGGACACCTG	
VL3	**GACATCGTCATGTCACAGT**CTCC	
VL4	**AAGTTTAAATTCTACTCACGTTTAAT**TTCCAGCTTGGTGC	
VL5	**ATTAAACGTGAGTAGAATTTAAACTT**TG	
VL6	AATGATTGTTGTGATGTTTGC	
VH2	**TCAAGAAGCCTCACCTCGG**AGTGGACACCTGTGG	
VH3	**CCGAGGTGAGGCTTCTTGA**GTC	
VH4	**CACCGGAGGCGACGGTGA**CC	
VH5	**TCACCGTCGCCTCCGGTG**AGTCCTTACAACCTCTC	
VH6	TTCAGCGTCCAGTGTCTG	
Primers for pSV1C3V_HWN19 (Fig. 2)		
TG17	AAGAAGAAGCCGGCTCGGCCC	*Ngo*MIV
TG18	AACGGCCGCTCGAGTTTACCCGGAGACAGGGAGAGG	*Eag*I, *Xho*I
WN11	ACTCGAGTCCGGAACTACTGTGGAGTCGCAC	*Xho*I, *Bsp*EI
WN12	AACGGCCGTTACCCTGCCTGAGTGGC	*Eag*I, stop
Primer used to confirm heavy and light expression in NS0 cells (Fig. 7)		
TG2	GAATGGCAAGGAGTACAAG	

[a] All primers are written 5' to 3'.

[b] Restriction enzyme (RE) sites and the stop codon are underlined once or twice as are the corresponding RE names and Stop.

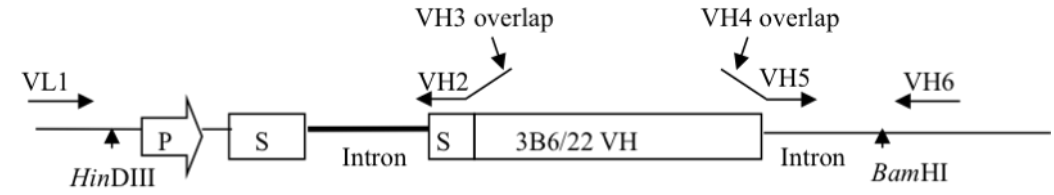
[c] Overlapping sequences are shown in bold type

Figures 1 and 2

Fig. 1.

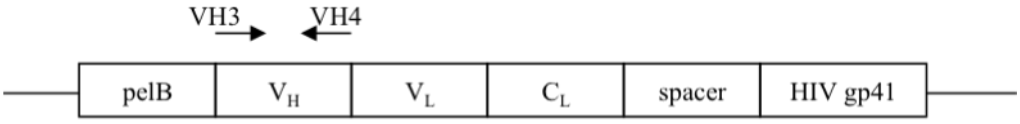
A. Generation of fragments for overlap PCR

Fragments 1 (Ig leader sequence) and 3 (3'region with exon splice site) amplified from pSVhygHuIgG$_1$



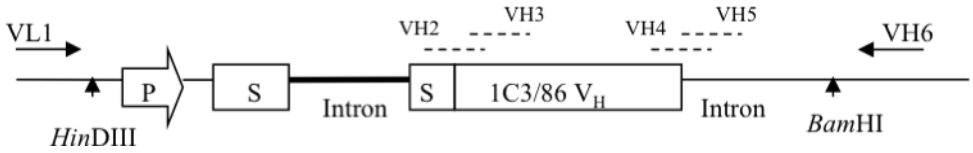
pSVhygHuIgG1

Fragment 2 (mAb 1C3 V_H sequence) amplified from pGC038C_L



pGC038C$_L$

B. Fuse fragments 1, 2 and 3 by overlap PCR



C. Replace the mAb 3B6 variable regions from pSVhygHuC$_k$ and pSVgptHuIgG$_1$ with the respective overlap PCR products to create pSV1C3V$_L$ and pSV1C3V$_H$.

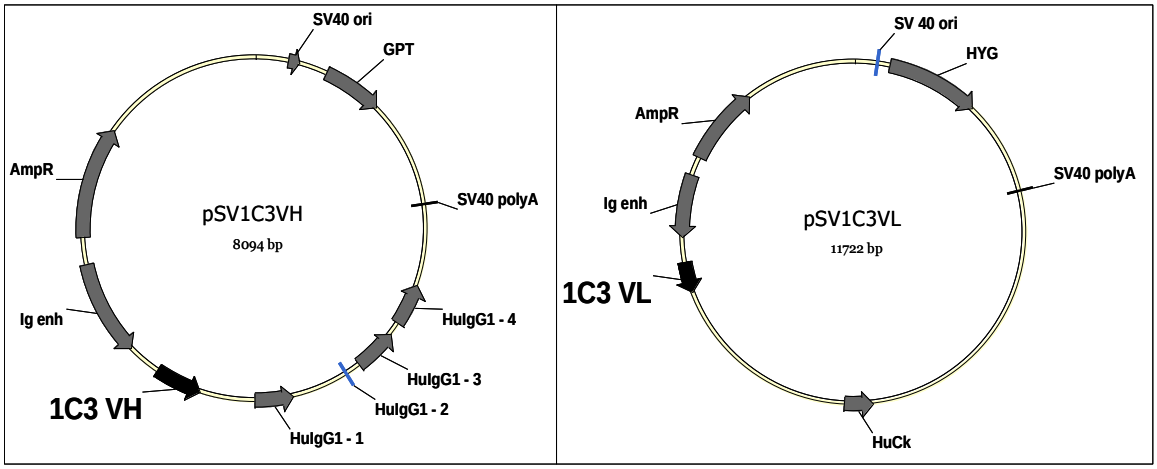
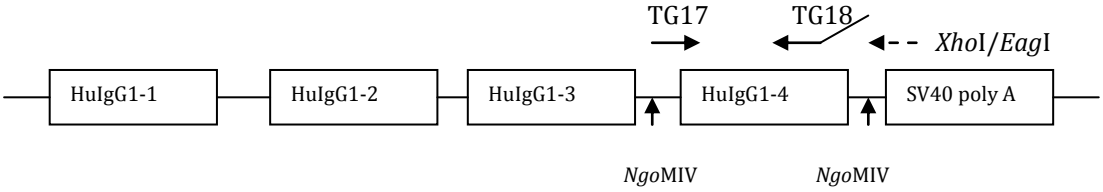
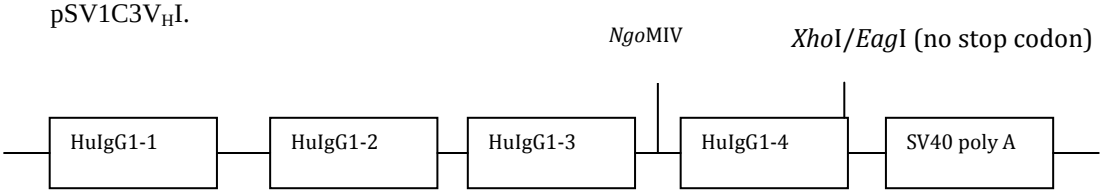


Fig. 2.

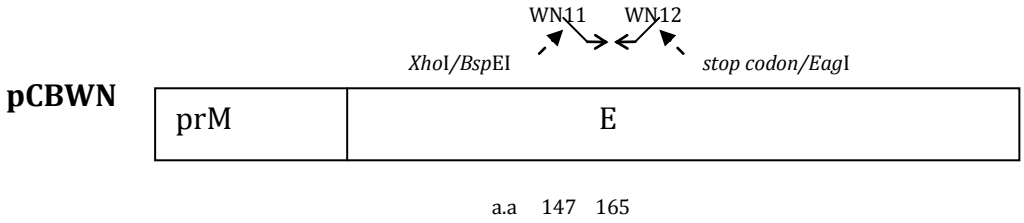
A. Amplify HuIgG$_{1-4}$ from pSVgptHuIgG$_1$, minus the 3' stop codon but including the 5' *Ngo*MIV site using TG17 and TG18 (incorporating *Xho*I and *Eag*I sites).



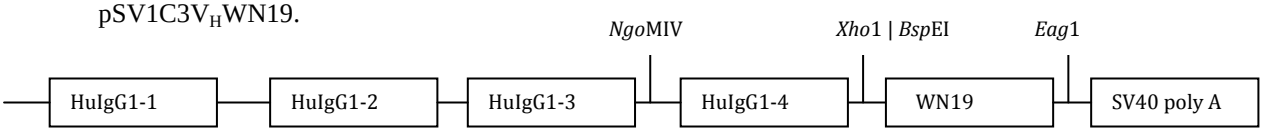
B. Insert *Ngo*MIV/*Eag*I digested PCR product into similarly digested pSV1C3V$_H$ to create pSV1C3V$_H$I.



C. Amplify peptide WN19 sequence from pCBWN using WN11 (incorporating *Xho*I and *Bsp*EI sites) and WN12 (incorporating stop codon and *Eag*I site).



D. Insert *Xho*I/*Eag*I-restricted product into similarly digested pSV1C3V$_H$I to create pSV1C3V$_H$WN19.



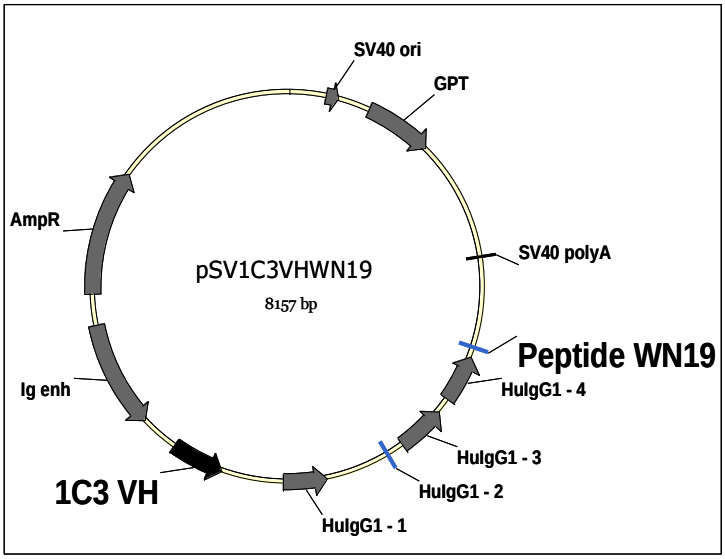


Figure 3

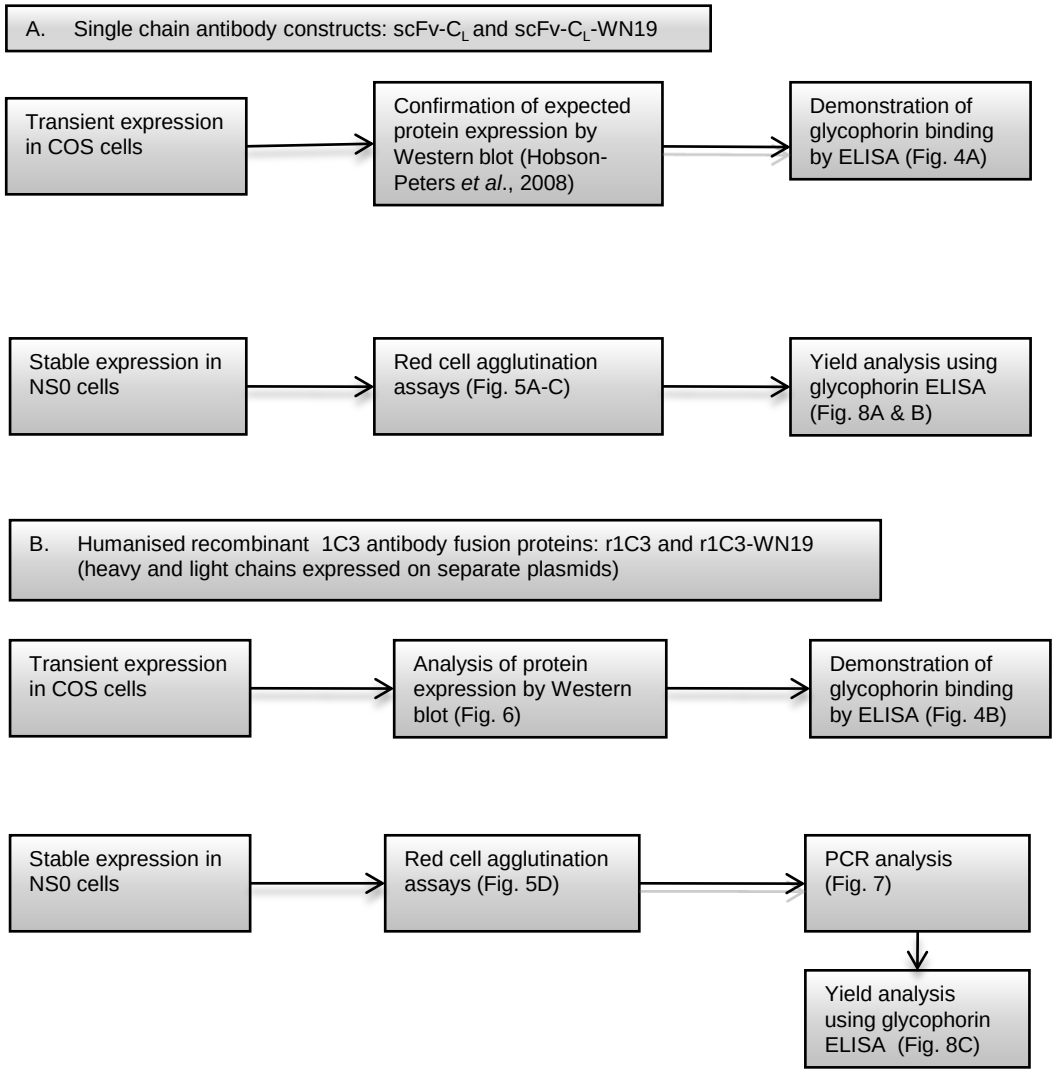


Figure 3

Figure 4

Fig. 4a

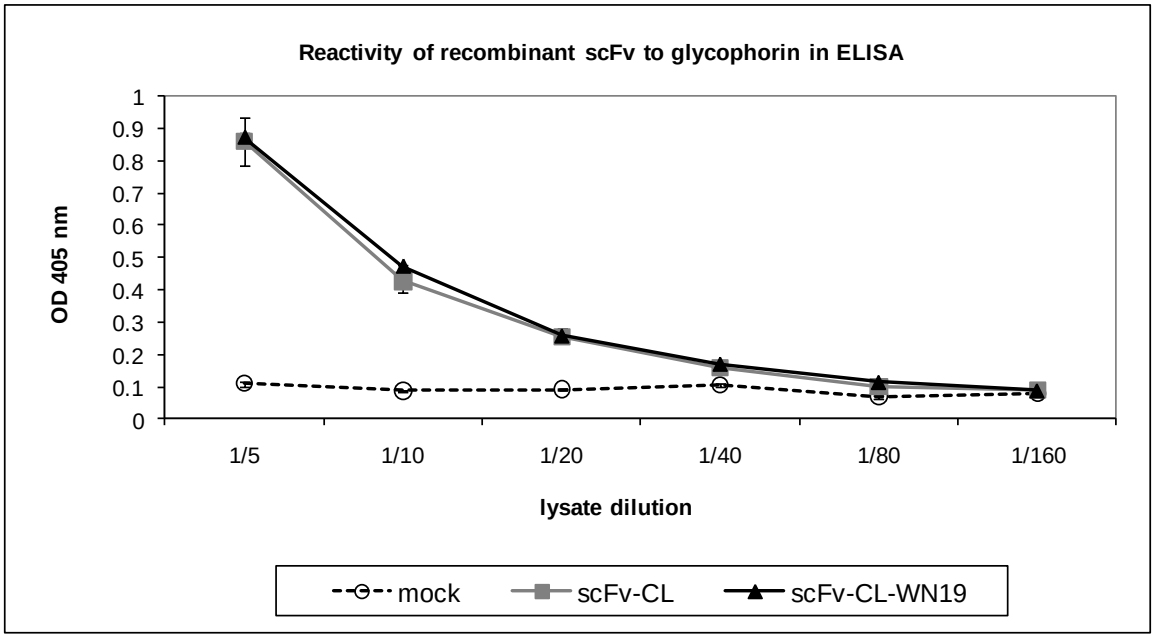


Fig. 4b

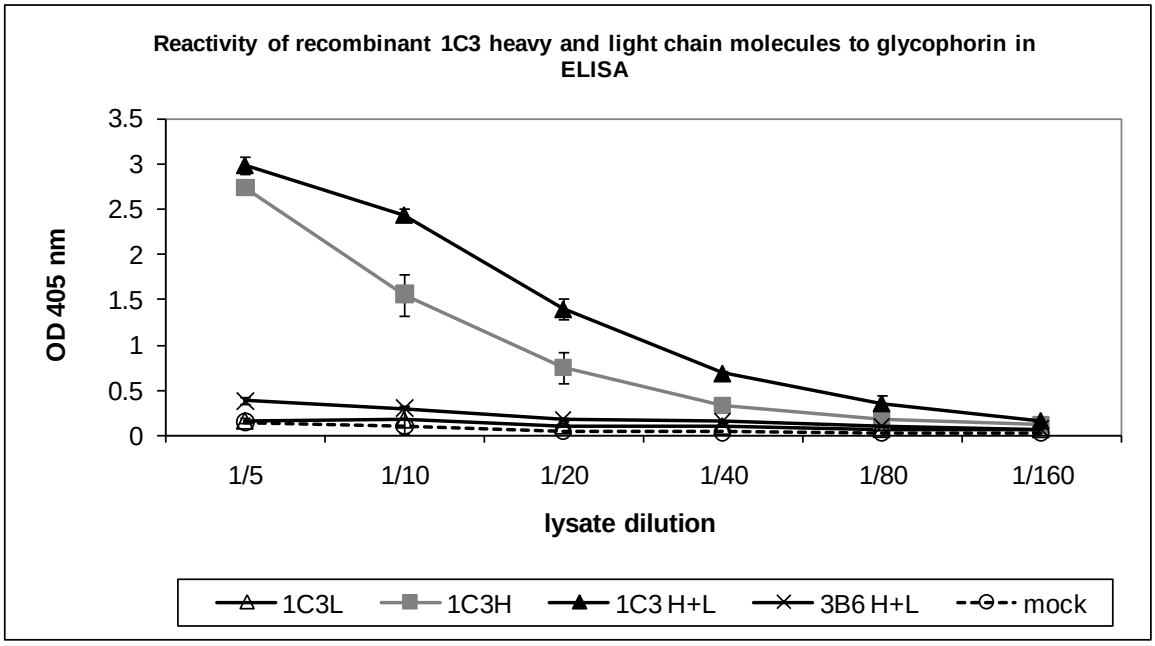


Figure 5 (colour)

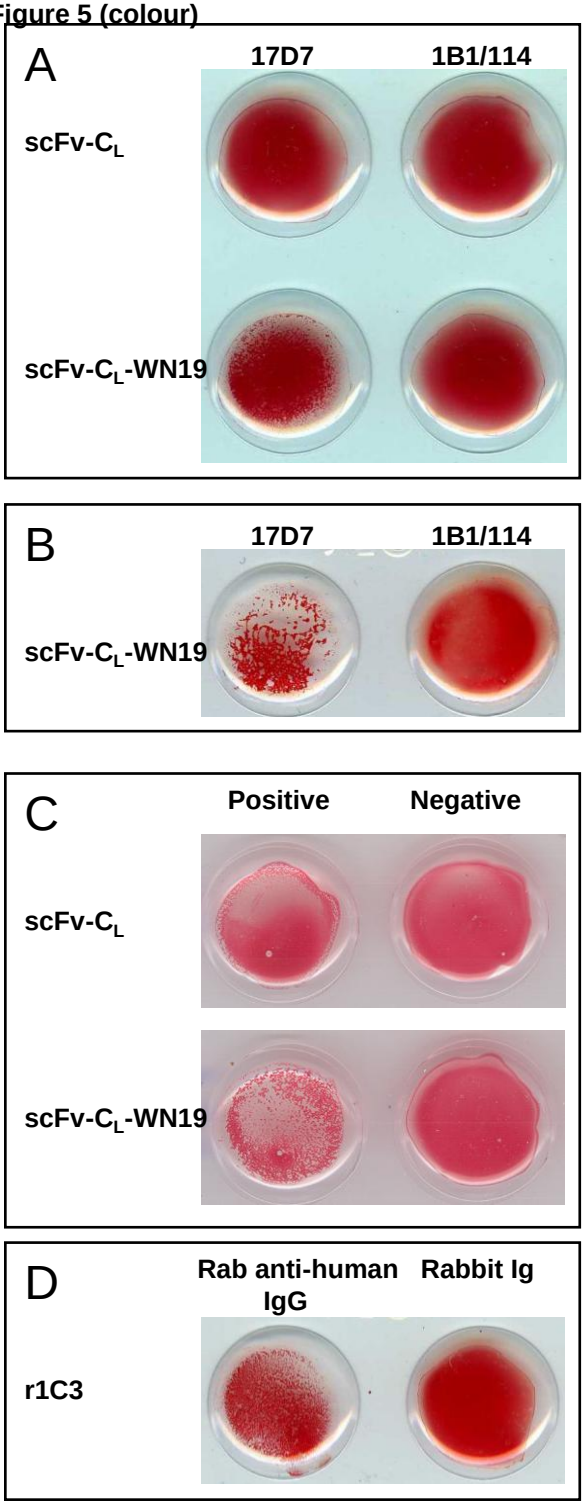


Fig. 5 Colour Figure

Figure 6

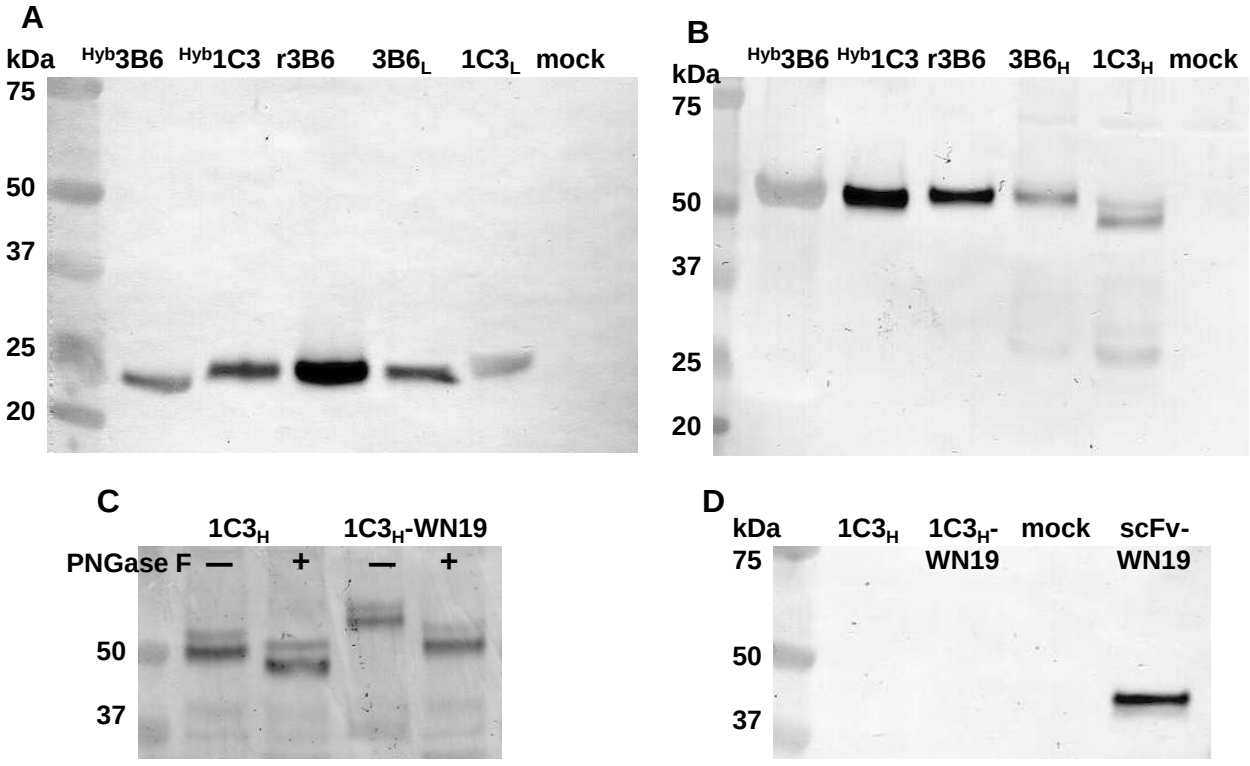


Fig. 6

Figure 7

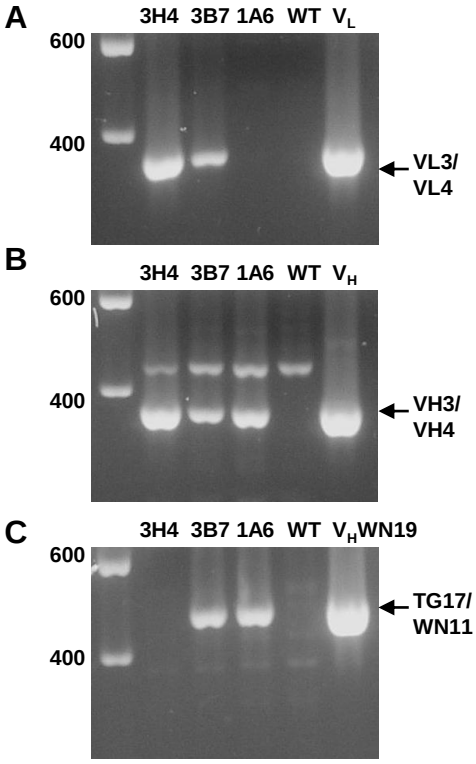
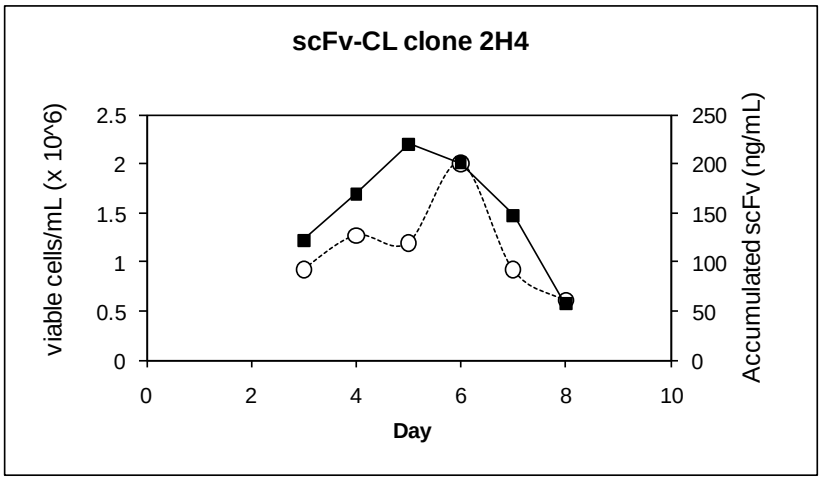


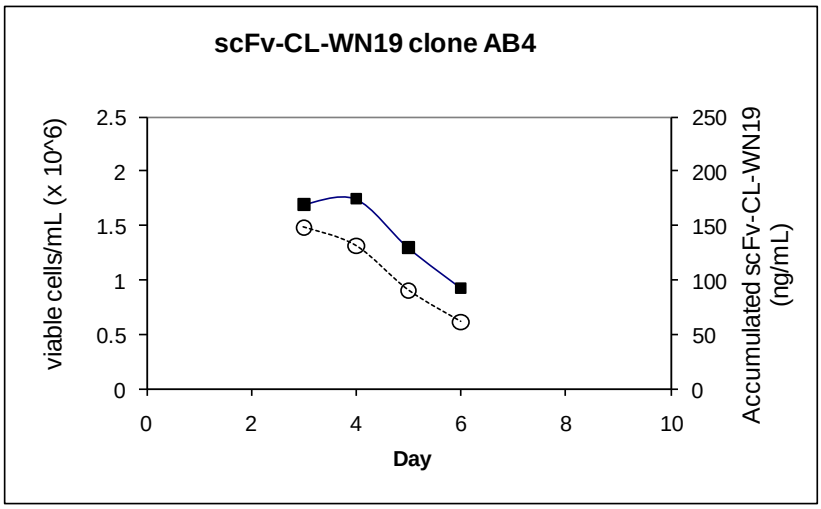
Fig. 7

Figure 8

a.



b.



c.

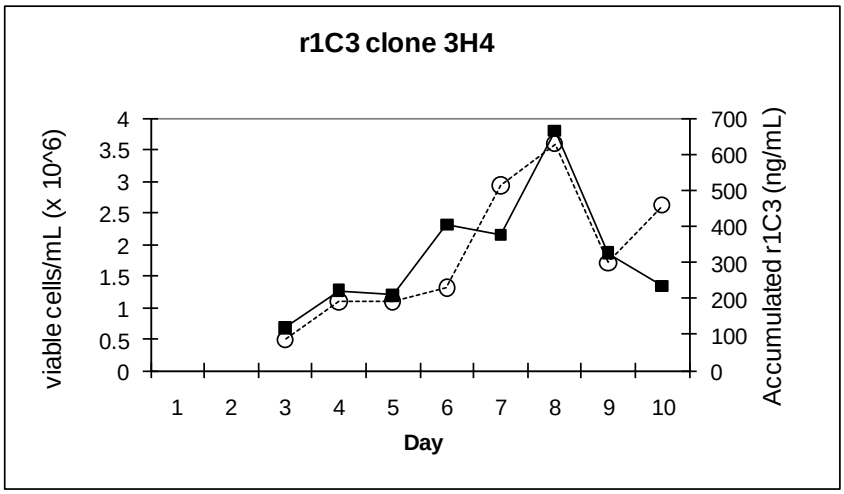


Fig. 8.

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