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Assays for Assessing the Compatibility of Therapeutic Proteins with Flexible Drug Containers

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1. Introduction

Biotherapeutics are among the fastest-growing segments of the pharmaceutical market. The packaging requirements for these therapeutics can be unique, primarily due to the multitude of factors that can influence the stability and overall potency of each particular therapeutic. Additionally, packaging has become a prominent concern in the healthcare industry due to the prevalence of medication errors, hospital acquired infections and potential for injury to the healthcare worker. The ability to provide these therapies in ready to use (RTU) containers would provide several advantages to both patients and clinicians: the RTU systems are closed containers, which minimize the risk of hospital-acquired infections; there are no reconstitution or admixture steps required, which minimize the risk of medication errors and healthcare worker exposure; and the RTU systems save time for the clinicians. However, the decision about drug formulation and packaging often needs to be made early in development when supplies of the drug are scarce.

Many of the biotherapeutics sold today are monoclonal antibodies. This circumstance lends itself well to the development of immunoassays for assessment of the activity of the particular therapeutic antibody. ELISA (enzyme-linked immunosorbent assay), also known as EIA (enzyme immunoassay), based assays are the most common approach for development of an immunoassay. This methodology has been extensively reviewed elsewhere (Wild 2001; Lequin 2005). These assays are usually performed in 96-well plates, but advances in automated liquid handling and spectrophotometric and fluorescent plate readers provide for formats as large as 1536 wells. The assays are typically structured in three basic formats, depending on the design of the assay. These include: 1) antibody capture assays, or solid-phase coated with antigen; 2) antigen capture assays, or solid phase coated with antibody; and 3) sandwich assays, which leverage an antibody pair, with one antibody coating the solid phase and the other binding the antigen in solution. The choice of assay format is primarily dictated by the analyte to be detected. In cases where the analyte is a small molecule that is either intrinsically fluorescent or has a distinct absorption spectrum, an antigen capture assay might be most applicable. Alternatively, if an antibody pair is available for an analyte, the sandwich ELISA is most commonly used. Since biotherapeutics

are often antibodies themselves, this poses more of a challenge and usually an antibody capture assay is most appropriate.

Traditional ELISA/EIA assays are only a subset of potential immunoassay applications. These plate-based assays can also be leveraged in a competitive format, to allow for comparison of a standard to a test article directly in a binding reaction (as opposed to interpolation from the response curve of a known standard in a traditional ELISA). Competitive binding reactions can also be utilized in non-plate assay systems as well. Cell-based assays, using the same direct binding or competitive binding principles, can be used to assess the binding of antibodies or ligands to cell surface receptors. Often these assays provide a more physiological approach to the assessment of the bioactivity of the therapeutic. However, this technique usually requires chemical modification of the antibody or ligand to include a fluorescent tag or radioisotope for detection, as the traditional ELISA colorimetric signal generation via horseradish peroxidase (HRP) is usually not feasible with these types of assays. Alternative signal generation methods have been developed, such as electrochemiluminescence detection (Meso Scale Discovery), which provides a much greater dynamic range and sensitivity compared to HRP based signals (Zhao *et al.* 2004). Fluorescent bead-based technologies, such as those developed by Luminex and PerkinElmer's AlphaScreen® are also alternatives to standard solid phase ELISAs (Kellar *et al.* 2006; Eglen *et al.* 2008). These are analogous to ELISA sandwich assays, but use suspended beads as the solid phase in the assay, rather than the plate surface. A caveat to bead-based assays and electrochemiluminescence, however, is the requirement for specialized equipment to perform the detection step. Nevertheless, there are multiple approaches and assay formats that can be used in developing an immunoassay for the characterization of a specific biotherapeutic.

This study focuses on the development and use of biological assays for assessing the compatibility of therapeutic proteins with flexible drug containers, including the development of in-house immunoassays for two therapeutic antibodies, cetuximab and rituximab. Cetuximab (marketed under the trade name Erbitux®) is a humanized chimeric mouse monoclonal antibody directed against the epidermal growth factor receptor (EGFR). It was approved by the United States Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer in 2004, and also has indications for the treatment of head and neck squamous cell carcinomas. Binding of cetuximab to the soluble extracellular portion of EGFR (sEGFR) has been previously demonstrated *in vitro* and the crystal for the cetuximab-sEGFR complex has been solved (Li *et al.* 2005). Additionally, it has recently been shown that cetuximab is ineffective in patients with K-ras mutations, providing an effective screening tool for oncology patients (Ramos *et al.* 2008). Rituximab (marketed under the trade name Rituxan®) is also a humanized chimeric mouse monoclonal antibody, but it is directed against the CD20 cell surface protein. CD20 is a transmembrane phosphoprotein expressed on the surface of the B-cells of the immune system (Perosa *et al.* 2005). Rituximab is used medicinally for the treatment of non-Hodgkin's lymphomas (i.e. various B-cell leukemias and lymphomas) (Sacchi *et al.* 2001).

Both of these antibodies are used prevalently in their respective oncology settings, and therefore were good candidates to evaluate as model proteins. Here we present data from the development of two immunoassays along with the subsequent use of the immunoassays to support a full protein-container compatibility study.

2. Materials and methods

2.1 Cetuximab immunoassay

A recombinantly expressed sEGFR domain is commercially available (Fitzgerald Industries International) and we developed an antibody capture ELISA using this domain. Microtiter plates (Costar® high-bind 8-well strips) were coated overnight at 4 °C with 100 µL/well of 1 µg/mL sEGFR reconstituted in 200 mM Na₂CO₃, pH 9.6. The coating solution was subsequently discarded and the plate was washed once with PBS-T (Phosphate Buffered Saline with 0.5% Tween® 20). The plates were then blocked with 200 µL/well of PBS-1% BSA (bovine serum albumin), sealed and stored at 4 °C until use. BSA only control plates were prepared by blocking uncoated plate strips with PBS-1% BSA as above. The required number of plate strips were removed from storage at 4 °C and allowed to reach room temperature prior to use. Dilutions of cetuximab (from the 2 mg/mL formulation concentration) were prepared in a range of 1:10-1:4096000 by serial dilution with PBS-0.5% BSA. The plate strips were washed three times with PBS-T. The dilutions were then added to the strips, in duplicate or triplicate, at 100 µL/well. The strips were then sealed and incubated at room temperature for one hour. During the incubation, a 1:5000 dilution of goat anti-human IgG-HRP secondary antibody (Sigma) was prepared in PBS-0.5% BSA by serial dilution. The strips were then washed three times with PBS-T and 100 µL/well of 1:5000 secondary antibody was added. The strips were sealed and incubated for 1 hr at room temperature. The o-Phenylenediamine (OPD) substrate (Sigma) was then prepared by adding one OPD tablet and one buffer tablet to 20 mL of water. The strips were washed three times with PBS-T and 100 or 200 µL/well of OPD substrate was added. The strips were incubated 10 min at room temperature and the reaction was quenched with 50 µL/well of 1M H₂SO₄. The plate was then read on a plate reader at 490 nm. The cetuximab standard curves were fit using a four parameter nonlinear regression model.

Competition reactions using unbound sEGFR were also performed. A standard curve was prepared using serial dilutions of cetuximab ranging from 1:5000-1:320000 in PBS-0.5% BSA. A vial of lyophilized sEGFR (25 µg) was reconstituted at 100 ng/mL with water. Additional sEGFR stocks were prepared by serial dilution with PBS over a range of 20-0.0064 ng/µL. Competition reactions were prepared by combining 10 µL of sEGFR with 90 µL of cetuximab (diluted 1:50000 with PBS-0.5% BSA) for each concentration of sEGFR tested. The reactions were incubated for 10 min at room temperature. The entire volume of each reaction and the cetuximab standards were then used in the cetuximab ELISA procedure described above.

2.2 Rituximab immunoassay

Rituximab was labeled with fluorescein isothiocyanate (FITC; Sigma). Rituximab (0.7 mL @ ~2.8 mg/mL) was labeled with 20 µL of 10 mg/mL FITC at room temperature for 2.25 hrs. The free FITC was removed via gel filtration with an EconoPac DG10 (BioRad) using Tris Buffered Saline (TBS) as the mobile phase. The pooled antibody had a concentration of 1.22 mg/mL with an F/P ratio of 10.4. Rituximab was diluted serially with PBS-1% FBS (fetal bovine serum) to generate various dilutions. A 1:5000 stock of fluorescein labeled rituximab (FITC-rituximab) was prepared by serial dilution with PBS-1% FBS (fetal bovine serum) for

the competition assay. Whole blood was drawn from the same donor in heparin-coated vacutainers (BD) prior to each experiment. The competition experiments for each dilution of unlabeled rituximab were then prepared by combining 100 μ L of whole blood with 10 μ L of diluted unlabeled rituximab and 20 μ L of diluted FITC-Rituximab. The reactions were vortexed and incubated at room temperature for 30 min in the dark. All reactions were vortexed again after the incubation and 2 mL of 1x lysis solution (BD) was added to each tube. The reactions were then incubated for 15 min at room temperature in the dark and spun down for 5 min at 3550 RPM. The supernatants were decanted and the cell pellets were washed with 2 mL of PBS-1% FBS. After spinning down the cells again as above, the supernatants were decanted and the cells were resuspended in 500 μ L of PBS-1% FBS prior to analysis by flow cytometry on a BD FACScan cytometer. A forward-scatter and side-scatter gate was established to isolate the lymphocyte population, and the mean fluorescent intensity value for this gate was calculated for each competition reaction. A standard curve was generated by serial dilution of a control rituximab sample in the competition reaction described above. The resulting standard curve was then used to interpolate the effective concentration value of the rituximab test samples.

2.3 Assessment of flexible container compatibility

Flexible film pouches were constructed using plastic film material and filled with 2 mL of antibody solution (cetuximab was formulated at 2 mg/mL and rituximab was formulated at 10 mg/mL). Glass vials were also filled in the same manner to serve as controls. The containers were sealed in a laminar flow hood, using a bench-top impulse sealer for the pouches. After filling, the units were stored at the temperatures listed in Tables 2 and 3. Samples were removed from storage at the time points indicated and the contents of the pouches were analyzed. This analysis included standard physical and chemical testing, and running a bioassay to determine the activity of the protein (as described above for cetuximab and rituximab).

Container	Testing Schedule				
	Temp. (°C)	2 week	4 week	8 week	16 week
Film 1	5			X	2X
	25		X	X	X
	40	X	X	X	X
Film 2	5			X	2X
	25		X	X	X
	40	X	X	X	X
Glass Control	5			X	2X
	25		X	X	X
	40	X	X	X	X

Table 1. Testing Matrix for Cetuximab Samples

Container	Temp. (°C)	Testing Schedule					
		0 days	3 days	1 week	2 weeks	4 weeks	8 weeks
Film 1	5	X			X	X	X
	25			X	X	X	X
	40		X	X	X	X	
Film 2	5	X			X	X	X
	25			X	X	X	X
	40		X	X	X	X	
Film 3	5	X			X	X	X
	25			X	X	X	X
	40		X	X	X	X	
Film 4	5	X			X	X	X
	25			X	X	X	X
	40		X	X	X	X	
Glass Control	5	X				X	X

Table 2. Testing Matrix for Rituximab Samples

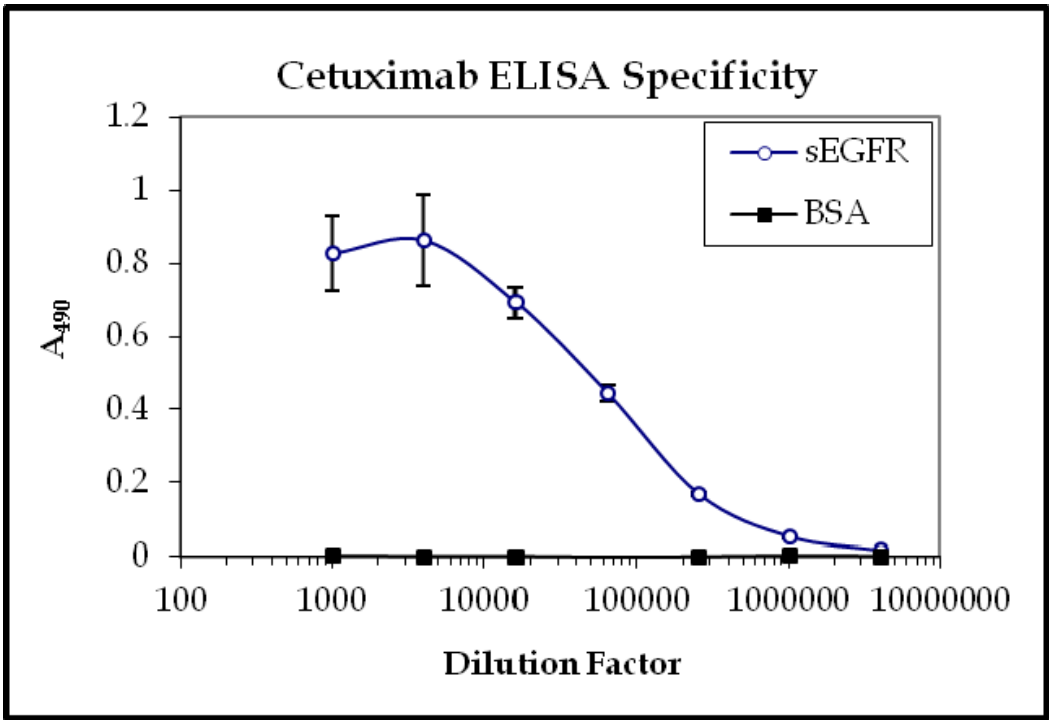


Fig. 1. Specificity of the cetuximab for sEGFR versus BSA

3. Results and discussion

3.1 Cetuximab immunoassay development

An immunoassay was developed for cetuximab, using commercially available sEGFR as the bound antigen for antibody capture. As shown in Figure 1, cetuximab has a specific response to sEGFR-coated strips with minimal background binding to BSA-only coated strips. A typical sigmoidal response was observed over a dilution range of 1:1000-1:4096000 of cetuximab.

The precision of the cetuximab ELISA was then examined over three independent experiments. Quadruplicate 1:50000 cetuximab dilutions (serially diluted with PBS-0.5% BSA) were prepared and analyzed in each experiment. The dilutions of the cetuximab standards were also varied across these experiments to determine the optimal range of concentrations for maximum linear response. All standards were run in triplicate and all test samples (1:50000 replicate dilutions) were run in duplicate. The optimal range for the cetuximab standard curve was ~1:5000-1:2000000 (typical standard curve is shown in Figure 2). The standards also had well-to-well CVs < 15% in all three experiments. The cetuximab standard curves were fit as described in the procedure and the concentrations were calculated for the 1:50000 diluted samples. The intraexperimental replicate variance (%CV) for the quadruplicate 1:50000 dilutions ranged from 8.6-12.8%. Additionally, the interexperimental variance (%CV) for the average calculated concentration for the 1:50000 diluted cetuximab samples from the three experiments was 14.2%. The average concentration across the three experiments, 39.6 ng/mL, was very near the expected value of 40 ng/mL for a 1:50000 dilution of the neat cetuximab formulated at 2 mg/mL.

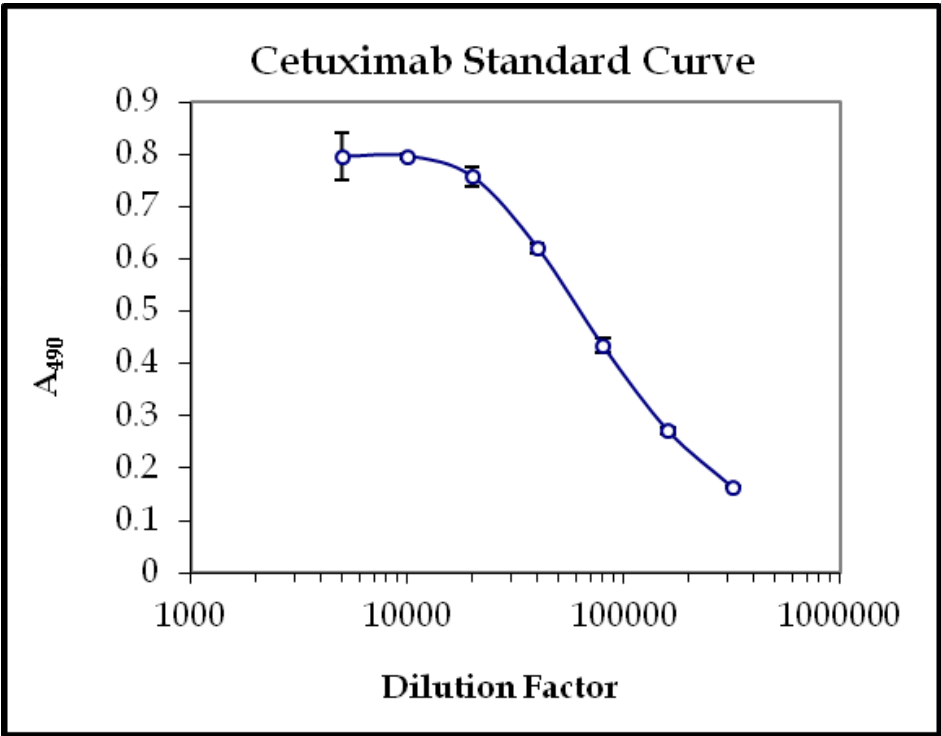


Fig. 2. Typical cetuximab standard curve for the sEGFR based ELISA

A competition experiment was also performed using free sEGFR in the ELISA assay as described in the Materials and Methods section. As shown in Figure 3, the percentage of cetuximab bound dropped to less than 5% with 10 ng/ μ L of free sEGFR and the observed IC_{50} was between 1-2 ng/ μ L or 12.5-25 nM. This result is comparable to the published K_d of 2.3 ± 0.5 nM for cetuximab binding to sEGFR via Biacore (Li *et al.* 2005).

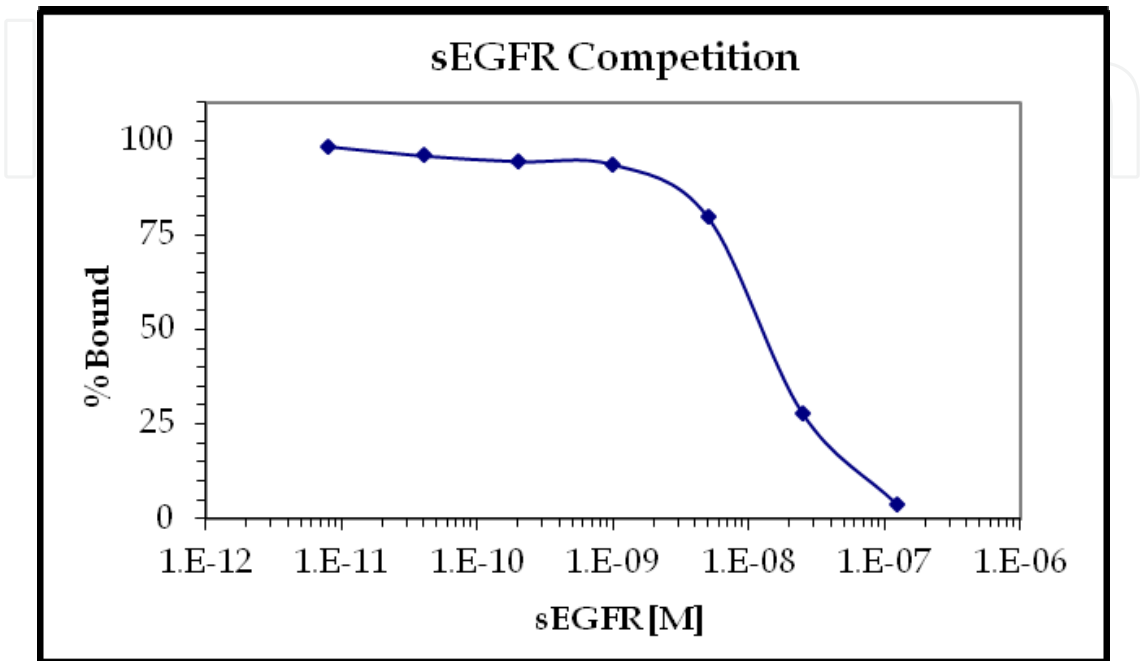


Fig. 3. Competition experiment using sEGFR titrated into the sEGFR ELISA

Cetuximab had a consistent response towards sEGFR in this ELISA based assay with minimal background binding to BSA. Across three independent experiments, the intraexperimental and interexperimental CVs were all < 15%, which is typical for most ELISA based assays. Additionally, free sEGFR was able to completely inhibit binding to the sEGFR coated plates and the observed K_d for sEGFR was similar to published results. Overall, the assay appeared to be adequate to serve as a bioassay for cetuximab.

3.2 Cetuximab container compatibility

Test articles were prepared consisting of pouches made of plastic films filled with cetuximab protein formulation (2 mL fill at 2 mg/mL). Additionally, glass vials were filled with cetuximab (2 mL fill) to serve as controls. The sampling time points and incubation conditions are summarized in Table 1.

The binding activity of cetuximab was monitored over the course of the study using the ELISA assay described here. The results are shown in Figures 4A-C, where the error bars represent plus or minus one standard deviation from the mean of triplicate assays of a single sample. The glass controls were used to normalize the ELISA data and these results were all well within the range of the assay variance (<15% CV). The physical and chemical test data (S. E. Lee, *et al.*, in preparation) showed that cetuximab solution held in plastic containers behaved similarly to cetuximab solution held in glass vials. These data suggest that cetuximab solution is compatible with both Film 1 as well as Film 2.

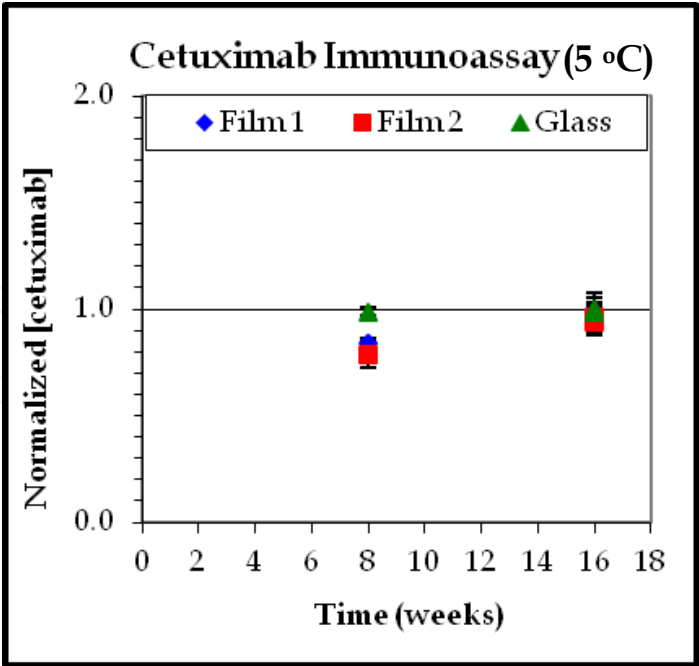


Fig. 4A. Immunoassay results for the various cetuximab test articles at 5 °C

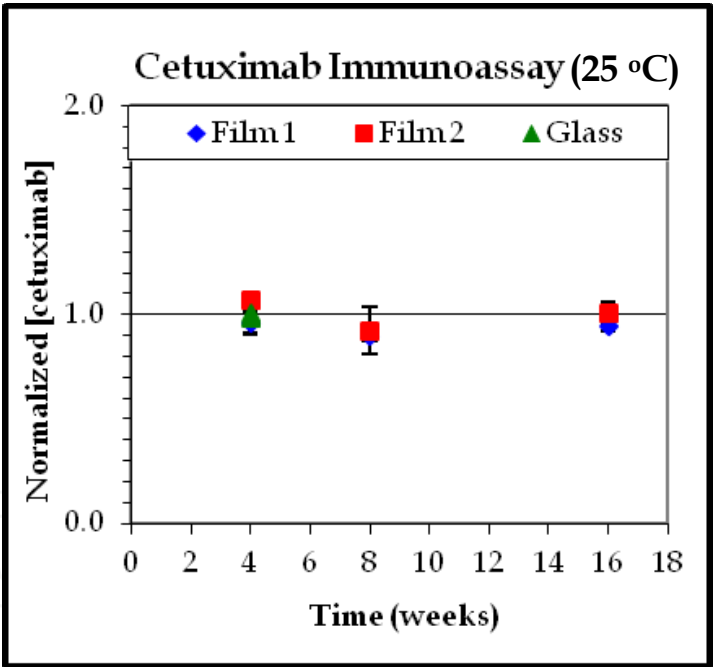


Fig. 4B. Immunoassay results for the various cetuximab test articles at 25 °C

3.3 Rituximab immunoassay development

Since CD20 extracellular domain was not commercially available for the development of an ELISA-based assay, a cell-based immunoassay was developed to evaluate the binding of rituximab to the CD20 cell surface receptor on B-cells. This assay format has the advantage of observing the direct binding of rituximab to the CD20 receptors on B-cells in the more physiological context of whole blood (as compared to ELISA-based approaches). Whole blood

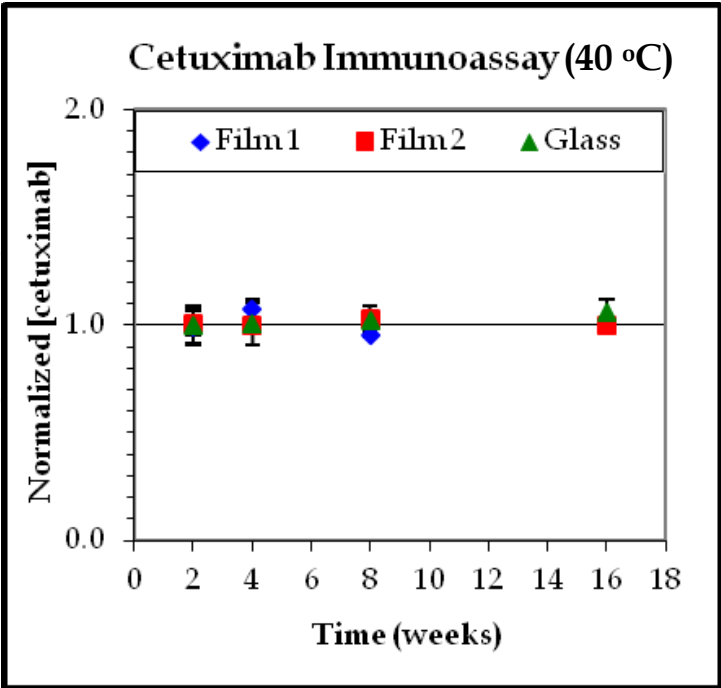


Fig. 4C. Immunoassay results for the various cetuximab test articles at 40 °C

was drawn from the same single donor at each time point throughout the study to serve as the source of B-cells. A competitive assay format was utilized for the immunoassay by titrating an unlabeled rituximab antibody standard against a constant concentration of FITC-labeled rituximab. A typical competitive response curve is shown in Figure 5, using a 1:5000 dilution of the FITC-rituximab. At each testing time point (as described in Table 2) a competitive standard curve was established based on the observed mean fluorescence intensity (MFI) for each dilution of the rituximab standard. The test articles were each diluted 1:2000 (to fall approximately at the midpoint of the standard curve) and the observed MFI value for each test article was used to interpolate a concentration value relative to the rituximab standard.

3.4 Rituximab container compatibility

To generate the samples for this study, rituximab solution was pipetted into pouches made from four different flexible films and then the pouches were sealed using a heat sealer. Care was taken to avoid dripping protein solution into the area where the final seal was formed. The sealed pouches were incubated at either 5 °C, 25 °C, or 40 °C, as indicated in Table 2. Glass controls were maintained at 5 °C for the duration of the study. Samples were removed from storage and the contents of the pouches were analyzed to determine the physical and chemical stability of the formulation and running an immunoassay to determine the activity of the protein.

Rituximab binding activity was assayed using the whole blood competitive binding immunoassay described here. The data shown here have been corrected using the apparent protein concentrations determined from SEC-MALLS data (S. E. Lee, *et al.*, in preparation). These data indicate that there is little decrease in binding activity over the course of the study and that there is no significant differentiation among the four film types tested in this study (Figures 6A-C). There was day-to-day variation in the normalized concentrations of the test samples, which is likely inherent to the competition assay used. At most time points,

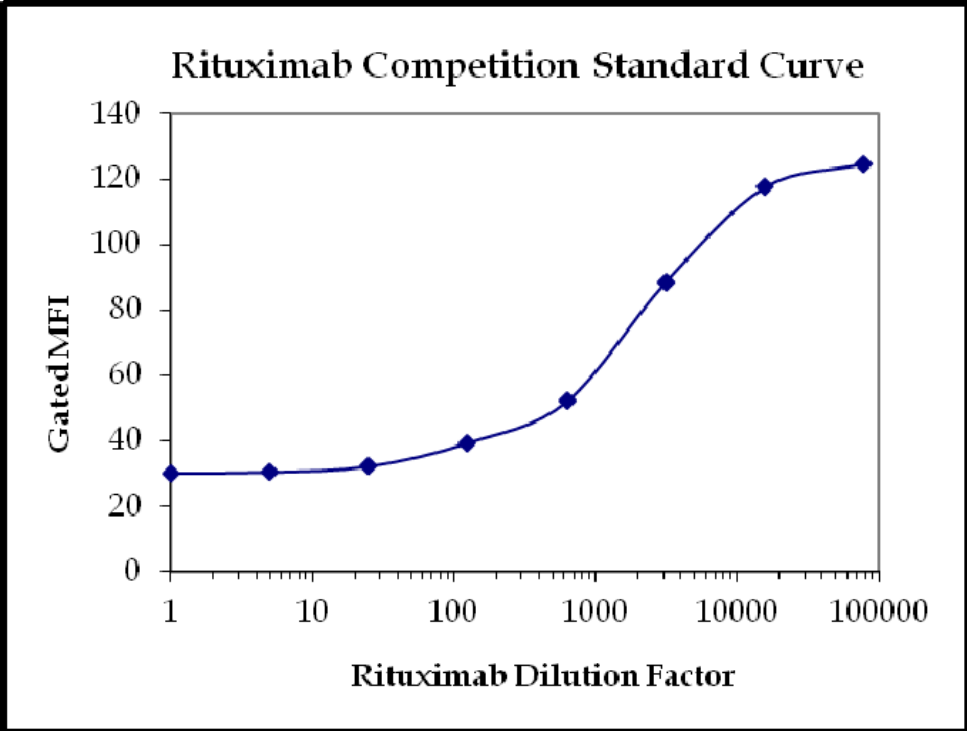


Fig. 5. Typical standard curve for the rituximab competitive cell binding immunoassay

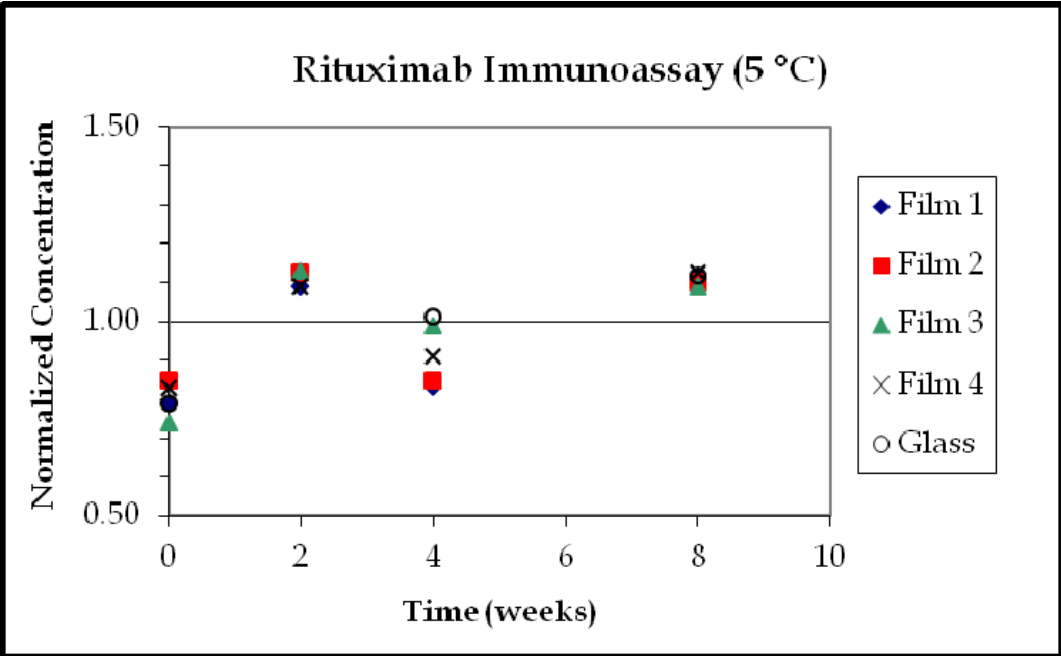


Fig. 6A. Immunoassay results for rituximab test articles at 5 °C (results normalized to the 5 °C glass controls)

all films were clustered in terms of effective concentration and the average effective concentration varied approximately $\pm 10\%$ from normal for the 5 °C and 25 °C storage conditions. This was not the case for the 40 °C storage condition, as other samples from the same testing interval had higher effective concentrations. These data indicate a slight downward trend in bioactivity in the samples stored at 40 °C for 4 weeks.

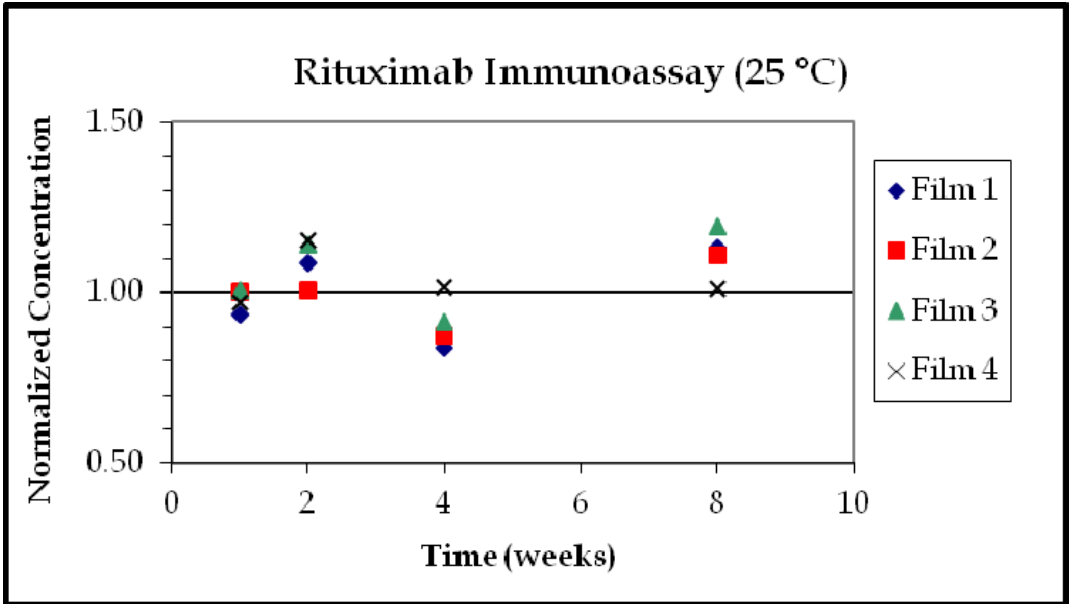


Fig. 6B. Immunoassay results for rituximab test articles at 25 °C (results normalized to the 5 °C glass controls)

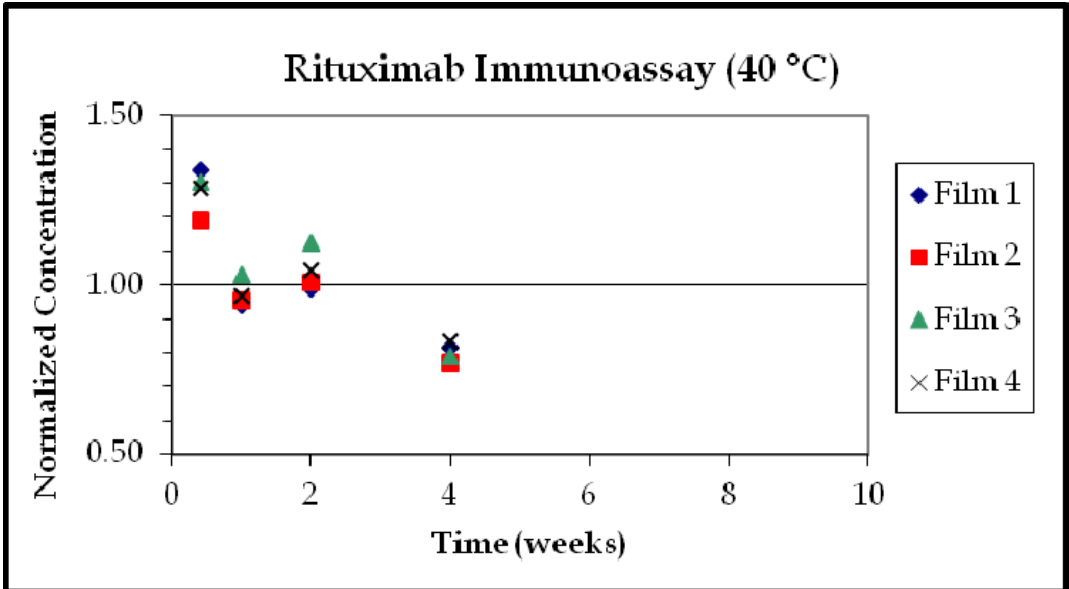


Fig. 6C. Immunoassay results for rituximab test articles at 40 °C (results normalized to the 5 °C glass controls)

The results of this study show that Rituximab solution is compatible with all four film types tested. Bioactivity assays confirm that the protein behaves similarly in all four film types over the course of the study, despite a slight decrease in activity upon storage for 4 weeks at 40 °C.

4. Conclusion

The primary aim of this study was to develop and implement protein-specific immunoassays to support the evaluation of the compatibility of two protein biotherapeutics with plastic RTU prototype containers. Here we have demonstrated, through the use of in-

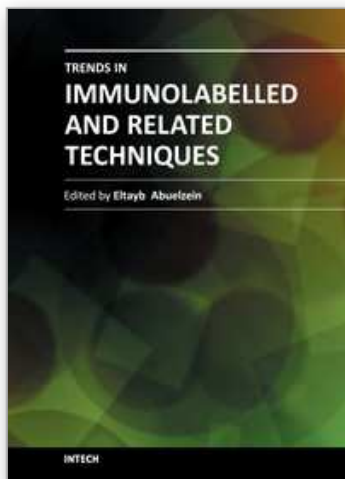
house developed immunoassays and standard chemical and chromatographic techniques, that flexible plastic containers can have equivalent performance to standard glass vials. This observation was true both in terms of the observed binding activities and the physical and chemical data collected (S. E. Lee, *et al.*, in preparation) for the two monoclonal antibodies tested, cetuximab and rituximab. Establishing this compatibility is essential to enabling a shift to this type of container system in the healthcare sector. Flexible plastic RTU containers provide a more convenient format for dosing to the patient, they can reduce medication errors by providing a ready to infuse format and also pose a lower risk of injury to both healthcare workers and patients. As the number of commercialized biotherapeutics increases, the need for these types of container systems becomes readily apparent. The methodology used in these studies can be used as a guideline for compatibility evaluations of other types of therapeutic proteins. As more types of biotherapeutic products make their way to market, there will be an increasing need for biological assays to assess their potency. The work described here illustrates the importance of using specifically tailored immunoassays to assess the activity of biotherapeutics selected for use as model proteins.

5. Acknowledgment

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Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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