GYROS PROTEIN Technologies

Application Report 207

Miniaturizing immunoassays for improved performance

Improve immunoassay performance

- **Increased dynamic range and faster analysis time**
- **Accuracy and reproducibility maintained with reduced sample and reagent consumption**
- **Matrix effects minimized**

Develop new immunoassays within days, not weeks

- **Existing ELISA assays easily transferred**
- **Choice of different immunoassay formats**
- **Unique Gyrolab® software tools speed up assay development and optimization**

Assay development in conventional immunoassay formats, such as ELISA, is a time- and reagent-consuming process due to long incubation times and limited flexibility in experimental set-up. Resulting immunoassays typically exhibit good overall performance, but may have limitations in measurement dynamic range and matrix compatibility.

Three case studies are presented that demonstrate how the automated, miniaturized assay format of Gyrolab immunoassay platforms enable fast and flexible assay development and improved assay performance. Working at nanoliter scale reduces consumption of sample and reagents up to one hundred fold when compared to ELISA.

Miniaturization improves assay performance

Transferring assays to Gyrolab platforms can improve overall assay performance, dynamic range, and analysis time, whilst still maintaining good quality data regarding accuracy and reproducibility.

The flow-through principle used in Gyrolab® Bioaffy™ CDs acts favorably to minimize matrix effects since low affinity matrix components do not have the opportunity to bind during sample application. As a result, the signal to noise ratio of the assay is improved. This generates a broader measurement range and reduces requirements for sample dilution in order to counteract various types of matrix effects, which are common in conventional assay formats.

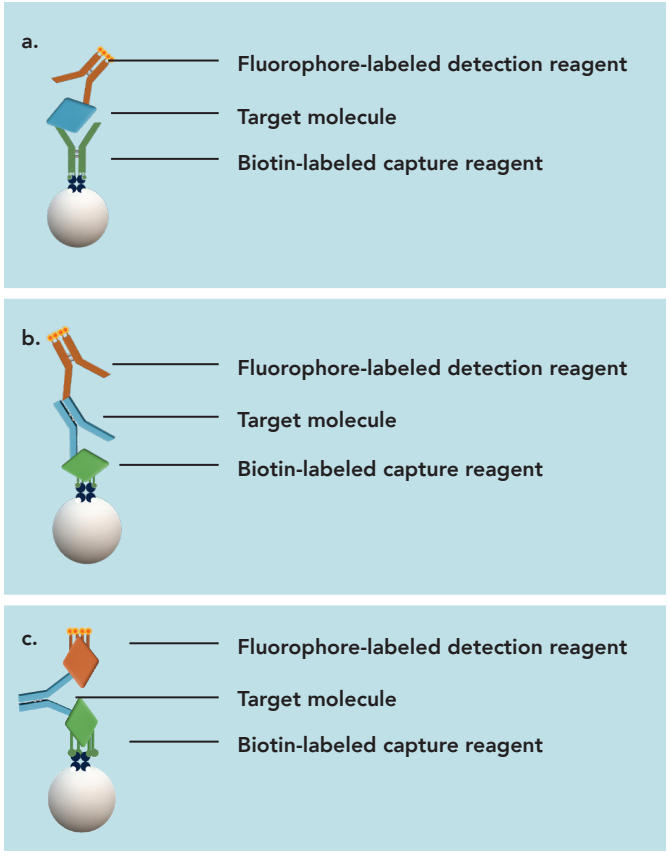

Figure 1 Examples of assay formats compatible with Bioaffy CDs. (a) sandwich immunoassay for antigen quantification, (b) indirect antibody immunoassay and (c) bridging immunoassay for antibody quantification.

Benefits of an open system

An open and flexible design facilitates quantification of any target protein for which an immunoassay can be developed. Assay development and optimization is performed efficiently using pre-programmed template methods, keeping usage of Gyrolab CDs to a minimum and easily selecting the most suitable reagents and experimental settings.

Compatibility with conventional immunoassay formats such as sandwich immunoassay (SIA), indirect antibody immunoassay (IAA) and bridging immunoassay (BA) enables miniaturization of most immunoassays (see Figure 1 a–c).

There are some basic prerequisites when selecting reagents to be used for immunoassays (see Table 1). Antibody pairs that are used in ELISA can normally be transferred provided that these prerequisites are fulfilled. For transfer to the Bioaffy CD format the antigen acts as both capture and detection reagent and must be labeled with biotin and fluorophor respectively.

Visualizing interactions speed up assay development

A unique combination of Gyrolab software designed for 21 CFR part 11 compliance assists in assay development. Gyrolab Manager enables a high level of flexibility for planning and setting up immunoassay runs at your desk, while Gyrolab Evaluator provides statistical data in tabular format and standard curves. Gyrolab Viewer graphically displays the fluorescence data from each column (see Figure 2), providing more qualitative information regarding affinity and non-specific interactions. The combined information from Gyrolab Evaluator and Gyrolab Viewer facilitates optimization and enables faster decision making when choosing assay conditions.

Guidelines for reagent selection

- Capture and detection reagents directed against different epitopes on the target protein
  - Unlabeled capture reagent available for biotin labeling
  - Unlabeled detection reagent available for fluorophor labeling
- Care should be taken regarding subclasses of immunoglobulins
  - Mouse or rat IgG1 has been found to generate less background than other subclasses
  - IgM should be avoided

Table 1 Prerequisites for reagents and optimal reagent pairs.

Reagent pair 'top-three' prerequisites for optimization	
Capture reagent	Detection reagent
1. Monoclonal antibody	Monoclonal antibody
2. Monoclonal antibody	Antigen-purified polyclonal antibody
3. Antigen-purified polyclonal antibody	Antigen-purified polyclonal antibody

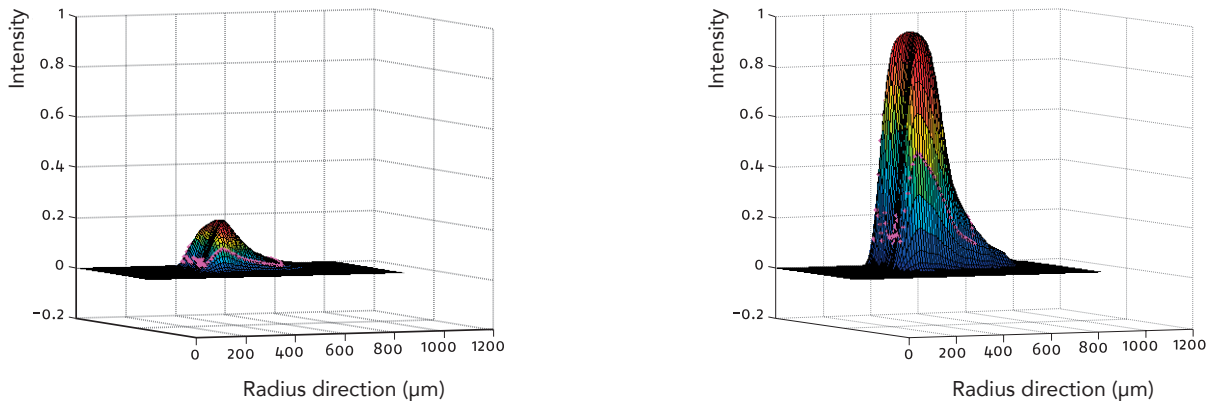

Figure 2 Gyrolab Viewer provides graphical representation of individual binding responses. Profiles of total fluorescence reflect the total amount of protein bound to each column. Liquid flows from left to right so that the fluorescent signal has its highest intensity where protein concentration is highest, i.e. at the top of the column. Sample protein: CHO host cell protein at 1.9 µg/mL (left) and 48 µg/mL (right).

Case study 1: Immunoassay that minimizes sample and reagent volumes for quantification of osteocalcin

Lund University, Department of Clinical Sciences, Malmö

The researchers at Lund University, Department of Clinical Sciences, Malmö, transferred their in-house developed ELISA-based sandwich immunoassay for quantification of osteocalcin to Gyrolab platform. Their aim was to minimize sample and reagent volumes whilst maintaining assay performance.

Osteocalcin is a small bone-specific protein (49 amino acid residues, MW ~5800) produced by osteoblasts during bone formation. The majority of osteocalcin is deposited into the extracellular matrix of bone, but a fraction of the secreted osteocalcin enters the blood, where it can be detected and used as a biomarker for bone turnover rate.

An ELISA assay based on two monoclonal antibodies (8H12/3H8) has been developed for detection of rat osteocalcin in serum and cell culture medium (Ivaska et al. 2004 J Biol Chem). This assay was developed on streptavidin-coated microtiter plates.

The capture monoclonal (MAb) binds to residues 7–19 in osteocalcin, while the detection MAb binds to residues 20–43 (Hellman et al. 1996 J Bone Miner Res). A human-specific modification of the assay (using a human-specific capture MAb) has been used in human studies (Kakonen et al. 2000 Clin Chem, Gerdhem et al. 2004 J Bone Miner Res).

Reagents

Hybridoma cell lines were cultured in a 'Tecnomouse' bioreactor at the University of Turku, and monoclonal antibodies were purified from the supernatant with Protein G affinity chromatography.

Biotinylated MAb 8H12 was used as the capture antibody (Hellman et al. 1996 J Bone Miner Res). 8H12 was biotinylated with 50-fold molar excess of biotin-isothiocyanate. The concentration of the capture antibody was 0.05 mg/mL. The capture MAb was identical to the one used in the osteocalcinspecific ELISA.

MAb 3H8 was used as detection antibody; 100 µg of 3H8 was Alexa-labeled using Alexa Fluor 647 Monoclonal Antibody Labeling Kit (Molecular Probes). The labeled antibody had a final concentration of 5.6 µM and the yield was 90%. The concentration was adjusted to 1 µM with PBS.

A synthetic osteocalcin peptide (49 residues, Glu at positions 17, 21 and 24 were g-carboxylated) was used as standard (Advanced Chemtech, USA). The peptide was diluted in TRIS-buffered saline supplemented with 7.5% BSA and stored at -85° C. The standard osteocalcin was diluted (1:1) in buffer and used at final concentrations: 0.075, 0.15, 0.3, 1.3, 3.3, 10.5, and 23 ng/mL.

Optimizing concentrations of labeled reagents

Dilution series of the osteocalcin standard were tested to optimize the concentration of detection antibody. The labeled MAb 3H8 detection antibody was used at four concentrations (25, 50, 75, and 100 nM) to quantify the signal from the different dilutions of standard osteocalcin. Measurements were carried out in triplicate for each dilution and each concentration of detection antibody. The detection limit ranged from 0.36 to 0.87 ng/mL with all combinations of detection antibody and PMT settings (1, 5, and 25 percent), as determined in Gyrolab Evaluator.

With all combinations, the response was linear between 1 and 23 ng/mL of osteocalcin (see Figure 3). Accordingly, it was decided to use the concentration of 25 nM for further assays as this concentration would require less reagent. The sensitivity of the assay might be improved if the concentration of capture antibody is increased. However, the range from 1 to 23 ng/mL is suitable for measurements of rat plasma from most combinations of strains and conditions and, therefore, the lower and upper limits of quantification of the assay were not investigated further.

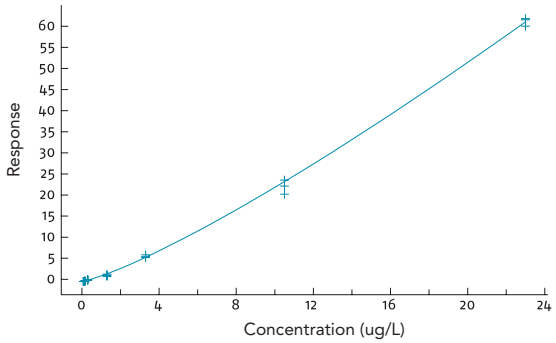

Figure 3 Standard curve for rat osteocalcin. The limit of detection for the assay is 0.7 ng/mL.

Assay performance

The sensitivity of the Gyrolab immunoassay was comparable with the ELISA (Ivaska et al. 2004 J Biol Chem), which requires 30 µL for triplicate measurements of rat serum or plasma as compared with 4 µL used in Gyrolab. Measurements of rat plasma from a number of rat strains and conditions displayed a major variation of osteocalcin concentrations between strains and age groups. The average concentration among the different combinations of Gk and F334 rat strains used for osteocalcin measurement in plasma was 50 ng/mL.

Conclusions from osteocalcin assay transfer

- With just two test runs, a sample and reagent-saving assay was developed for rat osteocalcin
- The transferred assay provided comparable sensitivity to the established ELISA sandwich immunoassay
- Sample volume requirements were reduced from 30 µL to 4 µL

Case study 2: Automated immunoassay that saves time and improves overall performance

Cambridge Antibody Technology (CAT)

CAT develops human monoclonal antibodies for therapeutic applications. The team was using a semi-automated ELISA and was seeking to automate and streamline their overall workflow. They wanted to speed up assay run time (currently three days with in-house developed ELISA) and assay development time (currently 2 weeks). At the same time, they wished to improve overall assay performance.

In the successful collaboration, an assay for quantifying Antibody X, a monoclonal antibody drug (human IgG4), was transferred from ELISA. To verify the specificity of the resulting assay, spiked human serum control samples were provided by CAT and quantified.

To allow comparison with the ELISA procedure, the indirect antibody approach with the antigen as capture reagent was attempted. The reagents provided by CAT were:

- Biotinylated Antibody X antigen
- Antibody X human IgG4
- Sheep anti-hIgG4, to be labeled with Alexa Fluor® 647 fluorophore (Invitrogen Corporation)

The assay development workflow for this assay is illustrated in Figure 4.

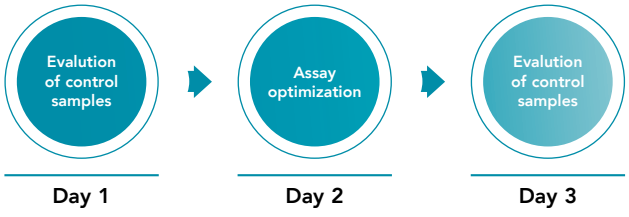

Figure 4 Timeline for development and optimization of the Antibody X assay. A total of 3 CDs were used.

Optimizing concentrations of labeled reagents

Titration of capture reagent

Two concentrations of capture reagent were evaluated, 0.05 and 0.1 mg/mL. The resulting standard curves (from Gyrolab Evaluator software module) are shown in Figure 5.

To maximize the measurement range of the assay, and to saturate the biotin binding capacity of the capture column, 0.1 mg/mL was chosen as capture reagent concentration.

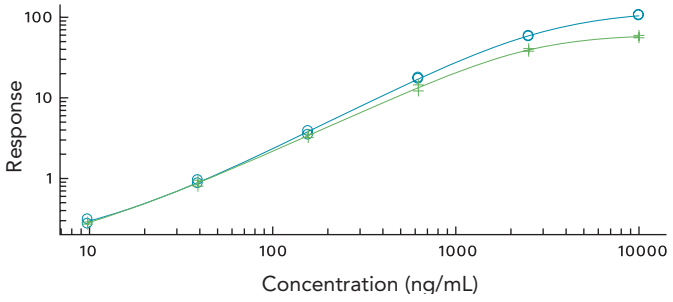

Figure 5 Standard curves for Antibody X using 0.05 mg/mL (crosses) and 0.1 mg/mL (circles) of capture reagent respectively.

Detection reagent

The concentration of detection reagent should be optimized so that it minimizes background noise and allows for quantification of high concentration samples without saturation of the fluorescence detector. Three concentrations of detection reagent were evaluated, 25 nM, 50 nM, and 100 nM. The resulting standard curves are shown in Figure 6.

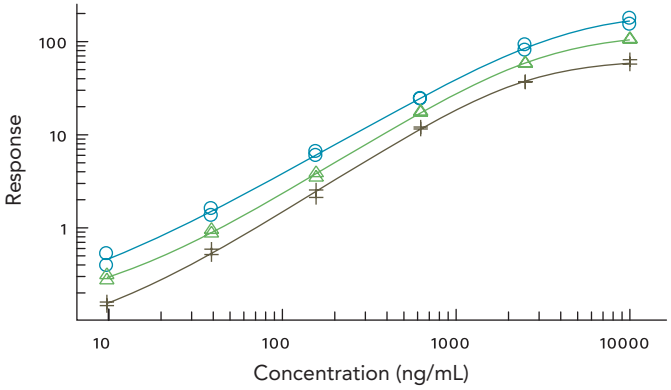

Figure 6 Standard curves for Antibody X using 25 nM (crosses), 50 nM (triangles) and 100 nM (circles) of detection reagent respectively.

Looking solely at the standard curves, the measurement range appeared to be more or less independent of concentration of the detection reagent. The main difference was the increasing signal level as the detection reagent concentration increased. When calculating the signal to noise ratio the 50 nM concentration of detection reagent gave the best assay dynamics. Similarly, when consulting the corresponding images (created in Gyrolab Viewer), it was evident that the highest evaluated reagent concentration (100 nM) began to saturate the fluorescence detector (see Figure 7). To maximize the signal and still avoid saturation, the middle concentration (50 nM) was chosen as detection reagent concentration.

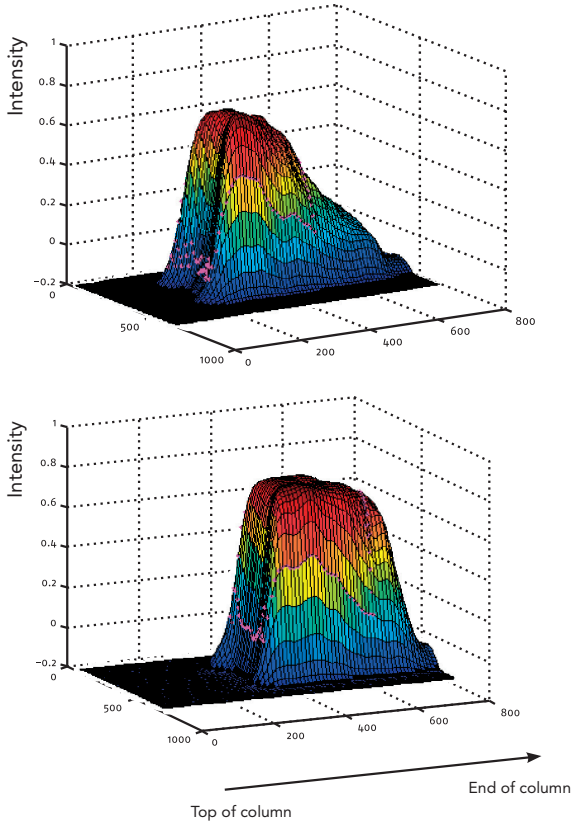

Figure 7 Images (created in Gyrolab Viewer) of the fluorescent signal for standard concentration 2500 ng/mL using detection reagent concentration 50 nM (a) and 100 nM (b) respectively.

Evaluation of control samples

Quantification of the control samples provided by CAT, using the Gyrolab assay, resulted in excellent agreement between expected and obtained concentrations of Antibody X (Table 2).

A comparison of the overall assay performance and time aspects is shown in Table 3.

Table 2 Expected and obtained concentration values in control samples.

Sample ID	Expected Antibody X concentration (ng/mL)	Gyrolab Antibody X concentration (ng/mL)
Control level 1	250	220
Control level 2	75	69
Control level 3	30	36

Table 3 Comparison of assay performance as part of the CAT study.

	ELISA	Gyrolab
Sample volume (µL)	50	3
Measurement range (ng/mL)	63–315	13–2500
Precision (% CV)	<25%	<12%
Assay time including sample preparation	3 days	3 hours
Assay development time	2 weeks	3 days

Conclusions from Antibody-X assay transfer

- The assay for quantifying Antibody X in human serum samples was successfully transferred using an indirect antibody immunoassay format
- Assay development and optimization of the Gyrolab assay was performed within three days, using a minimum of CDs - (compared to two weeks using current methodology)
- Overall Gyrolab assay performance was significantly improved compared to ELISA. The measurement range of the assay was increased by a factor of ten
- The total assay time was significantly reduced from three days to three hours
- Required sample volume was reduced by a factor of 15, from 50 µL to 3 µL

Case study 3: Automated immunoassay that increases dynamic range and reduces sample matrix interference

Pharmacokinetic assay

A third Gyrolab immunoassay was developed in collaboration with a research group performing pharmacokinetic studies of therapeutic antibodies. The group was using an in-house developed ELISA to quantify a biologic drug molecule in cynomolgus monkey serum. The researchers were looking to improve assay performance by increasing measurement range and reducing interference from the sample matrix (unknown components of the cynomolgus monkey serum).

In collaboration with researchers at Gyros Protein Technologies, an assay for quantifying the human monoclonal antibody drug molecule was transferred from an ELISA format to the miniaturized Gyrolab microfluidic format. Utilizing the antigen binding properties of the human monoclonal antibody, labeled target protein was used as both capture and detection reagent in a bridging immunoassay design.

Optimizing reagent concentrations

The bridging immunoassay format required optimizing the antigen density on the Gyrolab microchannel capture column by varying the ratio between biotinylated antigen and biotinylated BSA. This approach minimizes the risk of the drug molecule binding to two neighboring biotinylated antigen molecules, making detection of bound antibody impossible. Response values were evaluated while keeping the concentration of drug molecule and Alexa labeled antigen constant.

Figure 8 shows images (created in Gyrolab Viewer) of the fluorescent signal corresponding to the highest monoclonal antibody drug standard concentration and blank sample matrix, respectively, when titrating the ratio between biotinylated antigen and biotinylated BSA. The aim is to reach a sharp and narrow peak in the beginning of the column (i.e. in the middle of the 3-D image) with as high signal to noise ratio, and as low background as possible.

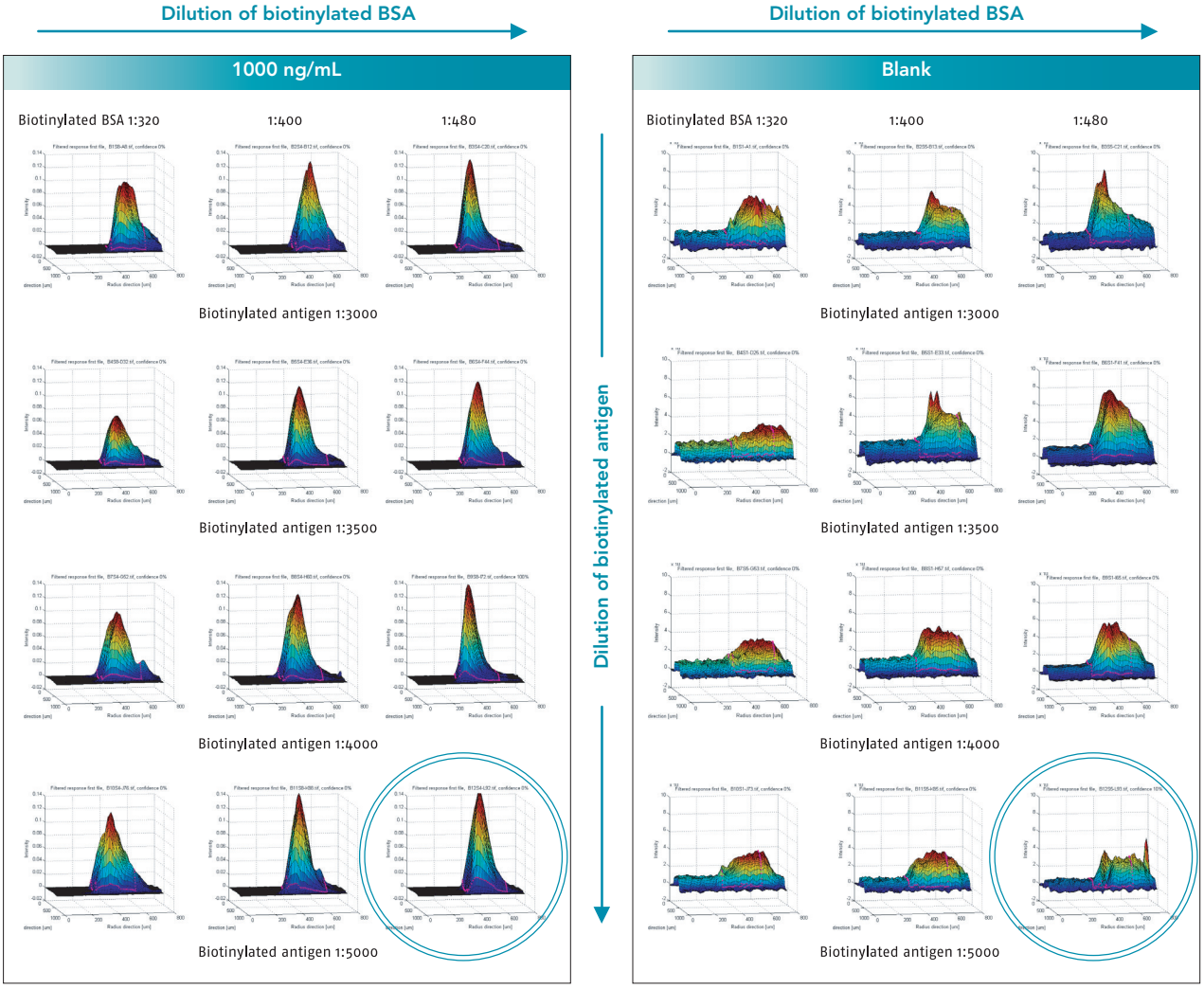

Figure 8 Images (created in Gyrolab Viewer) of the fluorescent signals from the blank (right) and the highest drug standard concentration (left), respectively, as the ratio between biotinylated antigen and biotinylated BSA is varied. (Note, different scales are used for the left side and right side graphs.) Marked with blue circles are images corresponding to the chosen ratio.

Quantification in cynomolgus monkey serum

Figure 9 shows the resulting standard curves for the transferred assay when diluting the drug molecule in pooled cynomolgus monkey serum, undiluted and diluted 1:4 in buffer respectively. When comparing the two curves there is no evidence of matrix effects from the serum components.

Five individual cynomolgus monkey serum samples, diluted 1:4 in buffer, were spiked with drug molecules in concentrations ranging from 3.9–1000 ng/mL. The resulting recovery values are shown in Figure 10.

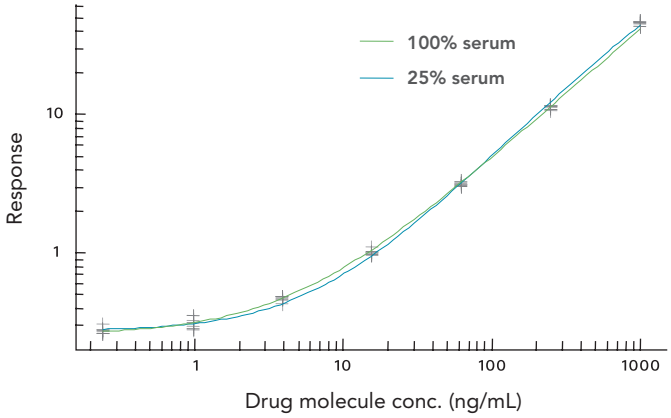

Figure 9 Standard curves for quantification of drug molecule generated with undiluted serum (green) and serum diluted 1:4 (blue) respectively.

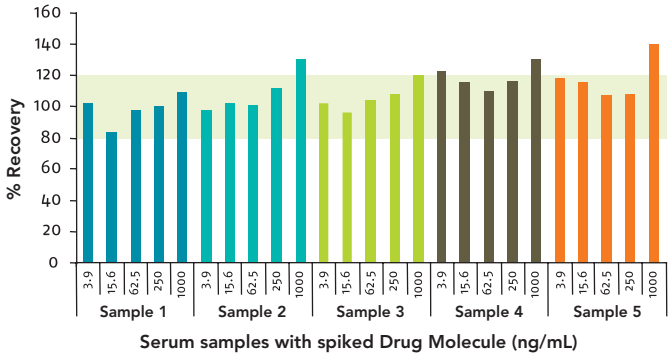

Figure 10 Recovery of drug molecule spiked into serum samples diluted 1:4.

Conclusions from the pharmacokinetic assay transfer

- Assays for studying the pharmacokinetics of monoclonal IgGs in cynomolgus monkey serum were easily transferred from ELISA to Gyrolab format
- The dynamic range of the assay was increased in the Gyrolab format to better fit analytical needs and recovery values were significantly improved compared to ELISA

Conclusions

The three successful collaborations in this report illustrate the versatility and speed of Gyrolab platform for miniaturizing immunoassays to reduce assay development and completion times and improve assay performance, whether they are sandwich immunoassays, indirect antibody immunoassays or bridging immunoassays.

Almost any existing ELISA assay can be easily transferred into the miniaturized format, providing that some basic prerequisites for reagents are met.

With the help of Gyrolab Viewer, a unique software tool for visualizing interactions, new assays can be developed and optimized in a matter of days.

The miniaturized and automated Gyrolab CD format offers inherent advantages over established plate-based technologies, providing benefits for workflow efficiency and improved assay performance:

- Automation and fast flow-through column interaction times reduce analysis time and increase throughput
- Nanoliter volumes reduce sample and reagent consumption without affecting accuracy and reproducibility
- Flow-through principle favors high-affinity interactions and minimizes matrix effects, improving assay performance

Gyros Protein Technologies would like to express their thanks to Lund University, Department of Clinical Sciences, Malmö, Sweden (www.lu.se) and Cambridge Antibody Technology, Cambridge, UK (www.cambridgeantibody.com) for permission to show their results.