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The development and validation of a sensitive, dual-flow cell, SPR-based biosensor immunoassay for the detection, semi-quantitation, and characterization of antibodies to darbepoetin alfa and epoetin alfa in human serum

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ABSTRACT

A surface plasmon resonance (SPR)-based biosensor immunoassay was developed and validated using the Biacore 3000 instrument to detect, semi-quantitate, and characterize serum antibodies against darbepoetin alfa (Aranesp®) and epoetin alfa (EPOGEN®). In this sensitive, dual-flow cell assay, epoetin alfa and darbepoetin alfa are covalently immobilized onto consecutive flow cells of a carboxymethyl dextran-coated sensor chip. Diluted human serum samples are injected sequentially over both surfaces. The binding of serum antibodies to the immobilized proteins are detected and recorded in real time based on the principles of SPR. Furthermore, antibody binding is confirmed with a secondary anti-human immunoglobulin antibody. Positive samples are further characterized to determine the relative concentration of the antibodies using an affinity-purified, rabbit anti-epoetin alfa antibody as a reference control.

The assay can detect 80 ng/ml and 100 ng/ml of antibody to epoetin alfa and darbepoetin alfa, respectively. The dynamic range of the assay is from 0.078 μg/ml to 10 μg/ml using a rabbit antibody with demonstrated accuracy and intra- and inter-assay precision. Approximately 80 serum samples can be analyzed on each sensor chip while maintaining a stable baseline and consistent immunological reactivity. The analysis of serum samples from subjects administered with epoetin alfa or darbepoetin alfa provided evidence that the assay can detect varying concentrations of antibodies of different off rates, isotypes, and IgG subclasses.

1. Introduction

Recombinant erythropoiesis stimulating agents (ESAs) have been used successfully to increase the production of red blood cells in patients with anemia resulting from chronic kidney disease (CKD) or chemotherapy. In rare cases, antibody-mediated pure red cell aplasia (PRCA) can occur from ESA therapy. Antibody-mediated PRCA develops when antibodies form to block or reduce the body's ability to make red blood cells, causing severe anemia. One cause of antibody-mediated PRCA occurs spontaneously without any prior exposure to ESAs. In other cases, antibody-mediated PRCA occurs when the patient develops neutralizing antibodies against the ESAs. Therefore, the potential to elicit a neutralizing antibody response by administering ESAs necessitates maintenance of a high level of pharmacovigilance. This includes the development and validation of immunoassays for anti-ESA antibody testing and surveillance to ensure patient safety.

The immunoassay is typically the initial method used to identify samples that may contain binding antibodies to the administered ESAs. If samples score antibody positive in the immunoassay, they are then tested in a cell-based bioassay for neutralizing antibodies. Throughout the early clinical development of epoetin alfa (EPOGEN®) and darbepoetin alfa (Aranesp®) by Amgen Inc., immunogenicity testing was supported with a validated radioimmunoprecipitation (RIP) assay. The method utilized ^{125}I-labeled epoetin alfa and a *S. aureus* suspension, which contains Protein A, to bind the anti-epoetin alfa or anti-darbepoetin alfa antibody-^{125}I-labeled epoetin alfa complex. This method is similar to two recently published methods [4,13], with the exception that these methods use Protein G sepharose as a means to isolate the immune complexes. Although the RIP assay is able to detect 10 ng/ml of specific

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antibody, it is labor-intensive, poor at detecting an IgM antibody, and generally lacks specificity.

In an effort to identify an assay platform that could detect varying concentrations of anti-epoetin alfa or anti-darbepoetin alfa antibodies of different off rates and isotypes, we considered an enzyme-linked immunosorbent assay (ELISA) [12]. The ELISA method is widely used, and has been described by others for the analysis of anti-ESA antibodies [15,6]. However, ELISA methods require multiple wash steps that can lead to a loss or underestimation of weakly bound or rapidly dissociating antibodies. These types of antibodies can be components of a primary IgM or a mature IgG response to drug and therefore may be clinically relevant.

Over the last decade, SPR-based biosensor technology has been used to detect antigen–antibody complexes [5,7,8] and to measure binding and equilibrium constants [10,11]. A variety of commercial SPR-based biosensors are available and have been reviewed elsewhere [3], but the Biacore family of optical SPR-based instruments are the most widely used. Over the past several years, numerous publications have documented the usefulness of Biacore instrument(s) in the detection and characterization of antibody responses [1,2,14,16]. Our laboratory has previously published the validation of a single-flow cell biosensor immunoassay with a sensitivity of 400 ng/ml of anti-darbepoetin alfa antibodies [9].

This work describes the development and validation of an improved SPR-based biosensor immunoassay method, extending the sensitivity of the darbepoetin alfa surface to 100 ng/ml and the implementation of an epoetin alfa surface to assess the potential anti-darbepoetin alfa antibody cross-reactivity to epoetin alfa. This dual-flow cell biosensor immunoassay is used by Amgen as the preferred method to detect and characterize anti-epoetin alfa and anti-darbepoetin alfa antibodies in human serum. During assay development, optimal conditions were established to create a robust and sensitive biosensor immunoassay. After exploring the many assay variables, the assay was fully validated to show that it consistently performs as intended.

2. Materials and methods

2.1. Equipment and materials

- Biacore 3000, sensor chip CM5, amine-coupling kit, P-20 surfactant (P-20), 10 mM sodium acetate (NaOAc) pH 4.0, and HBS-EP buffer (GE Healthcare Company-Biacore Group, Uppsala, Sweden).
- EPOGEN® (epoetin alfa) bulk standard, Aranesp® (darbepoetin alfa) bulk standard, and affinity-purified polyclonal rabbit anti-epoetin alfa antibody (Amgen Inc., Thousand Oaks, CA, USA).
- Pooled and individual human sera (Bioreclamation Inc., Hicksville, NY, USA).
- Affinity-purified goat anti-human IgG + IgM + IgA (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA); also reacts with Human IgE (data at Amgen Inc.).
- Sample diluent: 50 mM Tris (Sigma–Aldrich, St. Louis, MO, USA), 0.5 M sodium chloride (NaCl; Invitrogen Corp., Carlsbad, CA, USA), 5.48 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen), 0.01% P-20, pH 9.0, with 1% (w/v) carboxymethyl dextran (Fluka Chemical Corp., Ronkonkoma, NY, USA).
- Sample analysis running buffer: 25 mM Tris, 0.3 M NaCl, 2.74 mM EDTA, 0.005% P-20, pH 9.0.
- NaOAc, sodium meta-periodate ($NaIO_4$), sodium hydroxide (NaOH), hydrogen chloride (HCl), hydrazine, sodium cyanoborohydride ($NaBH_3CN$), and glycerol (Sigma–Aldrich).
- Sterile water (Baxter, Deerfield, IL, USA).
- Anti-human IgG + IgA + IgE antibody (MP Biomedical HQ, Irvine, CA, USA).
- Anti-IgM antibody (Fitzgerald Industries Inter., Concord, MA, USA).
- 10,000 molecular-weight-cut-off (MWCO) membrane Slide-A-Lyzer cassette (Pierce Biotechnology Inc., Rockford, IL, USA).
- N-ethyl-N′-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and ethanolamine hydrochloride (GE Healthcare Company-Biacore Group, Uppsala, Sweden).

2.2. Methods

2.2.1. Preparation of an affinity-purified rabbit anti-epoetin alfa antibody

New Zealand white rabbits were immunized by intramuscular injection with 200 μg of epoetin alfa in Hunter's Titermax adjuvant (CytRx Corp., Norcross, GA, USA) at the Montana State University Animal Resource Center. Three booster injections with 100 μg of epoetin alfa were administered, the first given 1 month after the primary injection, followed by two more booster injections 2 weeks apart. A production bleed (40 ml) was collected 2 weeks after the last booster injection followed by 2 more production bleeds taken 2 weeks apart. A total of 120 ml of rabbit antiserum was collected and pooled for affinity purification. Rabbit antiserum was fractionated over a Protein G column to isolate the immunoglobulin fraction. This fraction was then loaded onto an affinity chromatography column packed with epoetin alfa covalently coupled to sepharose beads for immunoaffinity purification against epoetin alfa. The anti-epoetin alfa antibody concentration was then determined by absorbance at 280 nm wavelength (A_{280}). The affinity-purified antibody was aliquoted and stored at −20 °C for use as the positive control antibody in the immunoassay.

2.2.2. Preparation of the epoetin alfa surface

Epoetin alfa (30.4 kDa) was covalently immobilized onto a sensor chip CM5 surface (flow cell 1 or 3) through its primary amines. Immobilizations were performed in HBS-EP buffer at a flow rate of 5 μl/min at 25 °C. A 35 μl injection of a 1:1 mixture of 0.2 M EDC and 0.05 M NHS in water introduced reactive succinimide esters onto the carboxylated dextran matrix to permit subsequent formation of covalent bonds with primary amines. A 20-min injection of epoetin alfa at 400 μg/ml in 10 mM NaOAc pH 4.0 was allowed to react with the activated surface. The unreacted carboxyl groups were quenched with 50 μl of 1 M ethanolamine hydrochloride pH 8.5. Finally, a 50 mM NaOH wash for 1 min completely removed any non-covalently bound protein. Immobilization levels of 1773.3 RU to 2537.0 RU were obtained.

2.2.3. Preparation of the darbepoetin alfa surface

Darbepoetin alfa (37.3 kDa) was covalently immobilized onto a CM5 sensor chip surface (flow cell 2 or 4) through the carbohydrate moieties. First, the sialic acid residues on the carbohydrate were mildly oxidized to introduce aldehyde groups for immobilization onto the sensor chip. To do this, darbepoetin alfa (1.99 mg/ml, >95% purity based on SDS-PAGE) was diluted to 1 mg/ml in 1 mM $NaIO_4$ in 0.1 M NaOAc pH 5.5, and was mixed and cooled on ice in the dark for 20 min. The oxidation of cis-diols to aldehydes by $NaIO_4$ was then stopped by quenching the unreacted periodate with a 5% glycerol solution. Next, the oxidized darbepoetin alfa solution was injected into a 10,000 MWCO membrane Slide-A-Lyzer and dialyzed against 0.1 M NaOAc pH 5.5. After determining the protein concentration at A_{280}, darbepoetin alfa was diluted to 50 μg/ml in 10 mM NaOAc pH 4.0.

To start the coupling procedure (performed at 5 μl/min at 25 °C), 35 μl of a 1:1 mixture of EDC–NHS was injected in order to activate

the carboxyl groups on the dextran of the sensor chip. A 7-min pulse (35 μl) of 5 mM hydrazine was then allowed to react with the esters. To prevent unwanted amine coupling, ethanolamine hydrochloride was injected to react with any residual esters. Next, 35 μl of oxidized darbepoetin alfa was coupled to the hydrazine groups. The formed hydrazone bond was reduced with 0.1 M NaCN in 0.1 M NaOAc for 20 min at a reduced flow rate of 2 μl/min to form a more stable hydrazide bond able to withstand acidic regeneration conditions. Finally, the surface was conditioned before use with three 30-s injections at 10 μl/min of 50 mM HCl with 5% P-20. Immobilization levels of 1905.6 RU to 2866.0 RU were obtained.

2.2.4. Analytical method for detecting anti-epoetin alfa and darbepoetin alfa antibody

Pooled normal human serum was used as a negative control in the assay. Positive controls were prepared by spiking rabbit anti-epoetin alfa antibody into pooled human serum at two final concentrations of 0.25 μg/ml and 10 μg/ml. A standard curve was prepared by spiking rabbit anti-epoetin alfa antibody into pooled human serum at a final concentration of 10 μg/ml and then serially diluted 2-fold to 0.039 μg/ml. Controls, antibody standards, and unknown samples were subsequently mixed 1:2 in sample diluent. All serum samples were filtered using a 0.22 μm vacuum filter plate (if microplate was used) or 0.22 μm SPIN X tubes by centrifugation. Additional solutions that are required include the secondary antibody and the regeneration solution. Affinity-purified goat anti-human IgA + IgM + IgG (H + L)-specific antibody was diluted to 50 μg/ml in sample diluent. Regeneration solution was prepared by making a mixture of 50 mM HCl and 5% P-20.

Samples were analyzed using a user-programmed wizard, a set of instructions that command the Biacore 3000 instrument for automated sample analysis. Each prepared sample was injected over the epoetin alfa surface first, for 2 min at a flow rate of 10 μl/min, and then continued across the darbepoetin alfa surface. The sample binding (in RU) to each immobilized ligand was recorded 60 s after the end of injection. Immediately after completion of the sample injection, the secondary goat anti-human IgA + IgM + IgG antibody was injected for 2 min at a flow rate of 10 μl/min. A confirmatory binding response was recorded 30 s after the injection. Finally, a 5 μl injection of regeneration solution was injected for 30 s to remove the bound antibodies. This was followed by a 2-min wait period of continuous buffer flow to allow the surfaces to stabilize before starting another sample and regeneration cycle of analysis.

2.3. Criteria for reporting results

2.3.1. Assay acceptance

The binding (in RU) of the negative control bracketing a set of samples must test below the threshold value for that surface (see Section 3.2.3). The binding of each replicate of the low positive control (PC01) bracketing a set of samples must be above threshold. The binding of each replicate of the high positive control (PC02) must be above threshold and the percent coefficient of variation (%CV) must be ≤20.

2.3.2. Defining sample results

If a serum sample demonstrates binding (in RU) less than the threshold value for its respective surface, the sample is reported "negative" for anti-epoetin alfa and darbepoetin alfa antibody. A serum sample with binding greater than the established threshold value for that surface is designated "reactive." For each reactive sample, the ratio of the confirmatory binding of the reactive sample over the median confirmatory binding of the negative control is calculated. If the ratio is <2.0, the sample is reported "negative", and if the ratio is ≥2.0, the sample is "positive" for anti-epoetin alfa or darbepoetin alfa antibodies.

2.4. Supplemental testing to characterize antibody-positive samples

A select number of clinical samples from patients administered darbepoetin alfa and confirmed to be positive for binding, non-neutralizing antibodies in the SPR-based biosensor immunoassay were re-tested to further characterize the isotype of the antibody and the relative rate of dissociation of the bound antibody. These tests are not part of the standard biosensor immunoassay described here, and the results are not part of the criteria in determining the antibody status of a sample. Samples must first test positive to be eligible for this supplemental testing. Both tests followed the same method listed above except where indicated. The results from these 2 supplemental tests are used to support the validation of the biosensor immunoassay for its intended use; specifically, to provide evidence that all 4 major human antibody isotypes and antibodies with relatively fast off rates can be detected.

2.4.1. Isotype determination

Clinical samples were tested according to the method described above except that the sample injection volume was increased to 50 μl. At this injection volume, the sample binding increased thereby improving the chance of confirming the antibody isotype. Immediately after sample injection, a 20-μl aliquot of anti-human isotype-specific antibody was injected followed by removal of bound antibodies with injection of the regeneration solution. The anti-human IgG, IgE and IgM antibodies were diluted in HBS-EP buffer to a concentration of 100 μg/ml; the anti-human IgA antibody was diluted to 200 μg/ml in HBS-EP buffer. A separate sample injection was used for each anti-isotype antibody test. The anti-isotype specific antibody must bind 100 RU or greater to the bound serum antibody for a positive identification of the antibody isotype. All 4 anti-human isotype antibodies were qualified in this Biacore assay for specificity and cross-reactivity (data not shown).

2.4.2. Determination of dissociation rate

Clinical samples were tested according to the method described above except that the sample injection volume was increased to 50 μl to increase overall sample binding; after the sample injection, the flow rate was increased too 30 μl/min. The loss of bound sample (in RU) over a 40-min period was then calculated per minute. The percent loss based on the total sample bound (in RU) was reported for each sample.

2.5. Validation

All of the validation parameters evaluated were performed following the method described above. The parameters evaluated for this antibody immunoassay included precision, specificity, assay cut point (threshold), limit of detection, accuracy, and sample stability. Additional parameters unique to the Biacore platform included immobilization reproducibility, baseline stability, and immunological reactivity of the immobilized drug.

3. Results

3.1. Assay development

Our prior development and validation of a single-flow cell, biosensor immunoassay achieved a limit of detection (LOD) of 0.40 μg/ml in neat human serum against darbepoetin alfa [9]. In order to improve the assay sensitivity in the current immunoassay, numerous factors were evaluated; sensitivity was most improved

Table 1
Immobilization reproducibility and range. The amount of epoetin alfa and darbepoetin alfa-immobilized on 5 different days using 4 different Biacore 3000 instruments throughout the assay validation were recorded (in RU). The mean, S.D., and %CV was calculated for the epoetin alfa and darbepoetin alfa immobilization. The highest and lowest immobilization per surface was used to establish the range.

Immobilization	Biacore instrument	Epoetin alfa (in RU)	Darbepoetin alfa (in RU)
1	1	1821.4	1905.6
2	2	1850.0	2202.6
3	3	2537.0	2392.5
4	4	2128.7	2288.3
5	1	1773.3	2866.0
	Mean	2022.1	2331.0
	S.D.	319.5	349.7
	Percent coefficient of variation	15.8	15.0
	Range	1773.3–2537.0	1905.6–2866.0

when the serum concentration was increased to 50% serum and the salt concentration and pH in the sample diluent and running buffer were increased.

Increasing the salt concentration and the pH decreased the non-specific binding (NSB) in a select number of serum samples previously demonstrated to have high NSB against epoetin alfa. The salt concentration was increased from 150 mM to 300 mM, and the pH was increased from 7.4 to 9.0 (data not shown). The improved signal-to-noise ratio means improved assay sensitivity. A change to TBS pH 9 buffer allowed the assay to tolerate a higher serum concentration. The rabbit anti-epoetin alfa antibody was spiked into neat serum and then diluted 1:2 or 1:10. Higher sample binding was detected at the low ng/ml antibody concentrations for both the darbepoetin alfa and epoetin alfa surfaces, thus demonstrating an improved LOD (data not shown).

3.2. Validation

3.2.1. Immobilization reproducibility and range

The routine coupling of epoetin alfa and darbepoetin alfa onto a dextran-coated sensor chip is a critical first step. As outlined in the methods, epoetin alfa is immobilized to the sensor chip using standard amine chemistry and darbepoetin alfa is coupled using aldehyde chemistry. Based on development data, the average amount of each immobilized protein was targeted for approximately 2000 RU.

Two parameters were validated: the amount immobilized, and the reproducibility of the epoetin alfa and darbepoetin alfa immobilization. Immobilization data from 4 different Biacore instruments using 2 different sensor chip lots were collected. Table 1 shows the amount of protein bound for each independent immobilization. Immobilization followed application of the regeneration solution to ensure removal of incomplete protein coupling. The mean RU was then calculated for each protein using data from 5 independent immobilizations. The %CV was determined to be 15.8% for epoetin alfa and 15.0% for darbepoetin alfa. The upper and lower immobilization amounts (in RU) from the 5 experiments with epoetin alfa and darbepoetin alfa were used as the immobilization range for each protein. Therefore, the immobilization range was determined to be 1773.3–2537.0 RU for epoetin alfa and 1905.6–2866.0 RU for darbepoetin alfa.

3.2.2. Baseline stability and immunological reactivity

A unique feature of the biosensor immunoassay platform is that the surface containing the immobilized drug is re-used to analyze multiple serum samples. Most other immunological methods rely on the reproducibility of identical surfaces (i.e. wells of a plate) rather than re-use of the same surface for a series of measurements. The number of samples analyzed per immobilized surface is dependent on the complete removal of previously bound serum proteins (baseline stability) and maintenance of immunological reactivity of the immobilized protein. To achieve this, a suitable regeneration solution must be identified. In general, antibody–protein interactions are susceptible to dissociation resulting from a sudden change in pH. A 50 mM HCl solution containing 5% P-20 was demonstrated to be the most effective regeneration solution based on its ability to minimize effects on the immobilized antigenic integrity and maximize immunological reactivity while efficiently removing all bound serum proteins, including the serum antibodies.

The extent of the baseline stability and immunological reactivity of each immobilized surface was evaluated using the rabbit anti-epoetin alfa antibody spiked into human serum. The analysis was performed up to 245 cycles by repeating the analysis of 20 negative control sample injections bracketed by an anti-epoetin alfa antibody spiked sample. Two data measurements were acquired from both the epoetin alfa and the darbepoetin alfa-immobilized surface: the baseline absolute response or stability prior to each sample analysis (in RU), which provides an indication of the material removed from the surface after regeneration, and the immunological reactivity approximately 1 min after the sample injection, indicating the amount of sample binding to the immobilized surface.

Results for the epoetin alfa-immobilized surface are shown in Fig. 1a. The rabbit anti-epoetin alfa antibody consistently bound to the immobilized epoetin alfa over 245 cycles, with a mean sample binding of 32.5 RU (S.D. ± 2.6 RU) and a %CV of 7.9. The negative control binding remained below the threshold of 7.9 RU throughout the 245 cycles. The baseline stability of the immobilized epoetin alfa was derived by recording the absolute response (AbsResp) throughout the 245 cycles. From the beginning of the 245-cycle run (22,938 RU) to the end of the run (22,941 RU), there was an overall gain of only 3.0 RU, or an overall change of <1% of the absolute response of the immobilized surface. The two measurements indicate that the epoetin alfa-immobilized surface retains consistent immunological binding using the positive control and maintains a stable baseline throughout 245 sample and regeneration cycles. Based on these data, a maximum number of 245 sample and regeneration cycles can be run per epoetin alfa-immobilized surface.

Results of the darbepoetin alfa-immobilized surface are shown in Fig. 1b. The low positive control binding to the immobilized darbepoetin alfa was recorded over 245 cycles, and had a mean sample binding of 14.8 RU (S.D. ± 4.5 RU) and a %CV of 30.2. Since the variability (%CV) was greater than 20%, it was recalculated to determine the maximum number of cycles that resulted in <20% CV. The positive control binding to darbepoetin alfa up to 97 cycles resulted in a mean sample binding of 19.3 RU (S.D. ± 2.6 RU) and a %CV of 13.3. The negative control binding remained below the threshold of 9.4 RU throughout the 245 cycles. The baseline stability of the immobilized darbepoetin alfa was derived by recording the absolute response throughout the 245 cycles. From the beginning of the 245-cycle run (23,996 RU) to the end of the run (24,203 RU), there was a mass accumulation of 207 RU on the darbepoetin alfa surface, or an overall change of 7.4%. The darbepoetin alfa-immobilized surface maintains consistent immunological binding of the positive control only up to 97 cycles; therefore, 97 cycles is the maximum number of sample and regeneration cycles that can be run for each darbepoetin alfa-immobilized surface.

3.2.3. Assay cut point (threshold)

The assay cut point or threshold is a statistically derived value of NSB. A response at or above the threshold value defines the sample as “reactive.” A sample-binding response (in RU) below the threshold value is reported “negative.” A threshold value must

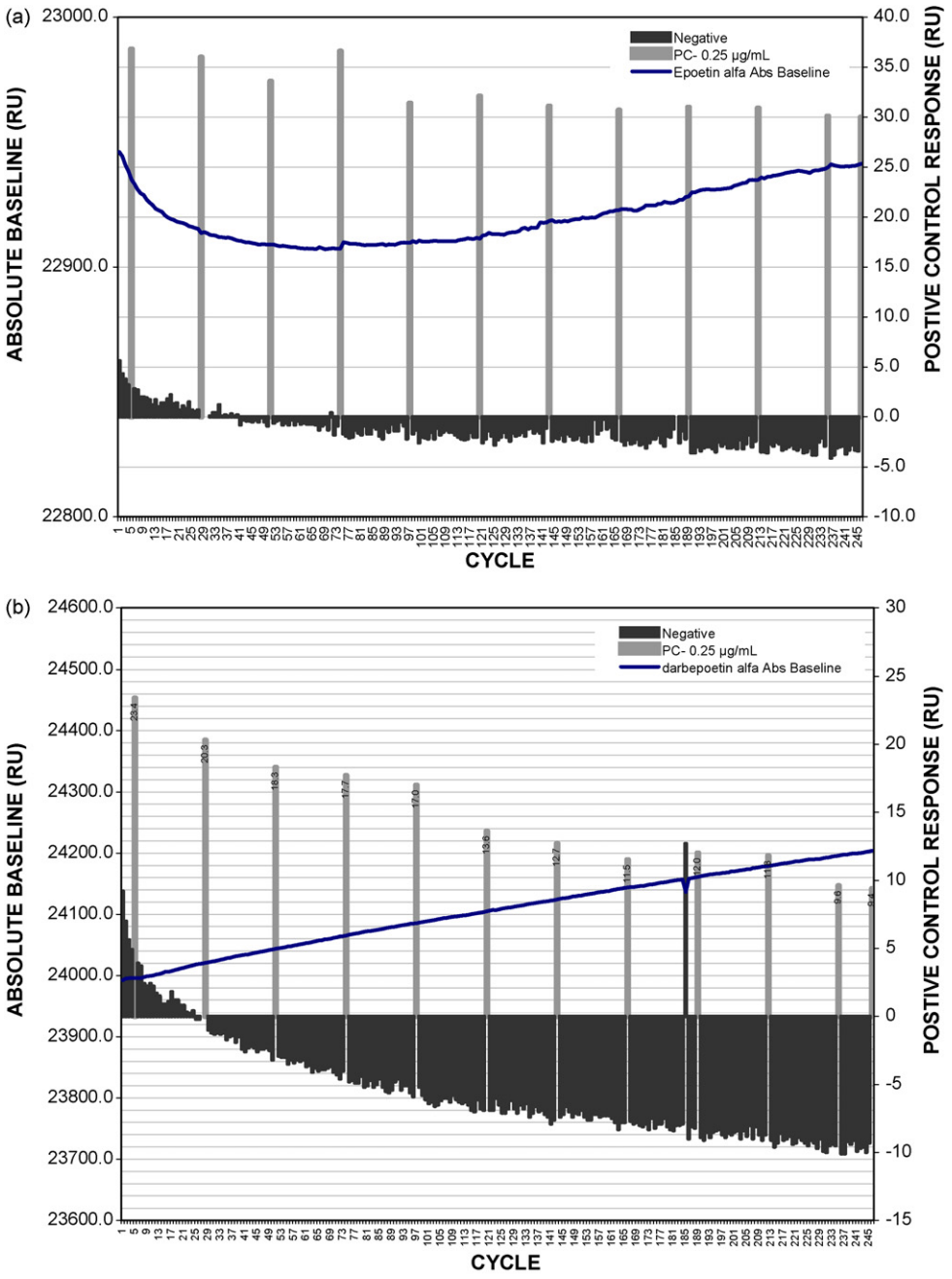

Fig. 1. Baseline stability and reactivity of epoetin alfa (a) and darbepoetin alfa (b). The absolute response (in RU), indicated by the blue line, was recorded over 219 samples and regeneration cycles to monitor the baseline stability of the immobilized epoetin alfa. In addition, the immunological reactivity of the immobilized epoetin alfa was monitored over 219 cycles by recording the binding of the negative and positive control. Negative and positive control binding is indicated by the bar graph.

be established for each immobilized drug surface. Therefore, the binding of 100 serum samples was analyzed and the sample binding to epoetin alfa and darbepoetin alfa was recorded. The mean sample-binding response of the 100 donor samples plus 2 standard deviations (S.D.) was then calculated. Sample-binding values greater than the mean + 3S.D. were considered outliers and removed from the threshold calculation.

Eighty-eight human serum samples were used to calculate the threshold value for detection of antibodies to epoetin alfa. As shown in Fig. 2a, the threshold value for identifying a sample as "reactive" for antibodies to epoetin alfa is 7.9 RU (note that the sample is "positive" only when bound analyte are confirmed to be antibodies). The sample-binding range was −4.6 RU to 20.1 RU. The mean sample binding was 0.1 RU (S.D. ± 3.9 RU).

Eighty-eight human serum donors were used to calculate the threshold value for detection of antibodies to darbepoetin alfa. As shown in Fig. 2b, the threshold value for identifying a sample "reactive" for antibodies to darbepoetin alfa is 9.4 RU. The sample-binding range was −5.9 RU to 21.5 RU. The mean sample binding was −0.2 RU (S.D. ± 4.8 RU).

3.2.4. Sensitivity and LOD

The LOD of the assay is the lowest concentration of antibody that can be reproducibly detected above the assay threshold. The rabbit anti-epoetin alfa antibody was spiked in human serum and serially diluted 2-fold from 10.0 μg/ml to 0.039 μg/ml. Samples were analyzed in triplicate and the mean sample binding (in RU) against the epoetin alfa or darbepoetin alfa surface was plotted against the

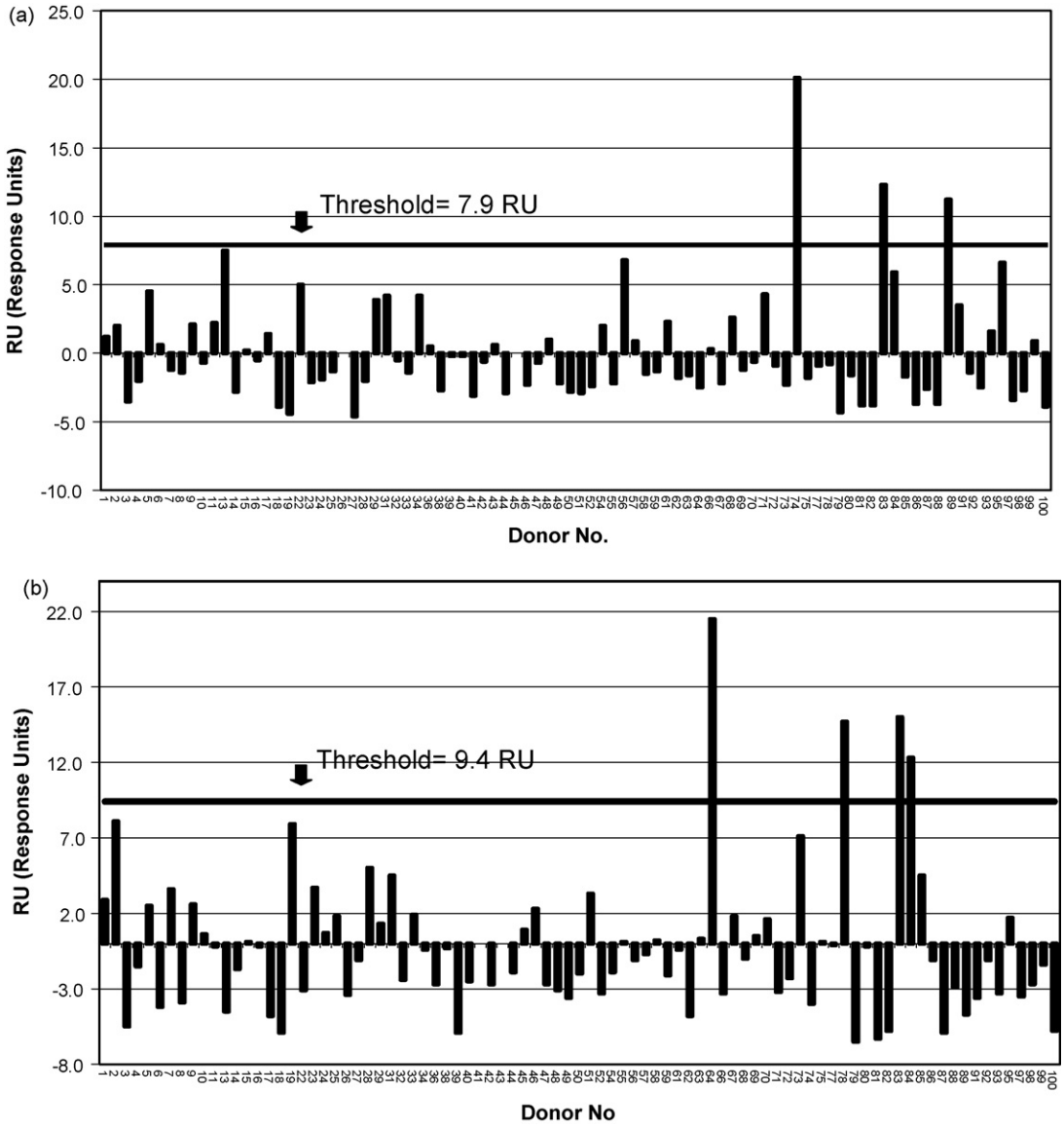

Fig. 2. Cut point (threshold) of epoetin alfa (a) and darbepoetin alfa (b). Human serum samples from 100 individual human donors were analyzed for binding to immobilized epoetin alfa (a) and darbepoetin alfa (b). The mean sample binding (in RU) plus 2S.D. was used to calculate the threshold of the epoetin alfa surface.

antibody concentration (Fig. 3). The assay range was linear from 10.0 μg/ml to 0.078 μg/ml based on a back-calculated value for each mean concentration falling within 80–120% recovery (see Table 3) and <20% CV (see Table 4). The assay was sensitive enough to detect 0.078 μg/ml antibodies above the respective threshold values for epoetin alfa and darbepoetin alfa.

To validate the LOD of the assay against epoetin alfa and darbepoetin alfa, 20 individual human donor serum samples were each spiked with anti-epoetin alfa antibody at a final concentration of 80 ng/ml in neat serum. Fig. 4a shows that all 20 samples recovered above the threshold value of 7.9 RU against epoetin alfa. This demonstrates that the epoetin alfa surface can detect as low as 80 ng/ml. However, only 80% of the samples spiked at 80 ng/ml recovered above the threshold value of 9.4 RU on the darbepoetin alfa surface. Consequently, the antibody concentration spiked into the donor samples was increased to 100 ng/ml and re-analyzed. Fig. 4b shows that all spiked samples recovered above the threshold value against both epoetin alfa and darbepoetin alfa. Therefore, the validated LOD for detection of antibodies against epoetin alfa and darbepoetin alfa was 80 ng/ml and 100 ng/ml, respectively.

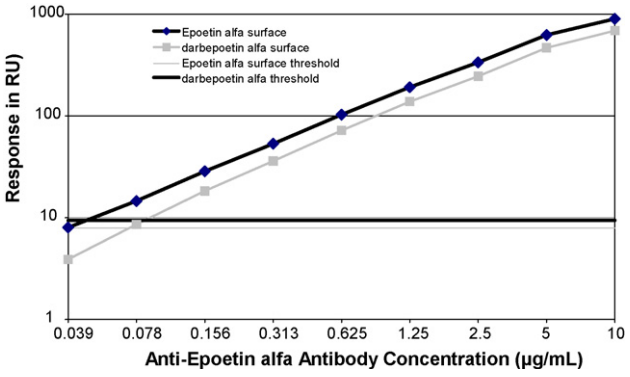

Fig. 3. Sensitivity. Affinity-purified rabbit anti-epoetin alfa antibody was serially diluted from 10.0 μg/ml to 0.039 μg/ml. Each relative antibody concentration was determined in triplicate. The mean sample binding (in RU) is plotted for each antibody concentration (in μg/ml) against the epoetin alfa surface (black line) and darbepoetin alfa surface (gray line). The data points were then modeled using a quadratic fit.

Table 2
Drug interference. Soluble epoetin alfa (1 ng/ml and 5 ng/ml) was spiked into human serum sample containing various concentrations of rabbit anti-epoetin alfa antibody. Samples were then analyzed and the binding (in RU) to epoetin alfa and darbepoetin alfa surfaces were reported. NC = negative control; PC = positive control; EPO = epoetin alfa.

Epoetin alfa surface		Darbepoetin alfa surface	
Sample ID	RU	Sample ID	RU
Start up	2.7	Start Up	8.6
Start up	3.7	Start Up	9.3
NC	−1.6	NC	2.9
PC- 100 ng/mL	18.4	PC- 100 ng/mL	22.6
PC- 150 ng/mL	25.7	PC- 150 ng/mL	29.7
PC- 200 ng/mL	34.3	PC- 200 ng/mL	36.5
PC- 250 ng/mL	41.9	PC- 250 ng/mL	43.4
PC- 100 ng/mL + 5 ng/mL EPO	17.4	PC- 100 ng/mL + 5 ng/mL EPO	24.2
PC- 150 ng/mL + 5 ng/mL EPO	19.6	PC- 150 ng/mL + 5 ng/mL EPO	23.3
PC- 200 ng/mL + 5 ng/mL EPO	26.4	PC- 200 ng/mL + 5 ng/mL EPO	30.0
PC- 250 ng/mL + 5 ng/mL EPO	35.1	PC- 250 ng/mL + 5 ng/mL EPO	38.2
PC- 100 ng/mL + 1 ng/mL EPO	16.0	PC- 100 ng/mL + 1 ng/mL EPO	26.0
PC- 150 ng/mL + 1 ng/mL EPO	23.0	PC- 150 ng/mL + 1 ng/mL EPO	33.7
PC- 200 ng/mL + 1 ng/mL EPO	31.5	PC- 200 ng/mL + 1 ng/mL EPO	39.5
PC- 250 ng/mL + 1 ng/mL EPO	39.5	PC- 250 ng/mL + 1 ng/mL EPO	44.7
NC	7.5	NC	6.1

3.2.5. Specificity

The assay specificity was evaluated for its ability to detect only anti-epoetin alfa and darbepoetin alfa in human serum. It is recognized that the protein constituents in human serum can vary within a patient population and interfere with the binding of specific antibodies. The LOD was established using serum samples from 20 individual donors (see section above). This provided evidence that variability in serum components do not interfere with detection of a true antibody positive. To further confirm the lack of an effect of serum components on antibody detection, 7 affinity-purified polyclonal antibodies against human proteins found endogenously in human serum were spiked 100-fold above the assay LOD; all tested negative (data not shown).

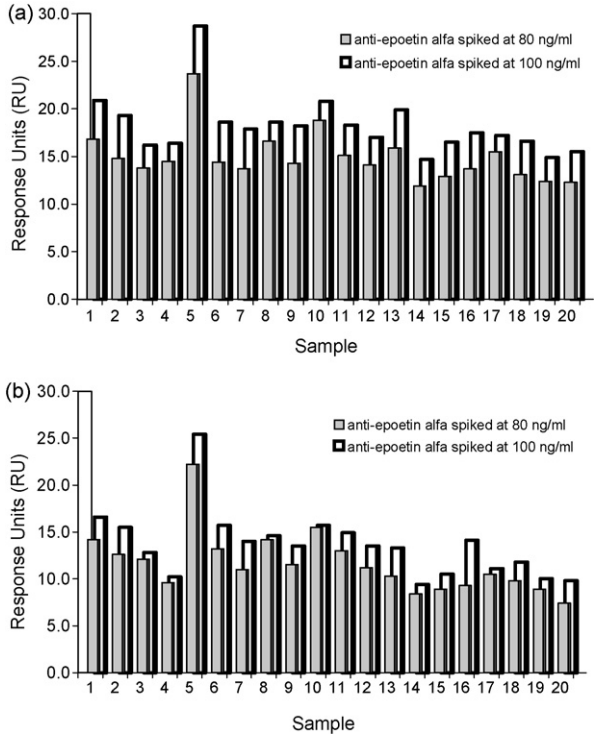

Fig. 4. Limit of detection (LOD) for the epoetin alfa surface (a) and the darbepoetin alfa surface (b). Twenty individual human serum samples were spiked with 80 ng/ml or 100 ng/ml of rabbit anti-epoetin alfa antibody. Spiked samples were run in duplicate and mean sample binding (in RU) was plotted against sample ID. The sample binding to the epoetin alfa surface (a) and the darbepoetin alfa surface (b) are presented.

3.2.6. Drug interference

The addition of soluble drug can inhibit the binding of specific anti-epoetin alfa and darbepoetin alfa antibodies. In separate experiments, epoetin alfa and darbepoetin alfa were added in 10-fold excess to a sample containing 10 μg/ml of rabbit polyclonal antibody. Sample binding (in RU) to both immobilized drug surfaces was measured. A 10-fold excess of soluble drug inhibits the binding of 10 μg/ml of rabbit anti-epoetin alfa antibody in human serum to immobilized epoetin alfa and darbepoetin alfa (data not shown).

To determine if trough levels (1 ng/ml and 5 ng/ml) of epoetin alfa and darbepoetin alfa can interfere with the detection of antibody at the LOD of the assay, rabbit anti-epoetin alfa antibody was spiked at 100 ng/ml, 150 ng/ml, 200 ng/ml, and 250 ng/ml into serum samples. Epoetin alfa and darbepoetin alfa were added to the antibody samples to a final concentration of 5 ng/ml, and sample binding was then measured. Table 2 shows that all four antibody concentrations spiked with 5 ng/ml of epoetin alfa or darbepoetin alfa tested were above the respective assay threshold.

3.2.7. Accuracy of the dose-dependent response

Accuracy represents the closeness of a test result to the theoretical value or concentration. A weighted quadratic function provided the best fit for the response versus antibody concentration in providing the most accurate and precise back-calculated (predicted) concentrations for the standards and test samples. The true range of reliable responses for the epoetin alfa and darbepoetin alfa surfaces was 0.078–10 μg/ml. Table 3a and b contain triplicate determinations of the eight back-calculated antibody concentrations. The mean and the percent recovery were calculated for each concentration. The predicted concentrations for each antibody concentration in the range from 0.078 μg/ml to 10 μg/ml were well within ±20% of nominal back-calculated value for anti-epoetin alfa antibody concentrations.

3.2.8. Precision

Precision, expressed in %CV, measures the closeness of replicate determinations. Both the inter-assay precision (the variation of replicates between assays) and intra-assay precision (the variation of replicates within an assay) were evaluated and results shown in Table 4a and b. Intra-assay precision was determined by assaying, in triplicate, various concentrations (ranging from 0.078 μg/ml to 10 μg/ml) of positive control antibody in 50% serum. This analysis

Table 3
Accuracy. Rabbit anti-epoetin alfa antibody was spiked into human serum at 10 μg/ml and 2-fold serially diluted to 0.078 μg/ml. Each concentration was analyzed in triplicate for binding to the epoetin alfa (a) and the darbepoetin alfa (b) surfaces. The mean antibody concentration and % recovery of the back-calculated values relative to the theoretical concentration are shown.

(a)

Target	0.078 μg/ml	0.156 μg/ml	0.313 μg/ml	0.625 μg/ml	1.250 μg/ml	2.500 μg/ml	5.000 μg/ml	10.000 μg/ml
Day	0.076	0.157	0.322	0.635	1.250	2.608	4.903	10.260
1	0.077	0.156	0.313	0.639	1.249	2.546	4.873	10.048
	0.079	0.157	0.309	0.634	1.249	2.530	4.808	9.947
Day	0.075	0.162	0.322	0.642	1.280	2.474	4.904	10.166
2	0.078	0.158	0.320	0.635	1.274	2.466	4.933	10.213
	0.079	0.161	0.319	0.635	1.264	2.498	4.959	9.796
Day	–	0.207	0.304	0.596	–	2.491	5.009	10.029
3	0.068	0.149	0.300	0.621	1.104	2.692	5.013	10.027
	0.068	0.140	0.433	0.623	1.203	2.561	5.019	9.882
Mean	0.075	0.161	0.327	0.629	1.234	2.541	4.936	10.041
% Recovery	96.0	102.8	104.6	104.6	98.7	104.6	98.7	100.4

(b)

Target	0.078 μg/ml	0.156 μg/ml	0.313 μg/ml	0.625 μg/ml	1.250 μg/ml	2.500 μg/ml	5.000 μg/ml	10.000 μg/ml
Day	0.073	0.145	0.297	0.651	1.312	2.642	4.858	10.748
1	0.074	0.148	0.310	0.643	1.304	2.505	4.804	9.804
	0.074	0.150	0.270	0.638	1.308	2.482	4.847	9.710
Day	0.080	0.162	0.320	0.629	1.265	2.512	4.972	10.045
2	0.080	0.158	0.314	0.626	1.265	2.467	5.008	10.097
	0.077	0.161	0.317	0.623	1.244	2.473	4.996	9.885
Day	0.079	0.150	0.304	0.594	1.260	2.511	5.054	9.996
3	0.075	0.153	0.294	0.610	1.255	2.517	5.042	9.993
	0.074	0.152	0.293	0.617	1.246	2.516	5.042	9.921
Mean	0.076	0.153	0.302	0.626	1.273	2.514	4.958	9.990
% Recovery	97.3	98.1	96.6	100.1	101.9	100.6	99.2	99.9

was conducted over 3 runs to measure the inter-assay precision. The mean value was used to calculate inter-assay precision. The intra-assay precision data shows that the %CV is below 20 at concentrations greater than the respective LOD of the dual-flow cell assay. The %CV ranged from 0.0 to 19.1 against epoetin alfa, and 0.1–7.6 against darbepoetin alfa. The inter-assay precision of the anti-epoetin alfa assay and the anti-darbepoetin alfa assay is also summarized. Again, the variability was <20% CV for the antibody control with concentrations greater than the LOD. The %CV ranged from 4.0 to 16.5 against epoetin alfa and 2.9–11.2 against darbepoetin alfa.

3.2.9. Stability of anti-EPO Abs in human serum

The freeze–thaw stability of anti-epoetin alfa antibody in human serum was assessed. The rabbit anti-epoetin alfa antibody was spiked into neat human serum, aliquoted into 5 tubes, and stored frozen at −20 °C. An individual aliquot was then subjected to 1, 2, 3, 4, or 5 freeze–thaw cycles. Each sample was then analyzed and the sample binding was compared to a sample that was frozen and thawed once. The antibody spiked into human serum can withstand up to 5 freeze and thaw cycles without any significant loss in immunological reactivity to epoetin alfa or darbepoetin alfa (data not shown).

3.2.10. Detection of human antibody isotype

To provide direct evidence that this immunoassay can detect all antibody isotypes (IgG, IgM, IgE and IgA), clinical samples were collected at 1 or more time point from subjects which previously tested to be positive for binding, non-neutralizing antibodies. Samples were then subsequently isotyped with anti-human IgG, IgM, IgA, and IgE antibodies. The sample binding (in RU) and confirmatory binding (in RU) were reported for each sample time point tested. In Table 5, at least 3 or more subjects had a sample time point that confirmed to be of the IgG, IgM, or IgA antibody class as indicated in bold. One subject (subject 1) was confirmed to be of the IgE class antibody.

3.2.11. Antibody dissociation

The real-time binding of serum antibody to the immobilized surface allows for the direct observation and acquisition of data regarding the association and dissociation of bound antibodies, particularly rapidly dissociating antibodies. A select number of clinical samples confirmed to be positive for binding, non-neutralizing antibodies and demonstrated to have a rapid dissociation of bound antibody are presented. Three clinical samples with an antibody concentration <1 μg/ml (donors 4–6) and three samples with an antibody concentration >1 μg/ml (donors 1–3) were compared to a high-affinity rabbit anti-epoetin alfa antibody. A measure of the relative dissociation of the bound sample over a 40-min time period is presented in Table 6 along with the sample RU binding before and after 40 min. All antibody-positive samples had >68% loss in binding after 40 min. All 6 samples had binding well above the assay threshold and demonstrated binding above the 80 ng/ml spike. The high-affinity rabbit anti-epoetin alfa antibody demonstrated little dissociation regardless of concentration: the 250 ng/ml sample demonstrated 14.1% dissociation, and the 10 μg/ml sample demonstrated had no detectable dissociation.

4. Discussion

Biopharmaceutical companies in general, including companies that have developed ESAs, have relied on traditional immunochemical techniques such as radioimmunoprecipitation (RIP or RIPA), enzyme immunoassay (EIA) or the ELISA for the detection of antibodies to their erythropoietic protein therapeutics. Here we present validation data to support the use of an SPR-based biosen-

Table 4
Intra-assay and inter-assay precision. Rabbit anti-epoetin alfa antibody was spiked into human serum at 10 μg/ml and 2-fold serially diluted to 0.078 μg/ml. Each concentration was analyzed in triplicate for binding to the epoetin alfa and the darbepoetin alfa surfaces over 3 days. The mean sample binding (in RU), S.D., and %CV was calculated for binding to epoetin alfa (a) and to the darbepoetin alfa surface (b).

(a)

Target	0.078 μg/ml	0.156 μg/ml	0.313 μg/ml	0.625 μg/ml	1.250 μg/ml	2.500 μg/ml	5.000 μg/ml	10.000 μg/ml
Intra-assay precision								
Day 1	8.2	18.4	39.0	77.6	151.4	304.7	533.8	921.6
	8.3	18.2	37.9	78.0	151.3	298.0	531.0	910.2
	8.5	18.3	37.34	77.4	151.3	296.2	525.1	904.6
Mean	8.3	18.3	38.1	77.7	151.3	299.6	530.0	912.1
S.D.	0.2	0.1	0.8	0.3	0.1	4.5	4.4	8.7
%CV	1.8	0.5	2.1	0.4	0.0	1.5	0.8	0.9
Day 2	9.6	19.1	36.4	70.6	137.0	254.7	467.8	808.1
	9.9	18.6	36.2	69.9	136.4	253.9	470.1	810.4
	10.0	18.9	36.1	69.9	135.4	257.0	472.2	789.6
Mean	9.8	18.9	36.2	70.1	136.3	255.2	470.0	802.7
S.D.	0.2	0.3	0.2	0.4	0.8	1.6	2.2	11.4
%CV	2.1	1.3	0.4	0.6	0.6	0.6	0.5	1.4
Day 3	–	25.5	35.1	63.9	–	242.0	455.7	803.5
	11.6	19.7	34.7	66.3	113.1	260.0	456.0	803.4
	11.6	18.8	47.9	66.5	122.6	248.3	45.5	794.8
Mean	11.6	21.3	39.2	65.6	117.9	250.1	456.1	800.6
S.D.	0.0	3.6	7.5	1.4	6.7	9.1	0.4	5.0
%CV	0.0	17.0	19.1	2.2	5.7	3.7	0.1	0.6
Inter-assay precision								
Day 1	8.3	18.3	38.1	77.7	151.3	299.6	530.0	912.1
Day 2	9.8	18.9	36.2	70.1	136.3	255.2	470.0	802.7
Day 3	11.6	21.3	39.2	65.6	117.9	250.1	456.1	8006
Mean	9.9	19.5	37.9	71.1	135.2	268.3	485.4	838.5
S.D.	1.6	1.6	1.5	6.1	16.8	27.2	39.3	63.8
%CV	16.5	8.3	4.0	8.6	12.4	10.2	8.1	7.6

(b)

Target	0.078 μg/ml	0.156 μg/ml	0.313 μg/ml	0.625 μg/ml	1.250 μg/ml	2.500 μg/ml	5.000 μg/ml	10.000 μg/ml
Intra-assay precision								
Day 1	5.8	13.7	30.2	68.2	137.8	272.3	480.1	932.7
	5.9	14.0	31.6	67.3	137.0	258.8	475.3	869.9
	6.0	14.2	27.2	66.8	137.4	256.6	479.1	863.5
Mean	5.9	14.0	29.7	67.4	137.4	262.6	478.2	888.7
S.D.	0.1	0.3	2.2	0.7	0.4	8.5	2.5	38.2
%CV	1.7	1.8	7.6	1.1	0.3	3.2	0.5	4.3
Day 2	7.2	14.8	29.4	57.7	114.8	223.0	421.4	767.4
	7.2	14.5	28.9	57.4	114.8	219.2	424.2	770.5
	7.0	14.7	29.1	57.1	113.0	219.7	423.3	757.8
Mean	7.1	14.7	29.1	57.4	114.2	220.6	423.0	765.2
S.D.	0.1	0.2	0.3	0.3	1.0	2.1	1.4	6.6
%CV	1.6	1.0	0.9	0.5	0.9	0.9	0.3	0.9
Day 3	7.2	13.8	28.0	54.7	115.2	226.1	439.9	811.4
	6.8	14.1	27.1	56.2	114.8	226.6	438.9	811.2
	6.7	14.0	27.0	56.8	114.0	226.5	438.9	806.2
Mean	6.9	14.0	27.4	55.9	114.7	226.4	439.2	809.6
S.D.	0.3	0.2	0.6	1.1	0.6	0.3	0.6	2.9
%CV	3.8	1.1	2.0	1.9	0.5	0.1	0.1	0.4
Inter-assay precision								
Day 1	5.9	14.0	29.7	67.4	137.4	262.6	478.2	888.7
Day 2	7.1	14.7	29.1	57.4	114.2	220.6	423.0	765.2
Day 3	7.2	13.8	28.0	54.7	115.2	226.1	439.9	811.4
Mean	6.7	14.1	28.9	59.8	122.3	236.4	447.0	821.8
S.D.	0.7	0.5	0.9	6.7	13.1	22.8	28.3	62.4
%CV	10.9	3.3	2.9	11.2	10.7	9.6	6.3	7.6

sor immunoassay as an alternative method. The SPR-based method meets the requirements achieved by the traditional immunoassays and affords a number of advantages in the detection and characterization of anti-ESA antibodies.

We present data to support the use of this SPR-based biosensor immunoassay platform for the intended purpose of detection, semi-quantitation, and full characterization of antibodies to both epoetin alfa and darbepoetin alfa. The selection of this biosensor

Table 5
Detection of anti-epoetin alfa antibody isotypes. Isotype analysis was performed on serum samples, positive for binding antibodies in the SPR-based immunoassay, taken from 12 patients administered an ESA. The sample binding and the confirmatory binding for each anti-human immunoglobulin reagent to each bound sample are summarized. Samples confirmed positive for a particular antibody isotype are in bold.

Subject	Time point	Sample response (RU)	IgE confirmatory response (RU)	Sample response (RU)	IgA confirmatory response (RU)	Sample response (RU)	IgM confirmatory response (RU)	Sample response (RU)	IgG confirmatory response (RU)
1	A	**150.0**	**106.3**	162.5	<100	162.9	<100	128.3	<100
2	A	212.2	<100	**212.6**	**135.1**	212.9	<100	213.0	<100
2	B	196.9	<100	**202.5**	**129.9**	199.3	<100	204.0	<100
3	A	216.8	<100	**238.8**	**118.4**	235.9	<100	236.0	<100
4	A	473.3	<100	**418.1**	**103.9**	416.4	<100	**415.1**	**326.9**
5	A	191.0	<100	197.2	<100	**192.6**	**140.3**	190.1	<100
5	B	170.6	<100	170.3	<100	**168.6**	**109.0**	162.9	<100
6	A	301.7	<100	301.1	<100	**299.3**	**206.5**	297.6	<100
7	A	552.3	<100	539.8	<100	**515.7**	**405.2**	555.9	<100
8	A	1575.7	<100	1601.9	<100	**1608.3**	**900.5**	1599.2	<100
9	A	156.5	<100	155.3	<100	154.8	<100	**153.6**	**166.5**
9	B	158.1	<100	160.1	<100	160.0	<100	**159.8**	**170.4**
10	A	930.9	<100	935.2	<100	933.5	<100	**933.5**	**985.7**
11	A	1470.4	<100	1438.1	<100	1462.5	<100	**1457.8**	**1602.9**
12	A	727.2	<100	724.7	<100	722.5	<100	**718.0**	**664.4**

immunoassay platform was driven by 3 main factors: the ability to use the dual-flow cell capability to simultaneously detect and confirm the presence and relative concentration of human antibodies to both darbepoetin alfa and epoetin alfa from a single sample analysis, the ability to readily determine antibody isotype using the validated screening assay in combination with qualified commercial reagents, and real-time monitoring to enable the detection of antibodies that rapidly dissociate. Therefore, a more detailed understanding about the antibody response to our ESAs can be obtained with minimal sample volume consumption and within the same day of initially detecting a antibody-positive sample.

Although the use of SPR-based technology is a unique analytical approach for the routine measurement of anti-ESAs in human serum, assay development and validation proved to be very similar to other, more traditional, immunoassays such as an ELISA. As stated previously, the initial development and validation of a single-flow cell, SPR-based immunoassay was previously described [9], with a detection limit of 400 ng/ml of serum antibody to darbepoetin alfa. This single-flow cell assay was extended to a dual-flow cell assay to test a single sample for antibodies with the capacity to bind both darbepoetin alfa and epoetin alfa. Although regulatory authorities generally accept this sensitivity in human serum, efforts to improve the assay sensitivity were initiated.

A general approach to increasing immunoassay sensitivity is to improve the signal-to-noise ratio. The SPR-based immunoassays are no exception to this approach. Increasing the salt concentration from 150 mM to 300 mM reduced the NSB in human serum samples, as did the pH change from 7.4 to 9.0. The shift in pH from 7.4 to 9.0 provided a more favorable net charge on the epoetin alfa and darbepoetin alfa-immobilized surfaces, resulting in consistently low NSB between individual samples. The combined addition of both reagents to the sample and running buffer significantly dropped the NSB while having little effect on the specific binding. These changes, as well as increasing the assay flow rate from 5 μl/min to 10 μl/min, significantly improved the overall performance of the assay.

In general, darbepoetin alfa is less immunologically reactive than epoetin alfa largely due to its 2 additional sugar moieties. By reducing the concentration of periodate from 10 mM to 1 mM, and the hydrazine concentration 10-fold to 5 mM, the immunological reactivity of the immobilized darbepoetin alfa was improved. The sample injection of 5 μl, 10 μl, and 20 μl at a 5 μl/min flow rate had little improvement between the 1-min, 2-min, and 4-min sample contact times, respectively. Two flow rates, 5 μl/min and 10 μl/min, were compared. The signal-to-noise ratio was optimal at 10 μl/min (data not shown). Finally, the quality of the positive control antibody preparation was critical to establishing the LOD. During assay development, various in-house and commercial monoclonal and polyclonal antibodies were tested (data not shown). The antibody preparation that demonstrated the highest binding at the lowest concentration was chosen (affinity-purified, rabbit anti-epoetin alfa polyclonal antibody) and used to generate the validation data.

As with all traditional immunoassays, the same validation parameters were also evaluated in this biosensor immunoassay. Rigorous stability testing was performed since the prepared drug surface is re-used to analyze multiple samples. The appropriate immobilization chemistries were determined for the attachment of epoetin alfa and darbepoetin alfa to the sensor chip, and we demonstrated that the immobilization of both drugs is reproducible (see Table 1). The first key validation hurdle was to demonstrate that both proteins maintain consistent immunological reactivity

Table 6
Detection of anti-epoetin alfa antibody-positive samples with a relatively fast dissociation. Clinical samples from 6 patients scored anti-epoetin alfa antibody positive in the SPR-based immunoassay were re-tested to determine the dissociation of antibodies. Samples were allowed to bind epoetin alfa and the amount bound (initial binding) was recorded. The sample flow rate was increased to 30 μl/min and the amount bound (in RU) after 40 min was recorded (final binding). The percent loss of antibody bound after 40 min was calculated (100 − (final binding/initial binding) × 100). Samples with final binding less than 20.8 RU (final binding of 80 ng/ml control after 40 min) are considered negative.

Samples	Anti-epoetin alfa antibodies (μg/ml)	Initial binding (in RU)	Final binding (in RU)	%Total RU loss
Donor 1	4.4	686.9	124.5	81.6
Donor 2	2.5	271.1	80.4	70.3
Donor 3	1.5	277.4	87.5	68.5
Donor 4	0.3	164.8	19.2	88.3
Donor 5	0.8	157.8	19.0	88.0
Donor 6	0.4	189.4	22.2	88.3
Low positive control	0.3	74.2	76.5	No detectable dissociation
High positive control	10.0	2598.3	2230.8	14.1

and stability over a number of sample binding and regeneration cycles. An acidic solution containing detergent was selected to efficiently dissociate antibodies from the surfaces. The epoetin alfa surface demonstrated consistent immunological reactivity through 245 cycles, while the darbepoetin alfa surface demonstrated consistent immunological reactivity through 97 cycles (see Fig. 1a and b); thus, the maximum number of cycles that can be run in this dual-flow cell assay is 97 cycles.

It is recognized that the analysis of 97 sample and regeneration cycles is somewhat restrictive, resulting in only about 80 samples analyzed per surface. Therefore, after some investigation, we determined that the accumulation beyond the 97 cycles on the darbepoetin alfa surface (see Fig. 1a) was due to the incomplete removal of serum components from the surface (accumulation), resulting in an unstable baseline. The result of this accumulation was an associated baseline drift downward with each cycle due to the dissociation of the bound serum components. Since the baseline was drifting downward, it resulted in a net negative RU for samples with little to no binding, such as the negative control, to the darbepoetin alfa surface. As a result of this observation, we determined that including an additional clean-up and wash step immediately after each regeneration injection helped to stabilize the baseline. A laboratory that cross-validated this assay included the new wash step and was able to achieve 217 sample and regeneration cycles on the darbepoetin alfa surface (data not shown).

A threshold value to discriminate a "reactive" from a "negative" antibody sample was determined. The NSB of 100 human donor serum samples to both epoetin alfa and darbepoetin alfa was measured. The validated threshold value was calculated to be 7.9 RU and 9.4 RU, respectively (see Fig. 2a and b). Using this threshold, the assay was determined to have an LOD of 80 ng/ml and 100 ng/ml of epoetin alfa and darbepoetin alfa antibodies in neat human serum, respectively (see Fig. 4a and b). This sensitivity is noteworthy, given this platform traditionally generates sensitivity near the 1 μg/ml range or greater using direct binding and dispels any notion that the Biacore 3000 platform is not sensitive enough in basic qualitative binding immunoassays.

Each sample that demonstrates binding (in RU) above the threshold is confirmed to be a human antibody by comparing the confirmatory binding of the sample to the negative control. The spiking experiment to assess the LOD of the assay presented in Fig. 4a and b provides evidence that antibodies can be detected in a variety of individual serum samples. Finally, the specificity of binding using a purified anti-epoetin alfa antibody was demonstrated by the addition of a 10-fold excess of soluble epoetin alfa and darbepoetin alfa in the sample. It was also demonstrated that trough levels of epoetin alfa and darbepoetin alfa ($\leq$5 ng/ml) do not interfere with the detection of antibodies at the assay LOD (see Table 2). Therefore, the SPR-based immunoassay demonstrates extremely high specificity.

The precision and accuracy data obtained with this biosensor immunoassay was typical of semi-quantitative immunoassays. Both the intra- and inter-assay precision was less than 20% CV (Table 4a and b) and the accuracy was within 95–120% recovery of the target concentration for all eight relative antibody concentrations tested (see Table 3a and b). Using the rabbit anti-epoetin alfa antibody spiked in human serum, consistent antibody binding to both epoetin alfa and darbepoetin alfa was measured when the serum sample was frozen and thawed up to 5 times.

A main reason for selecting the SPR-based biosensor immunoassay for the detection of binding antibodies was the ability to detect varying concentrations of antibodies with different off rates and isotypes. To provide evidence that this direct-binding immunoassay can indeed detect all 4 major antibody isotypes, binding data from clinical samples identified to have one of the 4 antibody isotypes is summarized in Table 5. As one would expect, the majority of antibody-positive samples are of the IgG and IgM type; four IgG and IgM-positive subjects, one IgE-positive subject, and three IgA antibody-positive subjects. The antibody isotypes of antibody-positive samples were readily determined using the same validated drug surface in combination with qualified commercial isotype reagents.

The identification of the antibody isotype is important because it distinguishes an early from a late, mature antibody response. It is recognized that patients confirmed to have antibody-mediated PRCA have antibodies of the IgG class, specifically of the IgG1 and IgG4 subclass [12]. It is hoped that with the use of methods such as this SPR-based biosensor immunoassay, that can detect all antibody types, we will be able to detect an early IgM antibody response if it is present. Since the RIP [13] detects antibody complexes with radiolabeled epoetin alfa tracer using Protein G beads, this method may not be able to detect an early IgM response or an IgE and IgA antibody response if present. It is possible that the bridging ELISA [6] can detect all 4 antibody isotypes, but specific isotype information on such samples that scored antibody positive have not been published.

Finally, the real-time detection of antibody binding using this SPR-based biosensor immunoassay can detect antibody populations in serum that bind but rapidly dissociate. Antibody-positive samples to epoetin alfa with relative antibody concentrations either in excess of 1 μg/ml or below 1 μg/ml, and that demonstrates fast dissociation from immobilized epoetin alfa, are presented in Table 6. These populations of anti-ESA antibodies would generally be difficult to detect with the ELISA [6] and the RIP [13] since both capture the end-point measurements after multiple incubation and wash steps. As almost 90% of the initial antibody binding from samples containing <1 μg/ml, and 70–80% of bound antibody from samples containing >1 μg/ml had dissociated using the biosensor method, the ability of the above described ELISA and RIP to detect this antibody population would be highly dependent on the total antibody concentration. Although it is recognized that the clinical significance of these rapidly dissociating antibodies is not known, we routinely detect these antibody populations at both baseline and after ESA administration, and they are of the IgG and IgM antibody isotype.

The previously described ELISA [6] and the RIP [13] methods for detection of anti-ESA antibodies have reported assay sensitivities of 1 ng/ml and 8 ng/ml, respectively. A benefit of having an immunoassay reach the single-digit ng/ml sensitivity is to identify more samples reactive in the immunoassay than would be subsequently tested in the bioassay for neutralizing activity. Based on the clinical data reported by Tacey et al., the prevalence of anti-ESA antibodies by the RIP in the general population is only 0.14% (8/5718). Housel et al. reported testing 1500 serum samples with the ELISA, but the prevalence of antibodies was not indicated. Despite the higher sensitivity of the RIP and ELISA relative to the biosensor immunoassay, a low prevalence of antibodies has been reported using the RIP assay. In contrast, the SPR-based immunoassay has detected up to 4% of all baseline samples positive for antibodies to either epoetin alfa or darbepoetin alfa with no associated signs of PRCA. A publication to describe these results is in preparation.

Based on the data presented here, the higher prevalence of anti-ESA antibodies in the biosensor immunoassay may be explained by its ability to detect all antibody isotypes. The RIP detects antibody complexes with radiolabeled epoetin alfa tracer using Protein G beads. Protein G binds IgG with high affinity but has weak binding to IgM and therefore may not detect it. Although the bridging nature of the ELISA may allow for the detection of all antibody isotypes, data has not been published. The higher prevalence of anti-ESA antibodies detected in the biosensor immunoassay may also be explained by the real-time detection of the SPR-based immunoassay, which can detect antibodies with a moderate-to-fast off rates as well as the

high affinity antibodies. The moderate-to-fast dissociating populations of anti-ESA antibodies would generally be difficult to detect with the ELISA [6] and the RIP [13] since both capture the end-point measurements after multiple incubation and wash steps.

A detailed analysis of antibody-positive samples at various antibody concentrations, isotypes, and relative dissociation rates must be tested and compared between the SPR-based immunoassay, ELISA and RIP to truly establish the limitations of each assay format.

In summary, the SPR-based biosensor immunoassay can simultaneously detect and confirm 100 ng/ml of specific antibodies to darbepoetin alfa and 80 ng/ml of human antibodies to epoetin alfa in human serum. The assay is very specific, detecting only antibodies above a validated threshold value. Both inter-assay and intra-assay precision were well below the typical performance specifications of 20% CV for immunoassays. The presentation of clinical data provides evidence that this immunoassay platform can detect all 4 major human antibody isotypes (IgG, IgM, IgE and IgA). The unique real-time monitoring of the biosensor immunoassay can detect serum antibodies bound to the immobilized ESA that rapidly dissociate from the drug surface. These populations of antibodies are typically not detected by methods that utilize wash steps in combination with end-point detection. Thus, using this SPR-based immunoassay, a very detailed understanding about the antibody response can be obtained. This SPR-based immunoassay has been used successfully to detect and characterize samples from patients with confirmed antibody-mediated PRCA [12]. Moreover, this biosensor immunoassay provided detailed information about the antibody response, such as the antibody isotype and the relative dissociation rate of these antibodies from the drug.

The collective dataset of the antibody responses measured in PRCA patients demonstrate that the predominant antibody isotype is IgG, specifically of the IgG1 and IgG4 subclass, and is generally associated with an excess of 1 μg/ml of antibody. The clinical significance of low-level antibody remains unclear. Since little is known about the early stages of the antibody response in antibody-mediated PRCA, valuable information can be obtained by using sensitive and specific immunological methods such as this biosensor immunoassay that can detect all isotypes and relative affinities of an anti-ESA polyclonal antibody response.

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