Research Article

Development, Validation, and Clinical Implementation of an Assay to Measure Total Antibody Response to Naglazyme® (Galsulfase)

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Abstract. Naglazyme® (galsulfase, rhASB) was developed as enzyme replacement therapy for mucopolysaccharidosis type VI. Naglazyme generated an IgG antibody response in most patients. To better characterize Naglazyme immunogenicity, a solution phase bridged immunoassay was developed to measure total antibody response regardless of isotype. Overnight incubation of serum dilutions with rhASB labeled with biotin and ruthenium-based tags allowed antibody–antigen complexes to form prior to capture on a streptavidin plate. Neat serum was tolerated in the assay, with a 1:10 screening dilution implemented for testing. At this dilution, the assay was sensitive to 75 ng/ml anti-rhASB. Titers were reported as the highest dilution factor with signal above a 95% confidence interval from naïve individual sera. Precise measurement of titers, within two consecutive dilution factors, was observed across analysts and days. Clinical samples showed similar positive/negative results between the IgG ELISA and the total antibody ECLA, although with an imperfect correlation. Improvements in assay performance and implementation strategy altered some positive clinical samples to negative and vice versa. Comparison of the titer readout for clinical samples with the screening signal illustrates a range of relationships for signal *versus* sample dilution factor, confirming that signal from a screening dilution cannot directly predict the reported titer.

KEY WORDS: bridging immunoassay; enzyme replacement therapy; immunogenicity; MPS VI; Naglazyme.

INTRODUCTION

All biopharmaceuticals have a risk of generating an immune response. This immunogenicity has the potential to impact safety and efficacy of the biopharmaceutical. One of the most severe safety events observed due to immunogenicity was the pure red cell aplasia seen in a small number of patients taking erythropoietin (1). Decrease in efficacy due to immunogenicity has been observed in a wide variety of products including interferon beta (2), factor VIII (3), and alglucosidase alfa (4). For most biopharmaceuticals, however, the immune response does not impact safety or efficacy (5). Due to the potential impact, the FDA is requiring immunogenicity evaluation for all biopharmaceutical products. One essential

component of any immunogenicity program is a screening assay to evaluate the presence of antibodies in clinical samples.

The relevant antibody isotypes and affinities to evaluate the impact on safety and efficacy can vary between individual biopharmaceuticals and patients. The goal in developing a screening assay or assays is to detect antibodies in patients across multiple isotypes, particularly IgM and IgG, and to maximize the likelihood of detecting both high and low affinity interactions. An assay that detects all antibodies regardless of isotype can be used to identify positive samples for further characterization, reducing the testing burden in those assays.

Recommendations for screening assay development have been published by a collaboration of industry and regulators (6). This white paper provides guidelines for current industry practices on assay development and discusses many issues that potentially apply across different biopharmaceuticals and is a useful starting point for new immunogenicity assay development. This manuscript describes an approach to develop a screening assay for Naglazyme® (galsulfase, recombinant human arylsulfatase B, rhASB).

Naglazyme has been developed as an enzyme replacement therapy for mucopolysaccharidosis type VI (MPS VI, Maroteaux–Lamy). Due to a lack of *N*-acetylgalactosamine 4-sulfatase (arylsulfatase B), MPS VI patients have lysosomal accumulation of dermatan sulfate that causes a progressive disorder with multiple organ and tissue involvement that

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ABBREVIATIONS: ECLA, electrochemiluminescent assay; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; MPS VI, mucopolysaccharidosis type VI, Maroteaux–Lamy syndrome; rhASB, recombinant arylsulfatase B, recombinant human *N*-acetyl-galactosamine 4-sulfatase, Naglazyme®; RT, room temperature; Ru, MSD Sulfo-TAG; SA, streptavidin; Ab, antibody.

often leads to death from disease-related complications between childhood and early adulthood.

During clinical trials, the majority of patients developed an IgG response against Naglazyme with no impact observed on safety or efficacy (7–9). To expand the immunogenicity evaluation, the FDA required a post-marketing commitment for the development of a new screening assay with increased sensitivity, broader isotype detection, and improved tolerance to free drug. Identifying the presence of a total antibody response is one component of an overall immunogenicity program that also includes identification of neutralizing antibodies and IgE detection.

To detect all antibody isotypes, a bridging immunoassay format was developed for Naglazyme. This format takes advantage of the multivalent interaction between antibodies and the antigen, in this case Naglazyme, by using the biopharmaceutical for both capture and detection and therefore has the potential to detect all antibody isotypes. Bridging assays in an ELISA format, using the biopharmaceutical as the coating reagent and a labeled biopharmaceutical as the detection reagent, have multiple challenges (10). High affinity antibodies may associate strongly with the coating reagent and not dissociate one binding site to concurrently bind the detection reagent, while low affinity antibodies may be lost in the wash steps.

The emergence of the electrochemiluminescence technology (MesoScale Discovery and Bioveris) has allowed development of a solution-phase bridging format that mitigates the difficulties with detecting high and low affinity antibodies (11). By labeling two separate preparations of Naglazyme with either a capture or detection moiety, the binding can be performed in solution phase and allowed to equilibrate prior to capture on a plate or bead. Since the binding occurs concurrently, the high affinity antibodies have equal opportunities to bind to both the capture and detection reagents. The single wash step after capture minimizes the loss of signal from low affinity antibodies.

The bridging ECLA described herein was developed to provide high quality data as the screening component of the immunogenicity program. Increased sensitivity permitted the detection of emerging immune responses. Use of titer as a reported value maintains precision across plates and analysts. The detection of multiple antibody isotypes allows use of this assay as an effective tool to screen samples for further characterization. Patient samples identified as positive can be tested for neutralizing antibodies or specific isotypes as appropriate in the overall strategy, with minimal risk of not further testing patient samples identified as negative. These key improvements increase confidence in the immunogenicity data and subsequent clinical correlations.

MATERIALS AND METHODS

Chemicals and Reagent Preparation

The ECLA was read on a Sector PR 400 Instrument from MesoScale Discovery (Gaithersburg, MD, USA). Plates and reagents for the electrochemiluminescent assay were purchased from MesoScale Discovery: MSD Sulfo-TAG NHS-Ester, Streptavidin Standard plate, 5% Blocker A, 5× phosphate buffer, and 4× Read Buffer T.

Immunosorp high-binding plates for the ELISA method were acquired from Nunc (Rochester, NY, USA). The secondary antibody was horseradish peroxidase-conjugated goat polyclonal IgG antibody to human IgG acquired from Cappel (Solon, OH). 3,3',5,5'-tetramethylbenzidine (TMB) substrate was acquired from BioRad (Hercules, CA, USA).

EZ-Link Sulfo-NHS-LC-Biotin was from Pierce (Rockford, IL, USA). Protein concentrations were measured using a BCA kit and biotin quantitation was performed using a EZ Biotin Quantitation kit, both from Pierce.

Naglazyme® (rhASB) was obtained from BioMarin Pharmaceutical Inc. (Novato, CA, USA).

Individual and pooled naïve human serum was purchased from Binding Site (San Diego, CA, USA) and BioReclamation (Hicksville, NY, USA).

Polyclonal sheep anti-rhASB (G192) was obtained from Covance (Denver, PA, USA) and polyclonal rabbit anti-rhASB (BP15, J8549, J8550) was obtained from Covance and Antibodies Inc. (Davis, CA, USA). In addition, fractions were isolated from pooled positive samples from cynologous monkeys and human. All antibodies were purified using Protein G affinity columns obtained from GE Healthcare (Piscataway, NJ, USA) followed by affinity chromatography with an rhASB column made using a HiTrap NHS-activated HP column from GE Healthcare. Polyclonal rabbit anti-laronidase (BP13) was obtained from Covance, and was purified using Protein G affinity column.

Labeling of Naglazyme®

RhASB was buffer exchanged into 10 mM sodium phosphate, 150 mM NaCl, pH 7.8, and concentrated to 2 mg/ml. The MSD tag (3–10 nmol/μl in water) and biotin (2 mg/ml in water) were added at 1.25 to 12-fold molar excess challenge ratios and incubated with gentle rocking at RT for 1 h prior to quenching with 20–25% (v/v) 2 M glycine. The reactions were buffer exchanged into product formulation buffer, and adjusted to 1 mg/ml as measured by the BCA method. Ru concentration was measured using the absorbance at 455 nm and biotin concentration was measured using the EZ Biotin Quantitation kit. Label ratios were calculated as [label]/[rhASB] and labeling efficiencies were calculated as label ratio/challenge ratio.

Total Antibody ECLA

A solution phase bridging ECLA was used to measure the relative levels of antibodies. Sample dilutions and stocks of Ru-rhASB and biotin-rhASB were prepared in 2% Blocker A. Equal volumes of 4 μg/ml Ru-rhASB, 4 μg/ml biotin-rhASB, and sample dilutions were mixed in a preincubation plate. Preparation of this mixture adds an additional threefold dilution factor. The preincubation plate was incubated overnight at RT with moderate shaking. The streptavidin assay plate was blocked overnight at 4°C with 150 μl/well 5% Blocker A. The block was shaken out of the assay plate and then 50 μl/well was transferred from the preincubation plate to the streptavidin assay plate. The assay plate was incubated 30 min at RT with shaking and washed three times with DPBS, 0.1% Tween-20, 0.05% ProClin 300. Just prior to reading the plate on the Sector PR400, 150 μl per

well of 2× Read Buffer T was added. The response was measured by the signal of the ECL reaction and compared against the signal for a pooled negative control.

IgG Sandwich ELISA

An indirect ELISA to measure IgG antibodies in human serum (refs 7–9), similar to an assay in cat serum (12). High-binding plates were coated with 4 µg/ml rhASB in 100 mM sodium phosphate, 150 mM NaCl, pH 5.6, and incubated overnight at 4°C. The plate was blocked with 2% BSA, 0.05% Tween-20, DPBS. Sample dilutions were prepared in the same blocking buffer with a minimum dilution factor of 50. Antibodies were captured from the serum matrix onto the microtiter plate *via* the binding interaction with rhASB coated on the plate. Horseradish peroxidase-conjugated goat polyclonal anti-human IgG at 1:1,000 dilution was used to detect antibodies captured on the plate through color development from 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Antibody titer, expressed as “OD per microliter serum”, was calculated for each dilution yielding an OD from 0.2–1.5 by multiplying the net absorbance by the serum dilution factor and then dividing by the volume of test sample in microliters. The lower limit of detection for this assay was 0.2 OD/µl serum, which corresponds to 1 µg/ml anti-rhASB IgG purified from positive patient samples.

Sample Preparation

Samples used for development and validation of the total antibody ECLA were purified antibodies spiked into naïve serum or buffer and subsequently treated in the same manner as clinical samples.

Sample Collection

Three clinical studies (ASB-01-04, ASB-03-05, ASB-03-06) were selected for reanalysis of serum samples (8,9). In these studies, all patients received the final therapeutic dose of weekly 4 h intravenous infusions of 1 mg/kg. Serum samples were collected at intervals of up to 12 weeks apart throughout the studies. The samples were frozen and shipped on dry ice, and subsequently stored at –70°C to –85°C. Clinical research followed the principles of the Declaration of Helsinki in 1984 from the World Medical Association. Protocols were approved by an institutional review board at each participating clinical site. Written consent was obtained from all parents or guardians before enrollment, and written assent was obtained from all patients.

RESULTS

Since the IgG ELISA for anti-rhASB antibodies was not appropriate for late stage clinical research and long-term monitoring of patients, the primary focus of this results section is on the total antibody ECLA assay.

Optimization of Challenge ratio for rhASB Conjugates

A final ratio of 1 to 2 labels per rhASB molecule was desired to ensure an acceptable signal level while minimally

altering rhASB to avoid masking epitopes. Initial labeling with biotin and Ru had an approximately 80% labeling efficiency at challenge ratios from 6 to 12. Further experiments with reduced challenge ratios of 1.25 and 2.5 had an approximately 60% labeling efficiency. The final challenge ratio of 2.5 was selected for subsequent assay development because this ratio generated a consistent label ratio of approximately 1.5. To test the robustness of reagent preparation, multiple small lots of reagent were made with a standard procedure and tested against dilutions of a known positive sample. Although variability in ECL signal was observed across the lots, the sensitivity was similar (data not shown). Three lots were used to test the stability under storage conditions, which was maintained across an 8 week period at 4°C, –20°C, and –70°C (data not shown). During in-study use, the reagent stability at –70°C is at least 12 months.

Optimization of Assay Conditions

In order to optimize assay performance, a variety of conditions were examined for reagent concentrations, incubation times, blocking conditions and sample diluents.

The concentrations of the Ru–rhASB and biotin–rhASB were evaluated between 1–16 µg/ml. Different combinations were compared for the best separation of low and high antibody concentrations *versus* the negative control. Using these criteria, concentrations of 4 µg/ml were selected for both Ru–rhASB and biotin–rhASB (data not shown).

To evaluate blocking conditions, signal from dilutions of a positive control were compared with and without blocking with 5% blocker A. Without blocking, the plates had significantly higher background for negative control serum coupled with dramatically reduced signal for positive control serum, so subsequent development continued with blocking of SA plates. The 5% blocker A was equally effective with a 1 h block *versus* overnight. Positive control dilutions prepared in buffer A or 2% blocker A were equivalent, so 2% blocker A was selected to minimize non-specific interactions for novel samples. Using 2× Read Buffer T yielded better separation of signal and background than 1× Read Buffer T.

Separating the total incubation time between the pre-incubation step and the SA plate incubation resulted in higher signals relative to background. This may be due to the differences in apparent affinity for Ru–rhASB in the solution phase relative to biotin–rhASB in the solid phase bound to the SA plate. The incubation time on the Streptavidin assay plate was optimized to 30 min, selected for lower negative control background but without much sacrifice on positive control signal. The preincubation was optimized to an overnight incubation for an increased signal at high dilutions without significant increase in background.

Robustness of Assay Performance

Small deliberate perturbations of assay conditions were evaluated using samples prepared at 100, 1,000, and 50,000 ng/ml anti-rhASB. The capture and detection reagent compete directly for any anti-rhASB antibodies present in the serum sample. The robustness of detection with 3.5–4.5 µg/ml Ru–rhASB and biotin–rhASB yielded accuracy of 90–135%, with higher variability at higher concentrations of anti-

rhASB. The 100 ng/ml anti-rhASB control, however, fell below cut point when the biotin-rhASB concentration exceeded the Ru-rhASB concentration. The 100 ng/ml anti-rhASB control was included on each sample plate to prevent reporting of false negatives due to errors in capture or detection reagent preparation.

The effect of different incubation times was investigated in human serum for both the preincubation step of serum with the capture and detection reagents (14–22 h) and the final incubation on the SA assay plate (25–35 min). The accuracy between the conditions was 83–108% across the entire range studied at all concentrations (data not shown).

Although robustness across plate lots was not formally performed during assay validation, in-study use has demonstrated good performance across multiple lots for the mid and high positive controls. Occasionally a plate lot had a different background signal requiring adjustment of the control range for the low positive control, but no impact has been observed on the cut point factor as a function of plate lot.

Matrix Interference

Curves of affinity-purified human polyclonal antibody were prepared from 6.25 ng/ml to 4 µg/ml in 10–100% human serum. The curves for signal relative to concentration overlapped for all serum percentages (Fig. 1). As expected, the signal is not directly proportional to the antibody concentration. The dilution of 1:10 was selected to conserve sample volume.

Samples were prepared at 100, 1,000, and 50,000 ng/ml anti-rhASB in three different serum lots and the average signals were compared. The CV% between multiple pooled serum lots was 1–23% and demonstrates that the assay is selective for anti-rhASB in samples from different individuals (Table I).

Since the bridging assay is capable of detecting multiple isotypes of antibodies and also antibodies from different species, the same reagents and format can be applied to additional matrices. This bridging assay was also validated in rabbit serum, rat serum, and human cerebrospinal fluid for additional GLP and clinical studies. In addition, the assay was developed for feline serum and cerebrospinal fluid for

Table I. Signal Variability from Different Serum Lots

Conc ^a	BRH71365 ^b	BRH76860 ^b	BRH76861 ^b	All lots ^c	
	Avg	Avg	Avg	Avg	CV%
100	199	258	271	243	16
1,000	679	968	1,096	914	23
50,000	24,175	23,599	23,524	23,766	1

^a Concentration of anti-rhASB in neat serum (nanogram per milliliter), subsequently diluted 30-fold in final assay format.

^b Lot of pooled serum.

^c Average and CV% for all lots are based on the average signals from each individual lot.

research studies. All matrices had similar matrix interference as observed for human serum.

Establishing Assay Cut Point

To establish the threshold for a positive result (cut point), the signal distribution for individual naïve sera was determined for each species. For human, 50 adult and 25 pediatric sera were analyzed. The naïve sera were run in triplicate in the same manner as unknown samples were screened. The average signal, CV%, and the difference relative to the pooled serum were calculated for each sample. The average difference and standard deviation was calculated from 75 individuals: -8.9 ± 11.9 . The one-tailed 95% confidence interval was calculated as 20 using the one-tailed student's $t=1.645$ (Fig. 2). The 95% confidence interval was used as a cut point factor, which was then added to the average from the pool on each plate to obtain the plate cut point.

The ECL signal from naïve pooled human serum was variable across assay plates. Therefore, the cut point was set individually for each plate to minimize the occurrence of false positives or negatives. Since the tight distribution is on the same order as instrument noise, a new cut point factor was set for each reagent pair (Ru-rhASB and biotin-ASB) to mitigate any small differences between reagent lots. In-study use indicates that this may not have been necessary since the

Fig. 1. Plot of signal versus concentration of affinity-purified human anti-rhASB constructed in 100% (diamond), 50% (square), 20% (triangle), and 10% serum (circle)

Fig. 2. Signal difference from the pooled naïve serum for 50 adult and 25 pediatric unaffected individuals (square) and pre-dose samples from patients (open circle)

Table II. Specificity of Antibody Assay

ng/ml ^a	Ab ^b	Signal	Pos ^c	Confirmatory signal ^d
0	NA	169	N	ND
50	G192	191	P	147
100	G192	198	P	181
500	G192	431	P	162
1,000	G192	884	P	167
5,000	G192	4,827	P	157
10,000	G192	9,505	P	203
50,000	G192	32,799	P	345
50,000	BP13	170	N	156

ND Not determined, P positive signal, N negative signal

^a Concentration of anti-rhASB in neat serum (nanogram per milliliter), subsequently diluted 30-fold in final assay format.

^b G192 is anti-rhASB affinity-purified. BP13 is anti-laronidase affinity-purified.

^c A positive signal is above cut point and a negative signal is below cut point.

^d Signal in the presence of 100 µg/ml rhASB to confirm specificity.

cut point factors have been within a range of 15–20 for five different reagent pairs.

Sensitivity

Purified antibodies were prepared at 50–100,000 ng/ml in naïve pooled human serum. The signal from 1:10 dilutions were compared to the signal from a 1:10 dilution of naïve pooled human serum. The limit of detection varied depending on the source of the polyclonal antibody, with better than 250 ng/ml detection for all four sources. The sheep polyclonal antibody G192 was selected for in-study quality controls because supply was more abundant. The detection of antibodies isolated from multiple species confirmed that the bridging assay was capable of detecting antibodies with different constant domains.

To establish the limit of detection (LOD), samples of anti-rhASB were prepared in neat serum at known concentrations (25–100 ng/ml). Five replicates for each concentration were analyzed and reported as positive or negative. The LOD was determined as the lowest concentration where four out of five replicates were positive. For human serum, the LOD in neat serum is 75 ng/ml.

Specificity

To test the specificity of the assay, samples of anti-rhASB (G192) were prepared in neat serum at known concentrations (50–50,000 ng/ml). The neat serum was diluted tenfold in blocking buffer and analyzed in the assay (Table II). For the antibody concentration range studied, the signal in the assay increased as the anti-rhASB concentration increased, indicating that the signal is proportional to analyte concentration. This antibody concentration range did not exhibit a hook effect in the ECLA.

To further evaluate the sensitivity, an antibody against laronidase was chosen since this biopharmaceutical also contains the mannose-6-phosphate post-translational modification found in rhASB that could potentially serve as an antibody epitope but would not be specific for rhASB. A sample

of anti-laronidase (BP13) was prepared at 50,000 ng/ml in neat serum and tested in the same manner as the anti-rhASB samples. The signal for this high concentration of antibody was below the cut point of the assay (Table II), demonstrating that the assay is specific for antibodies that bind rhASB.

Tolerance of Free Drug

Tolerance of free drug evaluates the ability of the assay to detect anti-rhASB antibodies in serum samples that also contain rhASB from prior dosing. This drug competes with the reagents in the screening assay and can reduce sensitivity of the method. The samples obtained for antibody analysis are typically acquired at day 7 post-infusion, immediately before the next infusion. Prior pharmacokinetic analysis has demonstrated that at the 5 h time point post-infusion after 24 weeks of continuous weekly infusion therapy, no patient had more than 663 ng/ml rhASB present in plasma and more than half had less than 100 ng/ml rhASB (13). The concentration at day 7, which is when the serum samples are collected, is anticipated to be below 1 ng/ml. To characterize interference from free drug in the ECLA, the decrease in sensitivity was measured in serum containing up to 1 µg/ml rhASB in the event that a patient has an antibody response that dramatically extends the PK curve or samples are acquired during or immediately after an infusion due to an adverse event.

Samples of anti-rhASB at known concentrations (100–2,500 ng/ml) were prepared in neat serum containing 0, 10, 100, or 1,000 ng/ml rhASB. The samples were analyzed to determine which samples were above the cut point (Table III). The presence of 1,000 ng/ml rhASB decreased the assay sensitivity to 250 ng/ml anti-rhASB in neat serum. This decrease in sensitivity is acceptable as the sensitivity is still within industry practices and this drug concentration is higher than is anticipated to be present in any patient samples.

Confirmatory

Since the assay cut point was selected at a 95% confidence interval, a false positive rate of 5% is anticipated in the screening assay. To minimize the chance of reporting a false positive, a confirmatory method was developed by comparing the signal change in the presence of excess rhASB (100 µg/ml). Although the assay is tolerant of free drug, the presence of 100 µg/ml rhASB would correspond to a 1 mg/ml

Table III. Interference from Free rhASB in Serum

Conc ^a	0 rhASB ^b	10 rhASB ^b	100 rhASB ^b	1,000 rhASB ^b
100	P	P	N	N
250	P	P	P	P
500	P	P	P	P
1,000	P	P	P	P
2,500	P	P	P	P

P Positive signal, N negative signal

^a Concentration of anti-rhASB in neat serum (nanogram per milliliter), subsequently diluted 30-fold in final assay format.

^b Concentration of rhASB in neat serum (nanogram per milliliter). A positive signal is a signal above cut point. A negative signal is a signal below cut point.

Fig. 3. Titer values for seven separate sample dilutions over five experiments for 100 ng/ml (white bars), 1,000 ng/ml (gray bars), and 50,000 ng/ml (black bars)

serum concentration, which is unlikely to occur in actual patient samples since that is the concentration of the drug formulation.

For a true positive, the signal is expected to drop in the presence of excess rhASB since the unlabeled rhASB competes with the labeled reagents to bind the antibody in the serum sample. To ensure that true positive samples near the cut point were not eliminated by the confirmatory assay, we propose a minimum decrease of (cut point factor)/cut point for identification of true positives. Although the exact value for the cut point shifts on a per-plate basis, the percent reduction required to confirm a positive result is approximately 9–10% across plates.

In repeated tests across analysts, the presence of 100 µg/ml excess free drug was able to decrease the signal by a percentage greater than or equal to this ratio for all but one positive control sample (Table II), so this criterion was used to establish true positives during sample analysis.

Inter-Assay Precision

Since results are reported as a titer value, the most critical measure of precision is the reproducibility of generating a titer result, not the raw signal precision. Samples at 100, 1,000, and 50,000 ng/ml were titrated with serial threefold dilutions on multiple dates by multiple analysts and the last positive dilution was recorded as the titer. Titers for each positive control fell within a range of two consecutive discrete values, demonstrating good precision for the method (Fig. 3).

Sample Stability

The sample handling conditions were studied to establish the optimal practices for storing samples between testing in the screening, confirmatory, and titer assay iterations as well as potential reanalysis. The samples at 100, 1,000, and 50,000 ng/ml anti-rhASB were subjected to up to five freeze-thaw cycles, storing the neat serum at 4°C for up to 8 days, and storing the 1:10 serum dilution at 4°C for up to 3 days. All samples were titrated with serial threefold dilutions and the last positive dilution was recorded as the titer. Titers for each control fell within a range of two consecutive discrete

values of the freshly thawed sample (Fig. 4), indicating that all handling conditions were acceptable to retain the analyte.

Clinical Sample Analysis

Due to the small affected population size, the cut point factor had been established using serum from naïve individuals from an unaffected population. The distribution of pre-dose serum from MPS VI patients was tested and compared against the distribution of unaffected individuals (Fig. 2). The difference between the patient population and normal individuals is statistically significant ($p=0.03$ for means, $p=0.06$ for variance), with the mean and standard deviation for the patient population greater than for the normal individuals. The use of a cut point established with normal individuals will generate a higher rate of positive samples than using a cut point based on the patient population. For these patients, four of 50 were positive in the screening assay (8%), with none confirmed positive. For this specific disease population, the use of unaffected individuals to set the cut point is an acceptable surrogate even though the risk of false positives is increased. In the reverse case, the patient population is not an acceptable surrogate for normal individuals as the rate of false positives would be decreased.

Of the 493 samples (across 48 patients) tested in both the total antibody ECLA and IgG ELISA, 246 (49.9%) were positive in both assays, 206 (41.8%) were below detection in both assays, 13 (2.6%) were positive in the IgG ELISA and below detection in the total antibody ECLA, and 28 (5.7%) were positive in the total antibody ECLA and below detection in the IgG ELISA.

For each discrete reported titer from the total antibody ECLA, the descriptive statistics were calculated for the associated reported results from the IgG ELISA (Table IV) and screening signals (Table V). Mean, median, and quartile values were calculated on the log transformed data and transformed back into the original readout. Although the ranges overlapped significantly, the mean and median for both readouts generally increased along with the increasing titer, with a few exceptions to the trend.

For samples that tested positive in the total antibody ECLA, the reported titer was compared to the difference of

Fig. 4. Titer values from stability experiments with up to five freeze-thaw cycles and 7 days storage at 4°C for 100 ng/ml (white bars), 1,000 ng/ml (gray bars), and 50,000 ng/ml (black bars)

Table IV. IgG Values (OD per microliter) Observed at Each Reported Total Antibody Titer

Total Ab titer	Number	Mean ^a	Min	25% ^a	Median ^a	75% ^a	Max
BLQ	13	0.2	0.2	0.2	0.2	0.2	0.3
30	2	0.1	0.1	0.1	0.1	0.1	0.1
90	3	0.1	0.1	0.1	0.1	0.1	0.1
270	5	0.2	0.1	0.1	0.2	0.4	0.5
810	8	0.2	0.1	0.1	0.1	0.3	1.3
2,430	7	0.5	0.2	0.3	0.5	0.8	1.0
7,290	31	0.7	0.1	0.3	0.6	1.6	14.9
21,870	41	1.1	0.1	0.4	1.3	3.4	13.0
65,610	85	0.8	0.1	0.2	0.3	5.2	42.8
196,830	65	3.0	0.2	0.3	5.0	24.2	54.4
590,490	22	6.8	0.2	0.4	28.2	40.5	64.9
1,771,470	5	19.1	0.6	4.5	50.1	50.1	52.3

^a Mean, quartiles (25% and 75%), and median were calculated on log transformed data and then transformed back into the original units (OD per microliter). For samples below the limit of detection (0.2 OD/μL) in the IgG assay, a value was assigned at half the limit of detection (0.1 OD/μL).

the signal from the 1:10 screening dilution from the pooled serum (Fig. 5). The signal from the screening dilution was not a validated readout for the assay, but was explored to understand the relationship between screening dilution and titer in clinical samples. Although for each screening dilution, there was an increase in the minimum measured titer value, there was still significant range for each screening dilution and vice versa. This variability supports the conclusion that across samples, a screening dilution can yield a different titer value depending on the concentrations and affinities of the contributing individual antibody clones.

For samples that tested positive in either assay, the reported titer in the total antibody ECLA was compared to the OD per microliter value from the IgG ELISA (Fig. 6), using surrogate values for negative results on the logarithmic scale. For each OD per microliter reported value, including the results below the limit of detection, a range of titers were reported from the total antibody ECLA. When samples did not fit the correlation, the total antibody ECLA titers were higher than would be predicted from the OD per microliter value reported from the IgG ELISA.

Since the relationship between the total antibody ECLA titer and the IgG ELISA OD per microliter (Fig. 6)

resembled the relationship between the total antibody ECLA titer and screening dilution (Fig. 5), the OD per microliter value from the IgG ELISA was also plotted *versus* the total antibody screening dilution (Fig. 7). A correlation between the two values is apparent, although a range of total antibody ECLA screening dilutions are observed for each reported IgG ELISA OD per microliter value.

DISCUSSION

Assay Validation

The total antibody assay met all design criteria (Table VI), with significant improvements over the previously developed IgG ELISA. We established a tight distribution of naïve sera, and used this distribution to establish the 95% confidence interval used as the cut point factor. The cut point factor was added to the average signal for the pooled serum on each plate to establish the cut point.

The assay specifically detected antibodies against rhASB with proportional response, and the limit of detection was 75 ng/ml in neat serum. The tolerance of free drug was sufficient to measure 250 ng/ml anti-rhASB at rhASB concentrations up to the C_{max} from pharmacokinetic analysis.

For all samples, reportable titers were within two consecutive threefold dilution factors across multiple sample analyses. The assay was robust to small changes in reagent concentrations and through all time ranges studied for the incubation steps. The sample handling was robust for the assay, with all tested permutations resulting in a titer within two consecutive threefold dilution factors as the control sample.

Summary of Differences in IgG and Total Antibody Assays

The IgG assay is an indirect ELISA format while the total antibody assay is a solution-phase bridging ECLA format. The switch to the ECLA format allowed binding of multiple antibody isotypes, increased sensitivity, and has the potential to allow detection of low affinity antibodies. The ECLA uses a cut point based on the normal distribution of naïve individuals while the ELISA uses the signal variability of a single pooled serum lot. The ECLA uses a confirmatory assay before proceeding to a titer assay, while the ELISA reports out an OD per microliter serum using raw OD values

Table V. Screening Signals Observed at Each Reported Total Antibody Titer

Total Ab Titer	Number	Mean ^a	Min	25% ^a	Median ^a	75% ^a	Max
30	2	4	3	3	4	5	5
90	3	35	5	5	79	111	111
270	5	224	46	91	385	423	432
810	8	194	4	27	410	907	1,052
2,430	7	2,575	1,162	1,320	3,031	4,627	5,159
7,290	31	2,660	4	1,381	3,581	8,266	21,873
21,870	41	4,420	89	1,318	7,329	17,788	59,569
65,610	85	2,874	27	490	2,151	27,744	195,077
196,830	65	10,183	139	1,203	17,713	78,122	277,040
590,490	22	21,559	204	2,794	79,778	151,480	208,331
1,771,470	5	84,956	8,278	27,868	93,880	246,373	364,501

^a Mean, quartiles (25% and 75%), and median were calculated on log transformed data and then transformed back into the original units.

Fig. 5. Data correlation for total antibody titer *versus* signal difference of the screening dilution relative to the negative control for samples that tested positive in both assays (diamond) and samples that were below detection in the IgG assay (open circle)

within a narrow OD range. In the total antibody ECLA, the reported value for titer is the last positive dilution, while in the IgG ELISA, the reported value is a type of relative mass unit (OD per microliter).

Differences Between Test Results from IgG and Total Antibody

Although the majority of the samples yielded the same result (positive *versus* BLQ) in the two assays, there were 8.3% of samples with different results. The differences between the assay formats suggest many possible explanations.

All samples that tested positive in the IgG ELISA but were below the limit of detection in the total antibody ECLA had results just above the IgG limit of detection (Fig. 6). Since the IgG ELISA did not have a confirmatory assay component, it is possible that these samples had a nonspecific signal from background that was responsible for the reported value. Another possibility is an overly restrictive confirmatory assay in the total antibody ECLA. None of these 13 samples

Fig. 6. Data correlation for total antibody titer *versus* IgG OD per microliter for samples that tested positive in both assays (diamond), samples that were below detection in the total antibody assay (open square, Y value set to 15 to allow graphing), and samples that were below detection in the IgG assay (open circle, X value set to 0.1 to allow graphing)

Fig. 7. Data correlation for *versus* IgG OD per microliter *versus* signal difference for total antibody screening dilution relative to the negative control for samples that tested positive in both assays (diamond) and samples that were below detection in the IgG assay (open circle)

generated a signal above the negative control in the total antibody ECLA, however, so the latter possibility did not appear to be true of this data set. Eleven of the 13 samples were from a single patient, so there may be a unique interference in the IgG ELISA assay from this patient.

Samples that tested positive in the total antibody ECLA but were below the limit of detection in the IgG ELISA had titer values spanning 30–65,610 (Fig. 6). Ten samples came from nine patients with antibodies detected one or two time points earlier in the total antibody ECLA *versus* the IgG ELISA. The discrepancy could be due to either an IgM response or an IgG response below the limit of detection either due to concentration or affinity. An additional 13 samples came from a single patient where the IgG assay did not detect antibodies until week 78. For this patient, the length of time suggests that the discrepancy is not due to IgM alone. Five samples came from late time points for three patients. Possible explanations are isotype switching to an IgG subclass detected less efficiently by the IgG ELISA secondary or a switch to lower affinity antibodies.

Across the samples that tested positive in both assays, the scatter appears to be an imperfect correlation between the total antibody ECLA and the IgG ELISA (Fig. 6). The relationship between total antibody titer and IgG OD per microliter bears a resemblance to the relationship between total antibody titer and total antibody screening signal (Figs. 5 and 6). The similar graphs are confirmed by the correlation between the total antibody screening signal and IgG OD per microliter (Fig. 7). This correlation is expected since both screening dilution and OD per microliter are types of relative mass measurements. For samples where the total antibody ECLA titer does not appear to correlate with the total antibody ECLA screening signal and IgG OD per microliter, the total antibody ECLA titer is higher than would be predicted solely from the screening dilution. This is suggestive of low affinity antibodies since in general, higher concentrations are required to generate an equivalent signal as high affinity antibodies, leading to a less steep curve for signal *versus* concentration.

The impact of assay design on clinical sample results could only partially be predicted from the validation results

Table VI. Summary of Assay Performance

Test parameter	Bridging ECLA	Indirect ELISA
Assay cut point factor	95% confidence interval based on 75 naïve samples (20)	OD > twice the plate blank
Limit of detection	75 ng/ml in neat serum	1,000 ng/ml in neat serum
Specificity	Average signal proportional to anti-rhASB concentration	Signal fell below background without rhASB coat, positive control serum, or conjugated secondary antibody
	Average signal for 50 µg/ml anti-Aldurazyme® below assay cut point	Not validated
Interference from drug	250 ng/ml anti-rhASB can be detected in up to 1,000 ng/ml rhASB	Not validated
Interference from serum matrix	Accuracy within 105–111% for 100 ng/ml 62–98% for 1,000 and 50,000 ng/ml in 0–10% serum Average signal proportional to concentration for anti-rhASB in 0–10% serum CV% 1–23% between three different lots of serum for 100–50,000 ng/ml anti-rhASB	Not validated
Precision	CV% of signal for intra-assay precision 1–4% for 100–50,000 ng/ml anti-rhASB CV% for inter-assay precision 6–19% for 100–50,000 ng/ml anti-rhASB Consistency in titer values did not vary more than one threefold dilution factor	CV% of signal for intra-assay precision 1–19% for three positive control samples CV% of signal for intra-assay precision 6–11% for three positive control samples Not applicable for OD per microliter readout
Robustness	Accuracy within 90–138% with 3.5–4.5 µg/ml biotin-rhASB and Ru-rhASB concentrations Accuracy within 83–108% for 14–22 h preincubation and 25–35 min capture incubation time Consistency in titer values did not vary more than one threefold dilution factors for up to five freeze-thaw cycles and 3 days storage at 4°C	Not validated Not validated Consistency in OD per microliter was within-15 to 2% relative to the fresh sample for up to three freeze-thaw cycles

due to the complexity of the polyclonal antibody response observed in humans. The imperfect correlation between total antibody ECLA screening signal and reported titer reiterates that although each sample is expected to have an antibody response proportional to the antibody concentration, the curve shapes through the dilution series may be quite different between individual patient samples.

CONCLUSIONS

To properly evaluate immunogenicity in the context of the overall clinical safety and efficacy, it is essential to understand the potential limitations of the assay during development and validation, and to avoid overinterpreting small differences in results from a semi-quantitative assay. This clinical data from two different screening assays illustrates some of the challenges facing interpretation of immunogenicity assays, which are semi-quantitative in nature.

The impact on data analysis cannot be fully predicted in the absence of the clinical safety and efficacy data. A major advantage for the total antibody ECLA assay is detection of any applicable antibodies regardless of isotype or subclass. Since the total antibody ECLA is used to identify samples for further characterization for neutralizing antibodies and IgE-specific antibodies, it is essential to ensure that the percentage of missed positive samples is as low as possible.

When evaluating immunogenicity impact on safety and efficacy, data can be compared within patients or between patients. Between patient correlations are less reliable than within patient trends since each individual will have a different mix of individual antibodies, although some study designs do not permit the long-term monitoring within single patients.

If between patient comparisons must be made, there is a risk of grouping patients incorrectly based on response level. The wide variability of total antibody titer values observed for comparable IgG ELISA values or screening signal underscores this challenge (Figs. 5 and 6). From either test, it would be inappropriate to make any rigid comparisons between relative levels of antibody formation.

The choice of immunogenicity assay also affected comparisons within individual patients. Since a few late time points for patients were positive samples in total antibody ECLA and negative in IgG ELISA, the IgG ELISA incorrectly evaluated the impact of immunogenicity by reporting negative seroconversion. Antibody detection in one patient was delayed by over a year in the IgG ELISA method relative to the total antibody ECLA. The delay in detecting the antibody response had the potential to mask serious antibody-mediated adverse events. Due to the imperfect correlation between the two methods, other patients may exhibit conflicting trends in the IgG ELISA and total

antibody ECLA which could incorrectly identify patient seroconversion or stability patterns.

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