Molecular Therapy
Methods & Clinical Development
Original Article

Validation of high-sensitivity assays to quantitate cerebrospinal fluid and serum β-galactosidase activity in patients with GM1-gangliosidosis

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GM1-gangliosidosis (GM1) is a lysosomal storage disorder caused by mutations in the galactosidase beta 1 gene (*GLB1*) that leads to reduced β-galactosidase (β-gal) activity. This enzyme deficiency results in neuronal degeneration, developmental delay, and early death. A sensitive assay for the measurement of β-gal enzyme activity is required for the development of disease-modifying therapies. We have optimized fluorometric assays for quantitative analysis of β-gal activity in human cerebrospinal fluid (CSF) and serum for the development of a *GLB1* gene replacement therapy. Assay analytical performance was characterized by assessing sensitivity, precision, accuracy, parallelism, specificity, and sample stability. Sensitivity of the CSF and serum β-gal activity assays were 0.05 and 0.20 nmol/mL/3 h, respectively. Assay precision represented by inter-assay percent coefficient of variation of the human CSF and serum was <15% and <20%, respectively. The effect of pre-analytical factors on β-gal activity was examined, and rapid processing and freezing of samples post-collection was critical to preserve enzyme activity. These assays enabled measurement of CSF and serum β-gal activities in both healthy individuals and patients with GM1-gangliosidosis. This CSF β-gal activity assay is the first of its kind with sufficient sensitivity to quantitatively measure β-gal enzyme activity in CSF samples from GM1 patients.

INTRODUCTION

GM1-gangliosidosis (GM1) is an autosomal recessive disorder that results from mutations in the human galactosidase beta 1 gene (*GLB1*), which encodes a lysosomal enzyme β-galactosidase (β-gal).[1–3] Bi-allelic pathological mutations in the *GLB1* gene result in a deficiency of β-gal enzyme activity and accumulation of undegraded and partially degraded β-gal substrates in the lysosomes of cells in the nervous system.[4–7] Based on literature reports, β-gal activities in GM1 patients may be less than 10% of the activity observed in healthy individuals.[1–3,8,9] GM1 manifests as a spectrum of neurodegenerative disease that affects both the central nervous system (CNS) and peripheral organs ranging from individuals with severe β-gal enzyme deficiency, having earlier clinical symptom onset and rapidly progressive disease (type 1), to those with less severe β-gal enzyme deficiency, having a later age of symptom onset with slower and more heterogeneous rates of progression (types 2 and 3).[1–3,6,7,10,11]

There is currently no FDA-approved disease-modifying treatment for GM1. Recently, encouraging results with *GLB1* gene replacement therapy using adeno-associated virus (AAV)-mediated gene delivery[12,13] and β-gal enzyme replacement therapy[14] have been reported in animal models. Human clinical trials using these and other approaches, such as enzyme enhancement therapy, substrate reduction therapy, and stem cell transplantation are in progress.[6,7] Passage Bio is conducting a clinical trial, Imagine-1 (NCT04713475 [ClinicalTrials.gov]; [EudraCTNumber: 2020-001109-22]) in GM1 patients, to assess the effect of delivering a functional copy of the *GLB1* gene to the brain through intracisternal magna injection (ICM). ICM delivery of AAV has been shown to result in widespread gene transfer in the CNS as well as some distribution to peripheral organs.[15] The ability to evaluate cerebrospinal fluid (CSF) β-gal activity in GM1 individuals before and after ICM injection is critical for ascertaining the success of gene delivery. It is also important to monitor β-gal activity in serum, in addition to CSF, to discern whether ICM delivery of *GLB1* into the central compartment increases β-gal activity in the periphery and therefore has the potential to correct the systemic enzyme deficiency in GM1. There are existing assays for the measurement of β-gal activity in various human tissues, including dried blood spots, leukocytes, or fibroblasts.[1,9,16] These assays are primarily used for the qualitative diagnosis of GM1, however, and do not allow for quantitative evaluation of β-gal activity within the CNS. Here, we describe the optimization and validation of two fluorometric assays to quantitate β-gal activity in human CSF and serum, with sensitivities sufficient to measure the residual β-gal activities in GM1 patients.

Received 28 March 2024; accepted 7 August 2024;
https://doi.org/10.1016/j.omtm.2024.101318.

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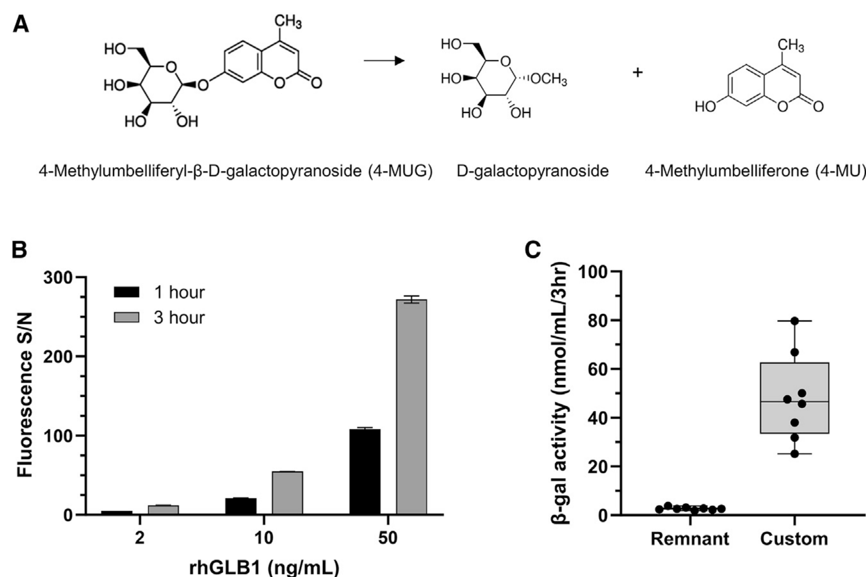

Figure 1. Design and optimization of the β-gal assays for human serum and CSF

(A) The fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG) is converted into the fluorescent compound 4-MU and D-galactopyranoside in the presence of active human acid beta-galactosidase (β-gal). (B) Recombinant human GLB1 (rhGLB1) at three different concentrations were incubated with 4-MUG for either 1 or 3 h to assess the effect of incubation time. Representative mean ± SD fluorescence signal-to-noise (S/N) results are depicted. (C) Remnant and prospectively collected (custom) serum samples (n = 8 per group) were tested to evaluate impact of post-collection handling and storage conditions on β-gal activity. Boxplots report the median enzymatic activity and ranges between the upper and lower quartiles. Error bars represent the full ranges of the data with individual values denoted with closed circles.

RESULTS

Design and optimization of human CSF and serum β-gal activity assays

To develop human CSF and serum β-gal activity assays, an assay format described previously for β-gal detection in mouse or human tissues[13,16] was adopted, using a 4-methylumbelliferone (4-MU)-linked enzyme substrate 4-methylumbelliferyl-β-D-galactopyranoside (4-MUG) and a low pH buffer (to mimic lysosomal pH). As the β-gal enzyme cleaves the 4-MUG substrate during incubation at 37°C, free 4-MU is released which fluoresces upon addition of a high pH stop solution (Figure 1A). Two calibration curves were used during assay optimization and validation: a 4-MU calibration curve to quantify 4-MU fluorescent product as a measurement of β-gal enzyme activity, and a recombinant human β-gal (rhGLB1) protein activity calibration curve to ascertain that the assay measured the activity of the target analyte, β-gal. During subsequent clinical sample analysis, the 4-MU standard curve was used to quantitate the sample β-gal enzymatic activity.

Assay buffers

For the serum β-gal activity assay, based on previous reports, two buffers were evaluated to optimize assay reaction conditions: a citric acid-sodium phosphate buffer (pH 4.2) and a citric acid-sodium chloride buffer (pH 4.2).[13,14,17,18] Both buffers exhibited similar signal-to-noise (S/N) ratios under preliminary assay conditions; the average relative percent difference in S/N ratios for the two conditions was $\leq$25%. Because of its higher buffering capacity, the citric acid-sodium phosphate buffer was chosen for use in the assay. Different stop solutions were tested and had no measurable impact on β-gal activity. The relative percent difference between signals for β-gal activity with stop solutions consisting of glycine pH 10 or carbonate pH 11.5 was <8%.

For the CSF β-gal activity assay, the same assay format, using the citric acid-sodium phosphate assay buffer (pH 4.2), was able to detect β-gal activity in CSF from healthy individuals (Figure S1). To maximize the ability to measure very low levels of β-gal activity in GM1 patients, we set out to develop a human CSF β-gal activity assay with improved sensitivity by increasing the volume of CSF sample added to the reaction mixture. The assay buffer pH was reduced to 3.5 to accommodate this sample volume change (human CSF pH is ~7.3[19]) and to maintain the assay reaction pH at 4.2–4.5.[16] Hereafter this assay using pH 3.5 buffer is referred to as the "CSF assay," while the assay using pH 4.2 buffer is referred to as the "serum assay." Comparison of 26 CSF samples analyzed using both assay conditions showed a positive correlation (Pearson's r = 0.9489; p = 0.0001; Figure S1).

Sample incubation time

To evaluate the effect of reaction duration, recombinant β-gal (rhGLB1) was incubated with 4-MUG substrate for 1 or 3 h. Representative results from the 1- and 3-h incubation periods are depicted in Figure 1B. Three hours of incubation provided a better S/N ratio for enzyme activity measurements and was used for all subsequent studies.

Evaluation of pre-analytical conditions and comparison of blood matrices

Remnant CSF and serum samples were evaluated during the early phase of this project. It was soon observed that β-gal activity was very low in remnant serum samples and barely detectable in remnant CSF samples. We hypothesized that special care may be needed during sample collection and storage since the acidic environment (in lysosome) is required to preserve β-gal enzyme activities. Therefore, we prospectively collected serum samples from volunteers at the laboratory for comparison. Whole blood was collected from eight healthy donors without any known allergies or health problems, or on any significant medications and immediately processed for serum.

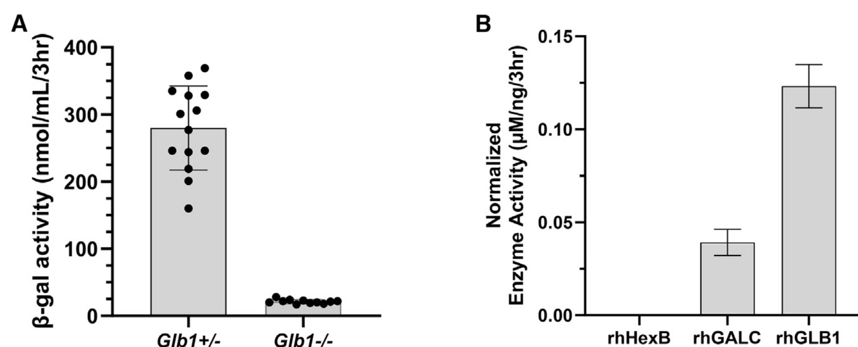

Figure 2. Specificity of β-gal enzyme activity
(A) Serum from heterozygous control (*Glb1*$^{+/-}$; n = 14, 8 male and 6 female) or *Glb1* knockout mice (*Glb1*$^{-/-}$; n = 11, 7 male and 4 female) were evaluated for β-gal activity. The mean ± SD as well as individual sample results (closed circles) are depicted. (B) Recombinant human hexosaminidase B (rhHexB), glucocerebrosidase (rhGALC), and β-gal (rhGLB1) were incubated with 4-MUG substrate to determine the ability of each enzyme to convert the substrate into detectable fluorescent 4-MU. Enzyme activity, quantified using the 4-MU standard curve, was normalized to the concentration of enzyme present in each reaction (ng/mL). The mean ± SD of normalized enzyme activity is presented.

These serum samples were then frozen at −80°C within 1 h of sample collection (see materials and methods for additional collection details). On average, serum from prospectively collected samples demonstrated 18-fold higher β-gal activity than that from remnant serum samples (Figure 1C). A prospective collection was also conducted through a commercial vendor following the same sample handling instructions, i.e., maintaining samples on ice and freezing them within 1 h post-collection. In contrast to the remnant CSF samples in which β-gal activity was barely detectable, β-gal activities were measurable in prospectively collected CSF samples (representative data is presented in Figure S1). These results suggest that sample collection and storage conditions are important to preserve the integrity of β-gal protein in serum and CSF. Therefore, in all subsequent assay optimization and validation studies, only samples prospectively collected following these special processing procedures were used. The instructions for these procedures were also documented in a sample management manual used for supporting the clinical studies.

To determine whether there were differences between β-gal activities in serum and plasma, paired samples were prospectively collected from eight healthy donors and β-gal activities measured for comparison. Overall, no significant difference was observed between these two blood sample matrices. The mean of β-gal activities in serum and plasma were 48.1 nmol/mL/3 h ± 17.9 (SD) and 52.1 nmol/mL/3 h ± 12.6 (SD), respectively. These results supported the selection of serum as the matrix for monitoring peripheral β-gal activity during clinical sample analysis, which was consistent with the sample type used during preclinical studies.[13]

Evaluation of assay specificity

Two approaches were used to assess the specificity of the β-gal activity assays. First, serum β-gal activity from *Glb1*$^{-/-}$ knockout mice was compared with that from isogenic heterozygous littermates. The average β-gal activity in serum samples from the *Glb1*$^{-/-}$ mice was <10% of that in samples from *Glb*$^{+/-}$ mice, 21.3 nmol/mL/3 h ± 3.1 (SD) versus 280 nmol/mL/3 h ± 62.5 (SD) (Figure 2A), which is consistent with a previous report.[20] Second, we evaluated whether the assay could detect enzyme activities of the only other known lysosomal β-galactosidase, galactosylceramidase (GALC), and another lysosomal enzyme, hexosaminidase B (HexB), using recombinant human proteins. Under the optimized assay conditions, rhGLB1 and, to a much lesser extent, recombinant human GALC (rhGALC), cleaved the 4-MU substrate; however, recombinant human Hexosaminidase B (rhHexB) did not. As shown in Figure 2B, at a given concentration of recombinant protein, the enzyme activity of rhGLB1 measured by this assay is approximately 3-fold that of rhGALC.

Effects of hemolysis and blood contamination on β-gal activity

Hemolysis may occur during blood sample collection, and CSF samples collected through lumbar puncture can be contaminated with blood. To evaluate the effect of hemolysis and blood contamination on β-gal activity, hemolyzed blood was spiked into individual human serum samples and pooled human CSF samples (contaminating blood cells in CSF are typically hemolyzed after a freeze-thaw cycle) at 1%, 2%, and 5% concentrations. This mimicked slight, moderate, and gross hemolysis, respectively, in accordance with a blood hemolysis visual reference chart. Hemolysis had a measurable inhibitory effect on serum β-gal activity levels, with an average decrease of 27%–53% in the presence of 1%–5% hemolyzed blood (Table 1). In pooled CSF samples with normal β-gal activity levels, enzyme activity was unaffected in the presence of up to 2% hemolyzed blood but reduced in the presence of higher blood contamination. In contrast, in pooled CSF samples where β-gal activity was substantially reduced (like CSF from GM1 patients), blood contamination demonstrably elevated the amount of measurable β-gal activity (Table 1). Therefore, assay interference is observed in both hemolyzed blood samples and CSF samples contaminated with blood.

Fit-for-purpose validation

Calibration curve, analytical range, and lower limit of quantification

Both 4-MU and rhGLB1 calibration curves were initially characterized for the CSF and serum β-gal assays. Representative standard curves for the two reference materials are presented in Figure S2. To evaluate the precision and reproducibility of these calibration curves, the 4-MU and rhGLB1 reference standards were prepared fresh daily in assay diluent and assayed in triplicate by 2 analysts over 3 days each for at least 12 independent runs. For the serum assay, the 4-MU reference standards demonstrated strong reproducibility

Table 1. The effects of hemolysis and blood contamination on β-gal enzyme activities in serum and CSF

		Serum		CSF			
Hemolyzed blood (%)	Assay diluent (nmol/mL/3 h)	Mean activity of healthy individual donor samples (nmol/mL/3 h)	% Difference	Mean of normal activity pools (nmol/mL/3 h)	% Difference	Mean of low activity pools (nmol/mL/3 h)	% Difference
0	<LLOQ	87.96	N/A	2.43	N/A	0.38	N/A
1	1.10	63.92	−27.33	2.69	10.7	1.15	205.03
2	1.97	56.92	−35.29	2.30	−5.35	1.08	185.45
5	2.22	41.14	−53.23	1.58	−34.98	1.53	304.76

Serum from healthy adult individuals, normal and low β-gal activity CSF pools, or assay diluent alone, were spiked with either 1%, 2%, or 5% hemolyzed whole blood. Each matrix condition was assayed in duplicate for β-gal activity in two independent runs. The mean β-gal activity (nmol/mL/3 h) of duplicates from the two independent analytical runs for each condition is presented. The percentage difference was calculated using the unspiked (0% hemolyzed blood) sample as the reference value. N/A, not applicable; LLOQ, lower limit of quantitation.

with percent coefficient of variation (%CV) of the individual calibrators ranging from 3.23% to 5.97% and the percent difference from theoretical (%DFT) averaging 1.59% across the 10 calibrators (Figure S2A). Similarly, %CV of the 9 individual calibrators in the rhGLB1 standard curve ranged from 0.63% to 2.11%, with an average 0.62% DFT (Figure S2B). The analytical range of the serum β-gal activity assay was established with the 4-MU calibrators run at lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), with acceptable accuracy and reproducibility predefined as <25% DFT and CV. Of note, the LLOQ corresponded to 0.20 nmol/mL/3 h and the analytical measurement range was 0.20–160 nmol/mL/3 h.

For the CSF β-gal assay, the 4-MU calibrators behaved similarly, with the %CVs of the 10 individual calibrators ranging from 0.14% to 3.34% across the 7 runs with an average 1.16% DFT; the 10 rhGLB1 calibrators ranged from 0.80% to 2.77% CV with an average 1.42% DFT (Figures S2C and S2D). The LLOQ for the CSF assay was confirmed to be 0.05 nmol/mL/3 h and the analytical measurement range was 0.05–40 nmol/mL/3 h.

Accuracy and precision

For both serum and CSF assays, quality control (QC) samples, described in the materials and methods, were assessed to characterize the accuracy and precision of the assays across the calibration curve range with results summarized in Table 2. Overall, intra- and inter-assay precisions (%CV) were <5% and <20%, respectively, for all QC samples tested across the two assays, providing evidence of acceptable precision of measurement (<20%) across the calibration curve range in each assay. The values for each enzymatic QC level were defined empirically using the average measured enzyme activity from the validation and served as the basis for accuracy determinations during sample analysis. Due to observed instability of the low rhGLB1 QC for the CSF assay in the low pH buffer, it was replaced with a 4-MU-based QC in-study, in tandem with other enzymatic QCs. As of the writing of this paper, over 100 CSF and serum samples have been analyzed in the ongoing Imagine-1 study sample analysis runs. The inter-assay precision of the QCs across these sample analysis runs was <15% for both assays and the accuracy performance was <10% DFT (Table 2).

Parallelism

Parallelism was performed to assess potential interference of endogenous matrix components with the measurement of β-gal activity. For the serum assay, six individual human serum samples were serial diluted 1:2 to 1:64, and β-gal activity was found to be equivalent in all sample dilutions (Figure 3A). The percent recovery for each dilution was within 20%, except for a single 1:64 dilution in one donor sample (Figure 3B). Consequently, a 1:2 dilution was selected as the minimal required dilution for this assay, with sample dilution acceptable down to 1:64. Parallelism also appeared to be observed down to at least 1:8 dilution if the serum assay conditions were used to measure β-gal activity in CSF samples (Figure S3A). For the optimized CSF assay with improved sensitivity, parallelism was not observed (Figure S3B). Therefore, CSF samples were analyzed without dilution. Due to the very low β-gal activity in CSF samples from GM1 patients, it was not required that samples be diluted for ongoing Imagine-1 clinical sample analysis.

Sample stability

Sample processing and storage stability were evaluated to guide sample management during clinical trials. Sample stability was evaluated under various storage conditions using endogenous QCs (eQCs) consisting of pooled serum or pooled CSF. Samples subjected to the testing conditions were compared with eQC aliquots maintained at −80°C and thawed once (baseline). As shown in Table 3, the samples were stable up to 4 h on ice with 94.9% and 83.8% β-gal activity (<10% CV) recovered in CSF and serum, respectively. Samples were also subjected to three freeze/thaw cycles. Serum and CSF eQC samples retained 92.4% and 98.0% of β-gal activity (<10% CV), respectively, after the three freeze/thaw cycles (Table 3). In addition, β-gal activities in serum and CSF were stable for up to 503 days (longest time point tested) when stored at −80°C. Of note, individual serum and CSF samples were also evaluated, and no marked change in β-gal activity was demonstrated up to approximately 11 (CSF) or 8 (serum) weeks of storage at −80°C (Figure S4).

Table 2. Accuracy and precision of the β-gal assays for human serum and CSF

Assay matrix	QC analytical level	Sample material	Enzyme activity (nmol/mL/3 h)	Validation		In-study analysis	
				Intra-assay precision (%CV)	Inter-assay precision (%CV)	Inter-assay precision (%CV)	Accuracy (%DFT)
CSF	low QC^	rhGLB1 spiked in assay buffer	0.15	4.18	6.10	N/A	N/A
	low QC*	4-MU spiked in assay buffer	0.15	N/A	N/A	10.30*	3.69*
	mid eQC	unspiked matrix pool	2.49	3.44	4.39	7.64	−6.58
	high eQC	rhGLB1 spiked in matrix pool	22.10	1.64	12.60	10.93	3.35
Serum	low eQC	diluted matrix pool	1.62	4.27	14.90	11.5	0.62
	mid eQC	diluted matrix pool	12.9	2.86	17.10	9.47	−1.55
	high QC	rhGLB1 spiked in assay buffer	120.00	1.55	12.20	10.9	4.17

Buffer quality control (QC) samples were freshly prepared by spiking 4-MU in assay diluent at 0.15 nmol/mL or recombinant human GLB1 (rhGLB1) in assay diluent at 0.45 ng/mL (low QC^, this QC was found to be unstable and not used during sample analysis) as low QC for the CSF assay or 120 ng/mL (high QC) for the serum assay. Pooled CSF matrix unspiked or spiked with rhGLB1 (15 ng/mL) were used as endogenous QCs (eQCs) for the CSF assay. Pooled serum matrix eQCs were diluted as described in the materials and methods. β-gal enzyme activity was quantitated using a 4-MU standard curve. The percent coefficient of variation (%CV) for intra- and inter-assay precision were calculated from replicate runs using ANOVA. The results for the 4-MU low QC (*) for the CSF assay are derived from sample analysis replicates (n = 20). For all enzymatic QCs, the inter-assay %CV was calculated across the runs and accuracy was determined as the percent difference from theoretical (%DFT) as defined empirically during the method validation runs. In-study QC data for the CSF and serum assays is compiled across ≥10 sample analysis runs with two replicates each. N/A, not applicable.

Assay robustness

As measures of assay robustness, enzymatic QC and calibrator curves were assessed for long-term performance. For both serum and CSF β-gal activity assays, trending analysis demonstrated strong longitudinal reproducibility of all QCs (Figures 4A and 4B).

4-MU is a hydrophobic molecule and difficult to dissolve even in DMSO. To reduce variation associated with preparing fresh 4-MU calibrator curves, bulk 4-MU calibrators were prepared as single-use aliquots and maintained at −80°C to be used during assay validation and sample analysis. The performance and stability of the frozen 4-MU calibrators were assessed. Calibration curves generated with the single-use frozen 4-MU aliquots, used over 15 months during multiple sample analysis runs, exhibited very low variability as depicted in Figure 4C. The calibration curves using frozen 4-MU aliquots also demonstrated a similar dose-response relationship to the curve using freshly prepared 4-MU calibrators. The frozen 4-MU calibrators were stable up to 519 days (longest time point tested) at −80°C (Table S1). Similar results were also observed for the CSF assay (Figure 4D). The consistent performance of enzymatic QCs over time demonstrates the quality of the assay's analytical performance and the stability of the QC samples. The use of single-use frozen calibrators enables batch sample analysis and comparison of data longitudinally which can be difficult to achieve with 4-MU-based activity assays.

Quantitation of serum and CSF β-gal activities in healthy adults and GM1 patients

For gene or enzyme replacement therapies, knowledge of normal reference ranges of the target protein is important to guide dose escalation, patient selection, and other clinical trial decisions. Toward understanding the normal reference ranges of β-gal activity, matched serum and CSF samples were prospectively collected from 30 healthy adult donors (aged 40–72 years) following the sample collection procedure described above and in the materials and methods. The β-gal activities quantified in these samples are shown in Figure 5A. In CSF, median β-gal activity was 2.73 nmol/mL/3 h (interquartile range [IQR]: 2.38–3.44). In serum, the median was 88.95 nmol/mL/3 h (IQR: 75.20–112.20). There was no correlation between CSF and serum β-gal activities (r = 0.12, p = 0.52; Figure 5B).

We were also able to measure β-gal activities from a limited number of untreated early- and late-infantile GM1 patients. The median levels of CSF and serum activities were 0.73 nmol/mL/3 h (IQR: 0.51–0.97; n = 10) and 6.48 nmol/mL/3 h (IQR: 5.07–8.85; n = 12), respectively (Figure 5C).

DISCUSSION

For over 50 years, fluorescent or mass spectrometry-based methods have been used to measure β-gal activity in human dried blood spot, leukocytes, and fibroblasts,[21–25] predominantly to aid disease diagnosis. A previous study has also described a method which measured β-gal activities in CSF samples from individuals with Parkinson's disease or other neurological disorders which are unlikely to have reduced β-gal activities.[26] It is unclear whether this assay has sufficient sensitivity to accurately measure β-gal activity in CSF from patients with GM1. Recent progress with enzyme and gene replacement therapy has highlighted the need to develop highly sensitive and precise assays for the quantification of β-gal enzyme activity in target tissues from GM1 patients at baseline and post-treatment. In this study, we conducted fit-for-purpose validation of two fluorescent methods for the quantification of β-gal activity in human serum and CSF. Analytical performance of these assays was characterized by evaluating assay sensitivity, precision, accuracy, parallelism, assay specificity, and sample stability. Together, the results demonstrated that these assays could serve as useful tools for functional β-gal measurement in the CNS and periphery to support clinical trials in search of therapies for GM1-gangliosidosis.

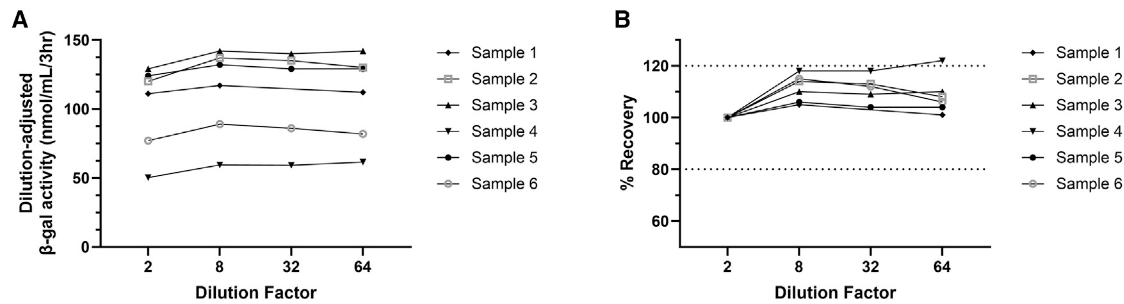

Figure 3. Parallelism of β-gal activity in human serum

Serum samples from individual healthy donors ($n = 6$) were serially diluted 2- to 64-fold to determine interference of endogenous matrix components with the measurement of β-gal activity. (A) The β-gal activity measured after correcting for the dilution factor is graphically depicted for each individual donor sample tested in the series. (B) The percent recovery of β-gal activity from each donor sample in the series is presented. Boundaries of ±20% recovery are presented as dotted lines.

This study presents an optimized and well-characterized fluorometric method with high enough sensitivity to quantitate β-gal activity in CSF samples from GM1 patients. Using this method, we were also able to detect significant increases in CSF β-gal activity after gene therapy (unpublished data). This is consistent with a pre-clinical study published earlier that showed, compared with samples from vehicle treated mice, a 4-MU-based fluorometric assay similar to the assays described here detected ∼3- to 100-fold increases in β-gal activities in CSF or serum from *Glb1*$^{-/-}$ mice treated with an AAV vector encoding the human *GLB1* gene.[13] The improved assay sensitivity for CSF measurements was achieved through the simultaneous use of higher CSF sample volume and an assay buffer with lowered pH in the assay system. The pH of human CSF is slightly alkaline, ranging from 7.28–7.32[19]; thus, the use of a higher volume of CSF in the reaction mixture may necessitate compensation by a lower assay buffer of pH 3.5 to maintain the acidic reaction conditions required for lysosomal enzyme activity.[27]

Preanalytical procedures, such as sample collection and handling conditions, are important for generating quality data in biomarker studies. In this study we observed that rapid processing and freezing of samples post-collection was critical to preserve lysosomal enzyme activity in CSF and serum. These observations are consistent with a previous report demonstrating progressive deterioration of β-gal activity in CSF maintained up to 48 h at room temperature or 4°C post-collection compared with flash frozen samples.[26]

It is common to encounter hemolyzed blood samples during clinical studies. Therefore, we assessed the effect of hemolysis on β-gal activity in serum. We observed that hemolysis led to reduced β-gal activity measurements in samples from healthy adults, indicating that hemoglobin may interfere with the assay. This was observed at all concentrations of hemolyzed blood tested.

The effect of blood contamination on CSF β-gal activity appears to be more complex. For CSF with normal β-gal activity levels, the presence of moderate blood contamination of up to 2% did not significantly impact measured β-gal activity. This finding is consistent with the report of Persichetti et al., which found no effect of blood contamination on CSF β-gal activity when erythrocytes were spiked into CSF at a concentration of up to 50,000 cells/μL (∼1% blood contamination).[26] However, we also observed that more substantial blood contamination (5%) in CSF did interfere with β-gal activity measurement (Table 1). Moreover, blood contamination in CSF that contained very low levels of β-gal activity (close to that observed in CSF from GM1 patients) led to an apparent increase in activity levels (Table 1). This may be, at least in part, due to the high levels of β-gal activity in blood or leukocytes.[28–30] Thus, our results suggest that CSF β-gal activity measurements may be influenced by multiple factors: (1) the concentration of hemolyzed blood present, (2) the enzyme activity within the CSF sample, and (3) the enzyme activity within the blood itself. Therefore, to obtain high quality β-gal activity data, it is important to minimize potential hemolysis during blood sample processing and avoid blood contamination in CSF sample collection.

It should be emphasized that although great care is needed to preserve β-gal activity during sample collection and processing, β-gal activity in CSF and serum is relatively stable once samples are frozen and stored at −80°C. We have demonstrated that β-gal activities in the two matrices are stable for over one year and repeated freeze/thaw cycles (up to three times) have no detectable impact (Table 3). In addition, we noted that β-gal activity in individual serum and CSF samples remained consistent when tested after storage at −80°C for up to approximately 8 or 11 weeks, respectively (Figure S4). While we did not run the exact same time points, this is in apparent contrast with a previous report which indicated instability of β-gal activity when CSF samples were stored at −80°C.[26] This apparent divergence may be due to differences in methodology and how the assay reagent and samples were handled between the laboratories. For example, to minimize analytical variability stemming from 4-MU standard curve reproduction, we prepared bulk 4-MU calibrators and stored them as single-use aliquots at −80°C for sample analysis in the Imagine-1 study.

There are two known lysosomal β-galactosidases encoded by distinctive genes, *GLB1* (EC 3.2.1.23) and *GALC*, also known as

Table 3. Stability determinations for endogenous QCs used in the serum and CSF β-gal assays

Assay matrix	Stability parameter	QC sample	Replicate precision (%CV)	Accuracy (%DFT)	Recovery (%)
CSF	thawed matrix (4 h on ice)	low eQC	3.10	−25.2	94.9
	3 freeze/thaw cycles		1.90	−22.8	98.0
	503 days at −80°C		2.94	21.71	N/A
Serum	Thawed matrix (4 h on ice)	mid eQC	8.51	−26.2	83.8
	3 freeze/thaw cycles		0.99	−18.3	92.4
	503 days at −80°C		0.19	7.53	N/A

Unspiked CSF matrix pool (low eQC) and serum matrix pool (mid eQC) samples were conditioned as described in the materials and methods and assessed for β-gal activity deterioration as compared with unconditioned eQC. The percent coefficient of variation (%CV) was calculated from triplicate values. Accuracy (%DFT) of β-gal activity measurement after a 4 h thaw or three freeze/thaw cycles was calculated based on eQC theoretical values established during method validation runs. The percent recovery of β-gal activity in the conditioned samples was determined relative to the activity measured in the unconditioned samples in the same analytical run. For measurement of long-term stability, observed β-gal activity was compared back to the theoretical activity to determine %DFT for each matrix as defined during method validation.

galactocerebrosidase (EC3.2.1.46).[31] The two enzymes have different tissue distribution and substrate preference. While GALC enzyme is enriched in the brain, it has been reported to have a strong preference for β-linked galactose conjugated proximally to glycolipids as seen in galactosylceramide; β-gal is enriched in liver and acts on β-linked galactose distal to the glycolipid moiety as seen in GM1 and GA1 gangliosides.[5,31] Tanaka and Suzuki reported that GALC activities were approximately four times higher in the brain than in the liver of healthy adults.[31] However, the relative protein abundance of GALC versus β-gal in the brain or CSF is not known and remains to be determined in future research. It is important to note that although our assays can detect activities from both rhGLB1 and rhGALC proteins, they preferentially measure rhGLB1 activity as we have shown that at a given concentration of recombinant protein, the enzyme activity of rhGLB1 is approximately 3-fold that of rhGALC.

The reference range for a biomarker is dependent on the method used and the sample matrix. Normal reference ranges for β-gal enzyme activity in human CSF and serum matrices are not currently available. Toward that goal, we measured β-gal activities in prospectively collected CSF and serum samples from healthy adults and found β-gal activities approximately 30-fold higher in serum than in CSF. These results are consistent with a previous report that β-gal enzyme is enriched in the liver relative to the brain.[31]

In GM1 patients, while β-gal activity is largely absent in general, residual β-gal activity and the presence of GALC enzyme has been described in previous reports.[9,31–33] Similarly, we detected low levels of β-gal activities in CSF and serum samples from early- and late-onset GM1 patients, and in samples from *Glb1*$^{-/-}$ mice. As expected, the median serum β-gal activity in the 12 GM1 patients was less than 10% of that in healthy adults. The median CSF β-gal activity in healthy adults was 2.73 nmol/mL/3 h which is comparable to what has been reported earlier in individuals with minor neurological disorders.[26] The median CSF β-gal activity in ten GM1 patients was 0.73 nmol/mL/3 h which is approximately 27% of the median level in healthy adults, and is higher than expected based on published reports that β-gal enzyme activities in infantile GM1 patients range from negligible to 1%–10% of normal levels.[1–3,8,9] It is worth noting that these previous reports describe β-gal activity levels that were measured in human dried blood spot, leukocytes, and fibroblasts,[21–25] and not in CSF. In addition, because the current assay can detect enzyme activities of both β-gal and GALC, the presence of GALC in the brain could have contributed to the higher-than-expected residual CSF β-gal activities detected in GM1 patients. To date, we are not aware of any β-gal activity assay completely differentiating the two β-galactosidases due to the lack of an enzyme substrate that is specific for β-gal, or an effective enzyme inhibitor for GALC.

A limitation of this study is that the assays described herein are not 100% specific for β-gal activity. The absence of a defined normal range for β-gal activity in the pediatric population poses another challenge. It is possible that CSF β-gal activities in healthy pediatric individuals are different than those in adults. A proper comparison of CSF β-gal activity between GM1 patients and healthy pediatric individuals can only be made when samples from these individuals become available. The interplay between β-gal and GALC in disease is also an area that needs further interrogation.

Conclusion

Highly sensitive and precise methods for the measurement of β-gal activity in CSF and serum are reported, enabling the quantitation of β-gal activity in GM1 patient samples. These assays may serve as a valuable tool for research on GM1-gangliosidosis and to support clinical trials investigating therapies for GM1 patients.

MATERIALS AND METHODS

Reagents

The following critical reagents were purchased from commercial sources: 4-MU (catalog no. M1381, Sigma, St. Louis, MO), 4-MUG (catalog no. M1633, Sigma), rhGLB1 protein (catalog no. 6464-GH-020, R&D Systems/Bio-Techne, Minneapolis, MN), rhHexB protein (catalog no. 8907-GH-020; R&D Systems/Bio-Techne), and rhGALC protein (catalog no. 6464-GH-020, R&D Systems/Bio-Techne).

Human samples

Remnant human serum and CSF were purchased from BioIVT (Hicksville, NY). Prospectively collected serum and plasma were obtained from volunteers from the PPD laboratory or BioIVT. CSF was prospectively collected from volunteers by BioIVT. For all prospective collections, individuals ≥18 years old without any known allergies or health problems, or on any significant medications, were eligible and

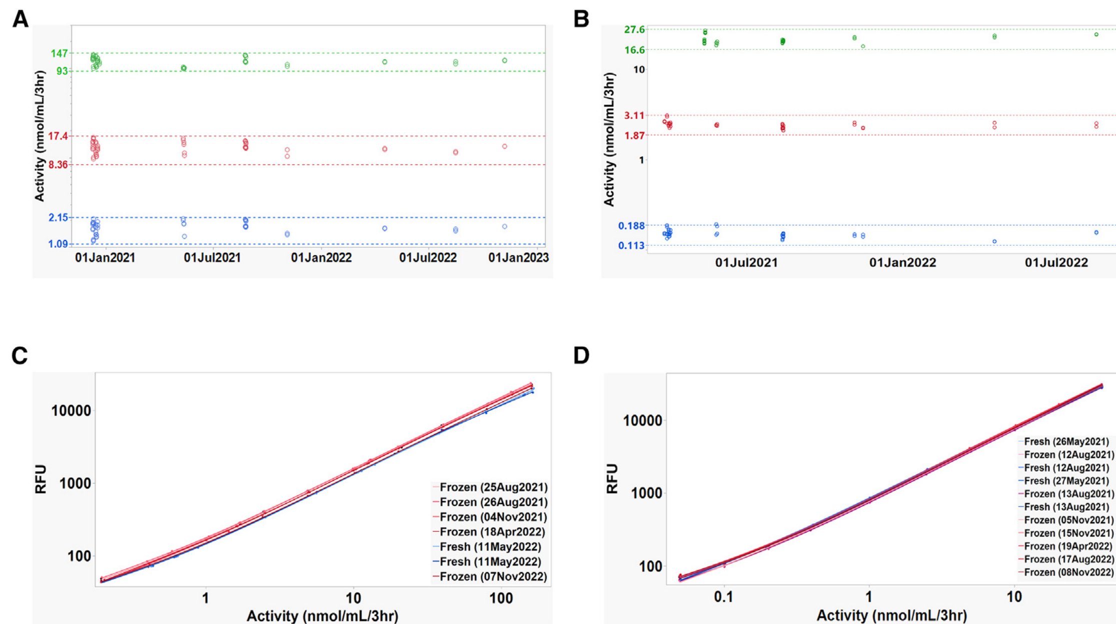

Figure 4. Assay robustness

Trending analysis to assess longitudinal QC performance for the serum (A) and CSF (B) β-gal activity assays. Results of rhGLB1 buffer QC (high QC [HQC]; open green circles) and the two endogenous QC, mid QC (MQC) (open red circles), and low QC (LQC) (open blue circles), were obtained in various runs over 2 years. The limits for each QC were defined by mean ± 2 SD results from validation runs and are denoted by the corresponding dotted lines (green, red, and blue). All individual QC data points fell within the limits defined for the corresponding QC level. Long-term stability of 4-MU calibrators prepared in bulk and stored at −80°C were characterized by monitoring for fluorescent activity over time for the serum (C) the CSF (D) assays. Independent runs using frozen 4-MU calibrators are depicted in red. Standard curves created using freshly prepared calibrators are included for reference and depicted in blue. Calibrator preparation and date of run are denoted.

consented to donation following each organization's institutional IRB. Serum or plasma were obtained from whole blood samples collected in the appropriate tubes following standard procedures. Blood was immediately processed for serum or plasma and frozen at −80°C within 1 h of blood collection. CSF was collected directly into glass or low protein binding microcentrifuge tubes (Eppendorf 022431102, Thermo Fisher Scientific, Waltham, MA) following standard lumbar puncture procedures under sterile conditions, then frozen at −80°C within 1 h of collection. Endogenous pooled matrix was prepared on ice by pooling human serum or CSF from individual donors.

CSF and serum samples were also collected from GM1 pediatric participants screened for or enrolled in the Imagine-1 trial (NCT04713475 [ClinicalTrials.gov]) with IRB approval and written consent from each individual. Children ranged from 6 to 31 months at the time of sample collection.

Mouse samples

Glb1 knockout mice (*Glb1*$^{-/-}$; RBRC00690) were obtained from RIKEN BioResource Research Center. Mice were maintained as heterozygous carriers *Glb1*$^{+/-}$ in a C57BL/6J background. Serum samples were collected from heterozygous littermates and *Glb1*$^{-/-}$ mice at age 4 months.[13] The facial vein was punctured with a lancet, and one to two drops of blood were collected in a 1 mL serum separator tube and allowed to clot for approximately 20 min at room temperature. Samples were centrifuged at 2,000 × *g* for 5 min, and serum was transferred to a polypropylene tube and frozen at −80°C.

Sample handling procedures

After the initial freeze post-collection, frozen samples were thawed and maintained on ice. Freeze-thaw cycles were limited ($\leq$3) during the study. A single freeze-thaw cycle was defined as freezing 12 h at −80°C followed by maintaining the sample on ice until full thaw was verified by visual inspection.

Assay procedure

β-gal activity was quantitatively measured from human CSF or serum using a fluorometric assay format. For the CSF β-gal activity assay, 45 μL CSF was combined with 30 μL of assay diluent (final concentration of 100 mM citric acid, 200 mM sodium hydrogen phosphate, and 0.1% BSA [pH 3.5]). For the serum β-gal activity assay, 15 μL serum was combined with 60 μL of assay diluent (final concentration of 100 mM citric acid, 200 mM sodium hydrogen phosphate, and 0.1% BSA [pH 4.2]). In each case, the substrate (4-MUG) was present in each well of a black plate at a final concentration of 600 mM. The plate was incubated for 3 h at 37°C and the reaction stopped with 1 M sodium carbonate (pH 11.5). Fluorescent 4-MU was detected using a

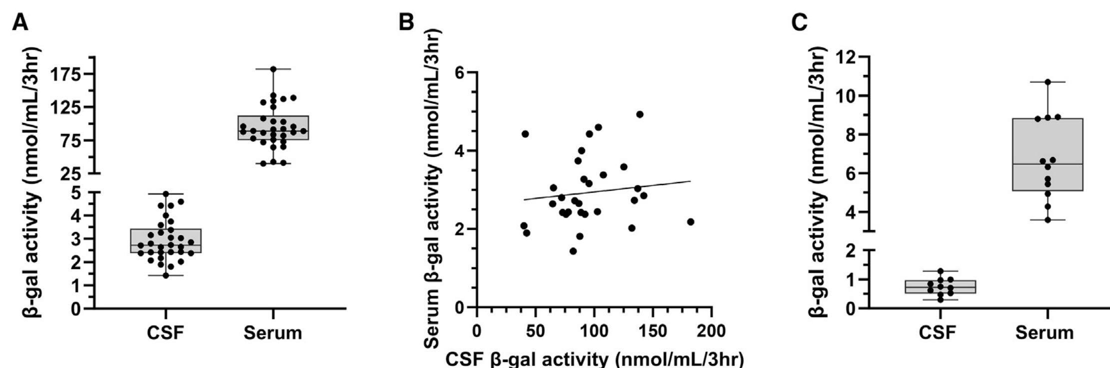

Figure 5. CSF and serum β-gal activities in healthy adults and pediatric GM1 patients

(A) Paired CSF and serum samples from adult healthy volunteers (n = 30) were assayed for β-gal activity. Boxplots report the median enzymatic activity and ranges of lower and upper quartiles. Error bars represent the full ranges of the data with individual values denoted with closed circles. (B) β-gal activity was not significantly correlated between paired CSF and serum samples for the 30 individuals (Pearson's r = 0.1217, p = 0.5218). C) CSF (n = 10) and serum (n = 12) samples collected from GM1 patients before treatment (i.e., baseline) in the Imagine-1 clinical trial were assayed for β-gal activity. Boxplots report the median enzymatic activity and ranges of lower and upper quartiles. Error bars represent the full ranges of the data with individual values denoted with closed symbols.

Tecan Spark microplate reader with excitation 360 nm/emission 460 nm β-gal enzyme activity was quantitated using the 4-MU standard curve, and data were presented as nanomole of 4-MU product released per mL (or μM) over a 3-h period. As described below, a combination of rhGLB1, 4-MU, pooled human CSF, and pooled human serum samples were used as QCs to ensure consistent performance of the assay. Except where noted, calibrators and samples were evaluated in duplicate (and considered one set or individual replicate), and the mean value calculated.

Reference standards and calibration curves

Ten 4-MU calibration standards were freshly prepared by 2-fold serial dilution to generate the nominal concentration range of 0.05–40 nmol/mL for the human CSF assay, or 0.200 to 160 nmol/mL for the human serum assay, respectively. These standards were prepared individually by fortifying assay diluent with 100 μmol/mL 4-MU reference standard stock solution dissolved in DMSO. In addition, rhGLB1 calibration standards were freshly prepared by 2-fold serial dilution of rhGLB1 with assay diluent to generate the nominal range of 0.100–150 ng/mL for the human CSF assay (10 standards), or 0.205 to 200 ng/mL for the human serum assay (9 standards), respectively. Each calibration standard was prepared independently by fortifying assay diluent with a 10 μg/mL stock solution of rhGLB1 protein. For sample analysis, the 4-MU calibration standards were run in duplicate per analytical run.

QC samples

eQCs samples were prepared from human serum or CSF pools generated using samples from individual donors. The CSF and serum matrix pools were aliquoted for single use and frozen at −80°C. For each analytical run, all eQCs were freshly diluted. Specifically, for the CSF assay, thawed CSF matrix pool was left unspiked or spiked with 15 ng/mL of rhGLB1 to generate mid and high eQCs corresponding to enzymatic activities in the mid- and high range of the calibration curve, respectively. Low QC was initially prepared by spiking rhGLB1 into assay buffer (pH 3.5) at 0.45 ng/mL during assay validation (Low QC; validation). However, this buffer QC was found to be unstable and was later replaced with 4-MU spiked into assay diluent at 0.150 nmol/mL (low QC; in-study analysis). For the serum assay, the serum matrix pool was diluted 1:8 for mid eQC and 1:64 for low eQC. Buffer QCs were prepared by spiking rhGLB1 into assay buffer (pH 4.2) at 120 ng/mL (high QC). Each QC sample was run in duplicate at the front and back end of each plate in an analytical run. Run acceptance during validation and sample testing was determined based on calibration curve and QC performance.

Assay characterization and validation

The following parameters were assessed to characterize the analytical performance of this method: assay specificity, LLOQ and ULOQ, assay accuracy and precision, parallelism, and sample stability.

Assay specificity

Specificity was evaluated using serum samples from *Glb1*$^{-/-}$ knockout and wild-type mice following the human serum assay format, as well as using recombinant human GLB1, GALC, and HexB proteins. For the latter, each recombinant human enzyme was resuspended in 30 μL of assay diluent pH 4.2 as required for the assay format.

LLOQ and ULOQ

The 4-MU and rhGLB1 calibration curves for each assay were run in triplicate over 3 days by 2 analysts for a minimum of 12 independent analytical runs to determine the assay dynamic range. The LLOQ was the lowest non-zero concentration level that was quantified with acceptable accuracy and precision (within 25% DFT and CV). The ULOQ was the highest concentration level that was quantified with acceptable accuracy and precision (within 25% DFT and CV).

Assay accuracy and precision

Accuracy and precision were evaluated across the calibration curve range by analyzing eQCs and buffer QCs. These enzymatic QCs, as well as 4-MU calibrators at the LLOQ and ULOQ levels, were run in triplicate over six independent runs, conducted by two analysts over 3 days. Assay precision was expressed as the %CV for each QC and matrix type, and accuracy was measured as the %DFT.

Parallelism

Human CSF or serum samples from individual donors were serially diluted 2-fold to a maximum dilution of 1:64 (serum) or 1:16 (CSF) to evaluate assay parallelism. The minimal required dilution for the serum assay was defined as 2 due to the modest suppression of β-gal activity observed when assaying neat serum. Samples in the high-sensitivity CSF assay were analyzed without dilution.

Sample stability

In brief, frozen aliquots of unspiked serum matrix pool (mid eQC) and unspiked CSF matrix pool (low eQC) were thawed on ice and tested immediately (unconditioned) or subjected to different handling conditions (conditioned), i.e., remained on ice for up to 4 h or subjected to three freeze-thaw cycles, to assess β-gal activity deterioration. Samples were also maintained at −80°C for 503 days to evaluate long-term stability. Before assaying, the serum matrix pool samples were diluted 1:8 (mid eQC). For each assessment, three independent replicates were run, and the %CV was calculated from the triplicate values. The accuracy or %DFT was calculated by comparing the QC sample β-gal activities with the theoretical activities defined during validation. The percent recovery of β-gal activity in the conditioned samples was determined relative to the activity measured in the unconditioned samples in a single analytical run.

Data analysis

A four-parameter logistic, 1/response^2 weighted regression algorithm was used for deriving sample data using PPD's laboratory information management system and ANOVA analysis as previously recommended.[34] Additional statistical analysis was performed using Microsoft Excel software or GraphPad Prism version 9.2.0 (GraphPad, San Diego, CA). Graphs were generated using GraphPad Prism or JMP version 15.0.0 (JMP, Cary, NC).

DATA AND CODE AVAILABILITY

The data supporting the studies presented in this paper are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS

The authors would like to thank Elyse Messick, Kathleen Brim, Samuel Schmidt, Joseph Green, and Scott Gilreath for technical assistance, Jiri Aubrecht for careful review of the manuscript, and Molly Hoke for editorial assistance.

AUTHOR CONTRIBUTIONS

Y.G.N. conceptualized the study. K.J.Q., C.V., C.D., C.W., and Y.G.N. designed the experiments and interpreted the data. C.J.H. and J.M.W. generated the mice used for the specificity assessment and contributed to the study design. C.V. conducted the experiments. K.J.Q. wrote the manuscript, with input from C.V., C.D., C.J.H., and Y.G.N. S.E.B., D.A.W., S.A.A.-Z., and J.M.W. provided intellectual contributions and reviewed and edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

At the time this project was conducted, K.J.Q., D.A.W., S.A.A.-Z., S.E.B., and Y.G.N. were full-time salaried employees of Passage Bio, Inc. and received stock options. C.V., C.D., and C.W. were full-time salaried employees of Thermo Fisher Scientific and have no conflicts of interest to disclose. J.M.W. is a paid advisor to and holds equity in iECURE, Passage Bio, and the Center for Breakthrough Medicines (CBM). He also holds equity in the former G2 Bio asset companies and Ceva Santé Animale. He has sponsored research agreements with Amicus Therapeutics, CBM, Ceva Santé Animale, Elaaj Bio, FA212, Foundation for Angelman Syndrome Therapeutics, former G2 Bio asset companies, iECURE, and Passage Bio, which are licensees of Penn technology. C.J.H. holds equity in a former G2 Bio asset-associated company. J.M.W. and C.J.H. are inventors on patents that have been licensed to various biopharmaceutical companies and for which they may receive payments.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101318.

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