High-Throughput Immunoassay for the Biochemical Diagnosis of Friedreich Ataxia in Dried Blood Spots and Whole Blood

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BACKGROUND: Friedreich ataxia (FRDA) is caused by reduced frataxin (FXN) concentrations. A clinical diagnosis is typically confirmed by DNA-based assays for GAA-repeat expansions or mutations in the FXN (frataxin) gene; however, these assays are not applicable to therapeutic monitoring and population screening. To facilitate the diagnosis and monitoring of FRDA patients, we developed an immunoassay for measuring FXN.

METHODS: Antibody pairs were used to capture FXN and an internal control protein, ceruloplasmin (CP), in 15 μL of whole blood (WB) or one 3-mm punch of a dried blood spot (DBS). Samples were assayed on a Luminex LX200 analyzer and validated according to standard criteria.

RESULTS: The mean recovery of FXN from WB and DBS samples was 99%. Intraassay and interassay imprecision (CV) values were 4.9%–13% and 9.8%–16%, respectively. The FXN limit of detection was 0.07 ng/mL, and the reportable range of concentrations was 2–200 ng/mL. Reference adult and pediatric FXN concentrations ranged from 15 to 82 ng/mL (median, 33 ng/mL) for DBS and WB. The FXN concentration range was 12–22 ng/mL (median, 15 ng/mL) for FRDA carriers and 1–26 ng/mL (median 5 ng/mL) for FRDA patients. Measurement of the FXN/CP ratio increased the ability to distinguish between patients, carriers, and the reference population.

CONCLUSIONS: This assay is applicable to the diagnosis and therapeutic monitoring of FRDA. This assay can measure FXN and the control protein CP in both WB and DBS specimens with minimal sample requirements, creating the potential for high-throughput population screening of FRDA.

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Friedreich ataxia (FRDA)10 (OMIM #229300) is an autosomal recessive neurodegenerative disease that presents with progressive ataxia, cerebellar dysarthria, sensory loss, and pyramidal signs (1, 2). Cardiomyopathy is apparent in most patients, and cardiac complications are a frequent cause of premature death (3, 4). The age of symptom onset can vary from childhood to adulthood. The estimated prevalence of FRDA is approximately 1 in 50 000 live births, making it one of the most common inherited ataxias. FRDA is caused by mutations in the FXN (frataxin) gene on 9q13 (5). Although effective therapies are presently lacking, several clinical trials are currently under way to examine the potential of antioxidants (i.e., idebenone), iron chelators (i.e., deferiprone), and other compounds to modulate symptoms or increase FXN concentrations (6). The impact of these promising therapies has been postulated to correlate with the timing of intervention (5, 7).

Most patients with FRDA are homozygous for expansion in the GAA trinucleotide repeat sequence in the first intron of FXN. These expansions impair gene expression, but up to 3% of FRDA patients have other molecular defects, such as point mutations within the FXN coding region and exonic deletions, which can complicate confirmation of a clinical diagnosis by molecular analysis exclusively (8). The FXN locus encodes a precursor polypeptide (FXN1–210) that is imported into the mitochondria and processed into the FXN pro-
tein, with 4 shorter isoforms (FXN⁴₂–₂₁⁰, FXN⁵₆–₂₁⁰, FXN⁷₈–₂₁⁰, and FXN⁸₁–₂₁⁰), of which FXN⁴₂–₂₁⁰ and FXN⁸₁–₂₁⁰ are the most abundant (5). FXN is important for the synthesis of iron–sulfur clusters and is critical for cellular iron metabolism (5). FXN mutations ultimately lead to a severe reduction in the FXN concentration and dysregulation of cellular iron homeostasis.

Molecular genetic analyses for detecting GAA-repeat expansions within intron 1 or mutations in the FXN coding sequence itself are currently used to confirm a clinical suspicion of FRDA. These assays are not applicable to population screening, however. In addition, protein-based assays are needed to monitor treatments aimed at increasing FXN concentrations, and such assays are currently under investigation (9). To address these emerging needs, recent efforts have been made to improve methods for measuring FXN concentrations in different tissues. Willis et al. manufactured and described a lateral-flow device that could measure FXN concentrations reproducibly down to 40 pg in lymphoblastoid cell lysates (10). Deutsch et al. demonstrated the dipstick’s clinical utility for whole blood (WB) and non-invasive buccal swabs by assessing a large cohort of FRDA patients, carriers, and unaffected individuals (11).

Lateral-flow devices are simple to use but lack the scaleability for application in a high-throughput testing environment. To address this limitation, Steinkellner and colleagues developed an electrochemiluminescence assay for a 96-well format to measure FXN concentrations in lymphocyte extracts (12). They also found a correlation in low FXN concentrations between skeletal muscle and blood mononuclear cell extracts from FRDA patients, indicating that WB concentrations of FXN could be used to assess the outcomes of clinical trials of new FRDA therapies (13).

We hypothesized that a duplex immunoassay for quantifying the concentrations of FXN and a control protein might be applicable to WB and dried blood spots (DBSs). Moreover, we investigated whether our approach could pave the way to a high-throughput format for population screening. We highlight a case report that describes successful use of this assay to assist in the diagnosis of an atypical FRDA patient without a full complement of GAA-repeat expansion alleles.

**Materials and Methods**

**SAMPLES**

Samples used for assay development were obtained in compliance with the Institutional Review Boards of Mayo Clinic and the Children’s Hospital of Philadelphia (CHOP). The latter collected 320 DBS samples with known FRDA status (affected, carrier, and normal). WB samples for reference values were collected at Mayo Clinic from consenting donors and deidentified residual clinical waste samples.

**ANTIBODIES AND REAGENTS**

Polyclonal, anti-FXN, rabbit detector antibodies (PAC 2517), and purified recombinant human FXN isoforms (FXN⁸₁–₂₁⁰ and FXN⁵₆–₂₁⁰) were generated and characterized as previously reported (14). Monoclonal anticeruloplasmin (anti-CP) mouse detection antibodies and purified recombinant human CP have also been characterized (15). Additional reagents were purchased from MitoSciences, including monoclonal, anti-FXN, mouse capture antibody (Anti-Frataxin antibody [17A11]), and recombinant human FXN protein. Polyclonal anti-CP rabbit antibodies were purchased from Cortex Biochem.

**REAGENT PREPARATION**

Microspheres were prepared by carbodiimide coupling of antibodies to xMAP® microspheres (Luminex), in accordance with the manufacturer’s suggested protocol (https://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/protein-coupling-protocol.pdf). We coupled 25 µg of anti-FXN antibody to 6 × 10⁵ microspheres (region 11, L100-C111-01) and 6 µg of anti-CP antibody to 6 × 10⁵ microspheres (region 8, L100-C108-01). Coupled-bead solutions were adjusted to 5 × 10⁵ beads/mL in PBS-BSA buffer (Phosphate buffered saline, pH 7.4, contains BSA; Sigma-Aldrich). The biotinylated detection antibodies were prepared by biotinylating 2.5 mg anti-FXN detection antibody and 1.6 mg anti-CP detection antibody with sulfo-NHS-LC-biotin according to the protocol supplied with the EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce). Biotinylated detection antibodies were adjusted to an Antibody Working Solution concentration of 1 µg/mL per antibody.

**SAMPLE PREPARATION**

Blood was collected by venipuncture into EDTA-containing tubes. Samples were aliquoted for sample-stability studies at refrigerated (4 °C), ambient (22 °C), and frozen (−20 °C) temperatures, as well as for freeze/thaw experiments. Additional blood samples were spotted onto Whatman 903 ProteinSAVER filter paper (GE Healthcare) for DBS preparation and stored frozen before analysis. FRDA patients participating in a longitudinal natural history study (2) provided blood samples, which were treated with EDTA and stored at −80 °C until used for DBS preparation. DBSs were also prepared from direct fingerstick, frozen EDTA-containing blood, or the remainder of original newborn-screening cards donated by patients and controls. These cards had been stored at ambient temperature or frozen, depending on the newborn-screening program. Once created or received, all DBS samples were stored frozen until analysis.
EDTA-containing blood samples and DBS eluents were spiked with recombinant human FXN$^{56-210}$ to a final concentration of 200 ng/mL and serially diluted to investigate recovery, linearity, limit of detection, and functional sensitivity.

**CALIBRATOR AND QC SAMPLES**

QC material was created by collecting 6 mL EDTA-anticoagulated blood from normal (high control) and FRDA (low control) donors. Blood from each donor was distributed into 1.5-mL microcentrifuge tubes (USA Scientific) by aliquoting 30 μL and storing the tubes at −80 °C until used. DBS QC material was prepared by distributing (via pipette) a single blood drop onto Whatman ProteinSaver 903 filter paper from the 6-mL EDTA-containing tubes of blood collected from normal (high control) and FRDA (low control) donors. DBS samples were dried for 4 h at ambient temperature and stored frozen. A 6-point calibration curve containing a mixture of recombinant human FXN$^{81-210}$ and recombinant CP was generated by serially diluting stock recombinant protein solutions and measuring their response with each analytical batch. The amounts of FXN calibrators per well were 63 pg, 31 pg, 15 pg, 8 pg, 4 pg, and 0 pg. The amounts of CP calibrators per well were 950 ng, 475 ng, 238 ng, 119 ng, 60 ng, 30 ng, and 0 ng. Recombinant human protein calibrator solutions were aliquoted and stored at −80 °C.

**ANTIBODY SELECTION**

We purchased 2 different anti-FXN mouse monoclonal antibodies from MitoSciences and purified 1 anti-FXN rabbit polyclonal antibody preparation from serum collected from a rabbit inoculated with recombinant human FXN$^{56-210}$, as previously described (14). These 3 antibody solutions were coupled to microspheres and biotinylated to assess the binding characteristics of 9 antibody pairs. The pair demonstrating the best analytical sensitivity and linearity was selected for further experimentation. In addition, a previous study indicated that FXN$^{56-210}$ and FXN$^{81-210}$ represent, respectively, the least and most abundant of the 4 mitochondrial FXN isoforms in different tissues, including lymphoblasts (14). Therefore, to detect total FXN concentrations we required that the antibody pair have identical affinities for the FXN$^{56-210}$ and FXN$^{81-210}$ isoforms. We tested anti-CP antibodies in the same manner and selected the pair with undetectable cross-reactivity to FXN. Both antibody pairs were optimized with standardized immunoassay procedures for measuring their working concentrations, as well as their affinities, sensitivities, and specificities. Each analyte immunoassay was developed independently in identical buffers to optimize analytical parameters. Both antibody pairs were tested for cross-reactivity among detection antibodies before combining the individual immunoassays into a duplex. Once combined, microsphere concentrations were adjusted to allow the Luminex LX200 instrument to count 100 beads from each set in a timely fashion.

**ASSAY PROTOCOL**

The assay protocol consisted of multiple steps. Wash Buffer was prepared by adding Tween 20 and sodium azide to PBS, pH 7.4, to a final concentration of 0.5 mL/L Tween 20 and 0.5 g/L sodium azide (Sigma-Aldrich). Assay Buffer was made by adding BSA, γ-globulins, Tween 20, and sodium azide to PBS, pH 7.4, to a final concentration of 0.5 g/L BSA, 0.5 g/L γ-globulins, 0.5 mL/L Tween 20, and 0.5 g/L sodium azide (all from Sigma-Aldrich). We placed one 3-mm DBS punch per sample into each well of a clear Nunc* MicroWell flat-bottom, sterile plate (Fisher). We extracted analytes by adding 200 μL Assay Buffer to every well, sealing the plate with an EZ Peel Sheet (BioSero), and shaking on an orbital shaker overnight at ambient temperature. We added EDTA-containing blood samples (15 μL) to microcentrifuge tubes containing 1 mL Assay Buffer for lysis. For the calibration curve, we serially diluted 200 μL of Recombinant Protein Standard Mixture (1.25 ng/mL FXN and 19 μg/mL CP in Assay Buffer) into microcentrifuge tubes containing 200 μL Assay Buffer. Once blood and protein calibration solutions were vortex-mixed, 50 μL of each sample were transferred into a well of a MultiScreen HTS-BV 1.2-μm filter plate (Millipore) that had been prewetted with 100 μL Assay Buffer. After an overnight incubation, 50 μL of each DBS eluent was added to the same filter plate. A Working Microsphere Mixture was made by diluting coupled anti-FXN and anti-CP microspheres in Assay Buffer to 5 × 10⁴ microspheres/mL. A 50-μL aliquot of the Working Microsphere Mixture was distributed to wells containing eluent. The 96-well plate was sealed and placed on a shaker for 2 h at ambient temperature. Each well was washed 3 times with 100 μL Wash Buffer with vacuum filtration. We added 100 μL of biotinylated detection antibody mixture to each well, sealed the plate, and incubated it for 1.5 h with shaking at ambient temperature. Wells were washed twice with 100 μL Wash Buffer with vacuum filtration. After the wash, we distributed 100 μL streptavidin-R-phycocerythrin conjugate (SAPE; Invitrogen) to each well, sealed the plate, and placed it on a shaker for 30 min at ambient temperature. We washed each well twice with 100 μL Wash Buffer with vacuum filtration and resuspended the microspheres in 100 μL Assay Buffer by gently them pipetting up and down with a multichannel pipettor before analysis. Data were collected and analyzed in multiplex acquisition mode on the Luminex LX200 instrument. Results were collected.
as median fluorescence intensities (MFIs) of 100 microspheres and exported into Microsoft Excel for calculating the number of nanograms of FXN per milliliter of blood and for data manipulation. For an analytical run to be deemed acceptable, we required that the coefficient of determination ($r^2$) be >0.99 for the linear regression of every FXN calibration curve, the QC samples (high and low controls) be within established limits, and the CP concentration of every sample be within its established interval.

**Results**

**DUPLEX ASSAY**

We previously developed an immunoassay for CP, a ferroxidase that plays a role in copper and iron metabolism, and characterized the antibody pair for this blood protein (15). We tested whether this pair could function within a duplex assay. The purpose of the CP analyte was to serve as an internal control for measuring FXN, and thus a common eluent volume was selected to best match the linear response of our anti-FXN antibody pair.

**CALIBRATION STUDIES**

The results from 4 repeat linearity experiments for recombinant FXN and CP spiked into DBSs and EDTA-containing blood eluents as measured with the duplex assay are summarized in Fig. 1. Saturation of the assay’s response to FXN began above 1000 ng/mL. Reference samples rarely demonstrated FXN blood concentrations >76 ng/mL (equivalent to 1250 pg/mL on the calibration curve); therefore, we adjusted our range for the daily calibration curves for FXN from 1250 pg/mL (or 63 pg/well) to 0 pg/well and for CP from 19 µg/mL (or 950 ng/well) to 0 ng/well.

While calibrating our duplex assay, we performed a study that compared fingerstick DBSs and EDTA-containing venous blood collected at the same time from 42 different individuals (see Fig. 1, A and B, in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue10). We observed a mean difference of 1%, with measured fingerstick FXN concentrations being generally slightly higher than for EDTA-containing blood. The observed difference in mean concentrations had no effect on determining our reference interval.

We compared the response of our duplex to that of purified solutions of the recombinant FXN isoforms, FXN56–210 and FXN81–210. Our analysis of the regression of MFI on recombinant FXN isoform concentration yielded a slope of 1.006 and an $r^2$ of 0.999 (see Fig. 2 in the online Data Supplement). FXN81–210 recovery was evaluated by analyzing solutions of DBS and WB eluents from FRDA samples supplemented with serially diluted known FXN81–210 concentrations. We added 3 FXN81–210 concentrations (5, 11, and 21 ng/mL) and analyzed these samples over 3 days. The mean FXN recovery was 99% (range, 78%–112%), and there was no difference in recovery between DBS and WB eluents.

The limit of detection was determined by measuring the mean MFI plus 2 SDs from 15 replicates of the zero calibrator. Our calculated FXN limit of detection was 0.07 ng/mL. The assay’s functional sensitivity was determined by analysis of 10 replicates of a solution containing FXN at 0.14 ng/mL. The 10 replicates had a mean FXN concentration of 0.14 ng/mL and a CV of 14%.

The intraassay CV was determined by measuring FXN in 20 replicates each of DBS and WB samples from a healthy adult, a carrier, and a patient in the same batch. The intraassay CVs are shown in Table 1. The interassay CV was determined by calculating the CVs for FXN concentrations from DBS and WB samples (n = 31) obtained from a healthy adult, an FRDA carrier, and an FRDA patient and analyzing the samples on 20 different days. The interassay imprecision for FXN is also shown in Table 1.

**REFERENCE INTERVAL STUDIES**

The age and sex distribution of reference individuals who donated samples at Mayo Clinic are listed in Table 1 in the online Data Supplement. Fingerstick and heel-stick DBS samples came from 43 adults and 41 newborns. FXN was measured for 125 adults (52 males and 73 females) from both DBS and EDTA-containing WB samples. In addition, FXN was measured in 157 DBS and EDTA-containing WB samples from children (98 males and 59 females; see Fig. 3 in the online Data Supplement). Adult and pediatric FXN concentrations ranged from 15 to 96 ng/mL (median, 33 ng/mL) for DBSs and WB and indicated no age dependency.

**CLINICAL VALIDATION**

To date, WB and DBSs have been analyzed from samples collected from 121 patients, 63 carriers, and 325 individuals from our reference population (288 from Mayo Clinic and 37 from CHOP). Patients’ GAA-repeat lengths ranged from 18 to 1550, and the range of summed GAA lengths was 330–2300 (median, 1433). Eighteen patients were characterized as having a late-onset form of FRDA, which we defined as any presentation after 40 years of age. The measured FXN concentration was inversely proportional to the sum of GAA-repeat lengths (Fig. 2), and patients with a disease onset at >40 years of age had shorter GAA-repeat lengths and had FXN concentrations in DBSs >5 ng/mL. Fig. 3 shows the ranges of FXN concentrations in DBSs obtained from our reference population, carriers, and patients and sorted by age of disease onset. FRDA patients were readily distinguishable from controls,
but we found that FXN concentrations overlapped for carriers and unaffected individuals, as well for carriers and patients with a disease onset of >40 years of age. In an attempt to improve the discrimination of FRDA and carrier status from the reference population, we used the ratio of the FXN MFI to the CP MFI. Fig. 4A shows that the MFI for FXN was lower for FRDA patients with an earlier age of onset, whereas the CP MFI intervals of...
the reference population, the carrier group, and the patient group overlapped completely (Fig. 4B). Both carriers and FRDA patients, however, had a median CP value that was almost double the MFI of CP for the reference population, regardless of age of onset. This observation triggered the assessment of the FXN/CP MFI ratio as a postanalytical interpretation strategy to improve the segregation of FRDA patients from carriers and controls when FXN concentration data alone remained inconclusive (Fig. 4C). Most FRDA patients were distinguishable from carriers with this ratio. To apply this tiered method to testing, we created an algorithmic tool that combined the absolute FXN concentration and the FXN/CP MFI ratio to calculate the diagnostic likelihood of FRDA. This tool was the product of multivariate pattern-recognition software developed under the auspices of a worldwide collaborative project to foster laboratory quality improvement of newborn screening by tandem mass spectrometry (16). Instead of a rigid cutoff value, the FRDA postanalytical interpretive tool compared the FXN concentration and the FXN/CP MFI ratio with the disease ranges of the true-positive cases used for clinical validation of the assay (see Fig. 4 in the online Data Supplement). We calculated a score based on the separation from the disease range and the degree of penetration within the disease range of each informative marker (16). Two

Table 1. WB and DBS intraassay and interassay imprecision.

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<td>CV, %</td>
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Fig. 2. Sum of GAA-repeat lengths vs. FXN concentrations for FRDA patients.
We summed the reported lengths of the GAA-trinucleotide repeat alleles for the participants with a disease onset younger than (black circles) or older than (gray circles) 40 years.

Fig. 3. Box-and-whisker plots showing the 1st and 99th percentiles (whiskers) and 10th and 90th percentiles (box) of the reference population range (reference interval, n = 319), carriers (n = 62), FRDA patients with an age of onset <40 years of age (n = 90), and FRDA patients with an age of onset >40 years of age (n = 27).
Open circles indicate the median of each interval.
separate tools were created to evaluate cases on the basis of age of disease onset. Fig. 4 in the online Data Supplement shows how the “FRDA <40 years tool” properly generated an uninformative score (lower than the value corresponding to the 1st percentile of affected cases) for 100% of 441 unaffected individuals and 15 carriers. On the other hand, 98% of FRDA patients with a presentation at <40 years of age and 86% of FRDA patients with a presentation at >40 years of age had an informative score and therefore an indication of disease. Since 2010, the prospective clinical use of this assay has led to the identification of 17 FRDA patients and 3 carriers from the 76 individuals tested.

CASE REPORT
As an example of the use of this assay, we had the opportunity to evaluate a 25-year-old woman with a 6-year history of myelopathic spasticity, peripheral neuropathy, and dysphagia. At the time of review, she required a walker for ambulation. Her family history was clinically relevant for a sister with similar gait difficulties and neuropathy from childhood. The patient’s medical history included genetic testing for a panel of ataxia syndromes, such as the FXN GAA-repeat expansion test, which identified a single expanded allele (900 GAA repeats) and led to the conclusion that she was an FRDA carrier. During reevaluation, the patient’s FXN blood concentration was 3 ng/mL (1st percentile of the reference interval, 17.3 ng/mL), corresponding to the 30th percentile of the FRDA disease interval (n = 90, patients <40 years of age). Detection of a low blood FXN concentration prompted additional molecular studies, and sequencing of the FXN gene identified a pathogenic mutation, c.398G>A (p.G130V), on the second allele, confirming a diagnosis of FRDA.

Discussion
FRDA is typically silent for several years after birth until a constellation of progressive deficits become apparent. Symptoms include muscle weakness, loss of voluntary-movement coordination, cardiac disease, skeletal deformities, vision and hearing loss, and diabetes. The phenotype progressively leads to wheelchair confinement, inability to perform independent daily activities, and ultimately death from cardiac and other complications. Effective management of FRDA is generally accepted to require both treatments to increase FXN concentrations and strategies to diagnose and treat patients as early as possible. Although current treatment remains primarily supportive, several clinical trials are under way. For example, idebenone and deferiprone have been shown to improve cardiac and neurologic function in symptomatic patients (17–19). As is the case with other progressive conditions, there is

Fig. 4. (A), Box-and-whisker plots of the FXN MFI ratio as measured for DBS punches with the Luminex assay. The 1st and 99th percentiles (whiskers) and 10th and 90th percentiles (box) of the reference population range (reference interval, n = 319), carriers (n = 62), FRDA patients with an age of onset <40 years of age (n = 90), and FRDA patients with an age of onset >40 years of age (n = 27). (B), Box-and-whisker plots of the CP MFI from the same samples as in (A). (C), Box-and-whisker plots of the FXN/CP MFI ratio from the same population as (A) and (B). Open circles indicate the median of each interval.
reason to believe that the initiation of treatment as early as the disease process as possible will lead to the greatest benefit and likelihood of reducing, if not preventing, morbidity and mortality (18). Therefore, the US Secretary’s Advisory Committee on Heritable Disorders in Newborns and Children has suggested including FRDA in future considerations of newborn-screening programs (http://www.curefa.org/FARA%20SACHDNC%20Public%20Comments.pdf). Confirmation of a diagnosis currently relies on molecular genetic testing of FXN, which is not amenable to high-throughput analysis. Our goal was to develop an approach that could take advantage of FXN as a potential FRDA biomarker and measure FXN in DBS extracts, the traditional specimen for newborn screening.

Our duplex immunoassay measures FXN and a control protein, CP. The simultaneous analysis of FXN and CP concentrations enhances the discrimination of affected individuals from carriers and distinguishing carriers from a reference population, and the results underscore the success of the multianalyte approach to improve FRDA detection. This type of multianalyte approach was previously shown to advance the interpretation of newborn-screening results for amino acid and acylcarnitine profiles (16). One shortcoming of our approach was the use of a cohort of previously diagnosed FRDA patients. Because such a cohort is likely to be under some form of intervention aimed at ameliorating symptoms and if such therapies is successful at increasing FXN concentrations, the FXN concentrations we measured may not be within the “true” interval expected for undiagnosed patients. Hence, it is uncertain whether the observed overlap in FXN concentrations between FRDA patients and carriers is a manifestation of the biochemical diversity of FRDA or an indication of the influence of therapy.

In summary, our immunoassay for measuring FXN concentrations in DBSs and WB is applicable to the diagnosis, population screening, and therapeutic monitoring of FRDA. The significant reduction in FXN concentrations in DBSs for patients that we observed is consistent with the reductions previously reported for multiple tissue types (10, 11, 13).

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