Comparison of Types of Relatively High-Throughput Frataxin Assays reported in the literature.

Friedreich's Ataxia is caused by low levels of the protein frataxin, a consequence of mutations in the frataxin gene. Many potential therapies disease aim to increase levels of frataxin, targeting the root cause of the disease. Thus, sensitive, accurate and quantitative measurement of frataxin is an important biomarker in drug development, while understanding protein levels is also important in basic research. Several frataxin assays have been developed with different characteristics, which can be used for different contexts of use both *in vitro* and *in vivo*. This table lists published assays with their characteristics and references. Please contact Jane Larkindale (jane.larkindale@curefa.org) if you would like an introduction to specific investigators who have developed or validated methods or would like more information.

	Dipstick	**Luminex**	**ELISA**	**ECLIA**	**Liquid Chromatography-High Resolution Mass Spectrometry**
Method	Willis et al, 2008 and Deutsch et al, 2010, Plasterer et al., 2013	Oglesbee et al, 2013	Plasterer et al, 2013, Koeppen et al. 2011	Steinkellner et al, 2010	Guo et al., 2018
Detection	Rapid, noninvasive lateral-flow immunoassay. Anti-frataxin mAbs were generated by immunizing mice (F_1 BALB/cJ × SLJ/J) with soluble, native recombinant human frataxin; amino acids 56–210 prepared as previously described [Cavadini, et al. Hum Mol Genet. 2002;11:217]. This construct corresponds to the 155 amino acid form of frataxin that was thought to be present inside mitochondria after removal of the mitochondrial targeting sequence (amino acids 1-41) by mitochondrial processing peptidase (MPP) followed by another MPP cleavage of 14 amino acids (42-55) from the precursor protein (Cavadini et al. J Biol Chem. 2000;275: 41469). The two mAbs selected as dipstick immunocapture (clone	Antibody pairs were used to capture FXN and an internal control protein, ceruloplasmin. 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 (Gakh et al. J Biol Chem. 2010;285:38486). Additional reagents were purchased from MitoSciences (no longer available), 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	Enzyme-linked immunosorbent assay. The assay system utilizes a mouse anti-frataxin antibody for the solid-phase (microtiter wells) immobilization. A rabbit anti-frataxin antibody was used as secondary antibody. Frataxin levels were normalized to the protein content in the samples. The protein content of the samples was determined by a Bradford protein assay (Bio-Rad, Richmond, CA).	Electrochemiluminescence assay (ECLIA) to measure frataxin protein levels. The primary mouse anti-frataxin monoclonal capture antibody was purchased from Chemicon (Clone: 1G2, #MAB1594) (Millipore); the secondary frataxin (H-155) rabbit polyclonal antibody was from Santa Cruz Biotechnology. The third antibody which was used as detection antibody was goat anti-rabbit HRP-Sulfo-TAG™ from Meso Scale Discovery (Gaithersburg, US).	The assay is based on stable isotope dilution immunopurification two-dimensional nano-ultrahigh performance liquid chromatography/parallel reaction monitoring/mass spectrometry.

	ID# 17A11AC7) and detector (clone ID# 18A5DB1) mAbs recognize frataxin in all assay formats (Western, immunofluorescence, dipstick, ELISA etc.).	Biochem.			
Uses	Diagnostic, FXN measurement in peripheral tissues, biomarkers, assessment of disease severity	Under validation for newborn screening, high throughput screening, available as a diagnostic test to be used as an adjunct to genetic testing			Can detect size of frataxin and absolute amounts.
Calibration	The relationship between frataxin loaded and dipstick signal was linear through 500 pg of recombinant frataxin and did not saturate until approximately 4,000 pg of recombinant frataxin. The assay's linear range could be expanded significantly by normalizing the frataxin signal to the signal of the "procedural control" (goat anti-mouse IgG, GAM) band, which is an internal positive control automatically run on each dipstick	Reference adult and pediatric FXN concentrations ranged from 15 to 82 ng/mL (median, 33 ng/mL) for dried blood and whole blood.		Recombinant frataxin can be measured over a wide range (0–2000 picograms (pg) per well, with $R^2 = 0.99$)	Calibration standards were prepared by spiking appropriate amounts of the frataxin standard to 500 µg frataxin-depleted platelet lysates or 500 µg of BSA to make the final concentrations of 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, 20, 40 pg/µg protein. The lower limit of quantification (LLOQ) of 0.08 pg/µg protein was defined as the lowest QC sample.
Reliability	Intra-assay (3.3–11.2% and 0.5–12.2%, control and patient, respectively), interassay (10.3% and 5.3%, control and patient, respectively), and intersample (17.4%, control) coefficient of variation (CV) values.	Intraassay (CV) values were 4.9%–13%. Interassay imprecision (CV) values were 9.8%–16%,	CV was 15.6% over a period of several weeks (intersample)	Intraassay: 1.4%-3.8% depending on tissue, Interassay: 1.8-7.2% depending on tissue	100% sensitivity and specificity for discriminating between controls and FA cases. Variance: intraday: (n=5) precision 5.1%, accuracy 96.8%; interday (n=5), precision 3.7%, accuracy 101.0%
Sensitivity	Accurately measures picogram levels of frataxin protein - 40 and 4000 pg/test or approximately 0.1 – 10 nM of sample.	The FXN limit of detection was 0.07 ng/mL, and the reportable range of concentrations was 2–200 ng/mL.		Pictograms, narrow range can be measured.	The lower limit of quantification is 0.08 pg frataxin/µg protein
Tissues that can be assayed	Buccal cells, Whole blood and lymphoblastoid lines have been tested.	Whole blood, dried blood spots		Multiple tissues, require invasive collection of tissues	Platelets, whole blood, likely other tissues. Can distinguish between FXN from human and other species (mouse and non-human primates)
Notes	Western blot	Very high	Can be done in 96	Labor intensive,	Quantitative.

	examination of lymphoblast whole cell extracts showed significant amounts of the intermediate and mature forms of frataxin (frataxin42–210 and frataxin56–210), while only trace amounts of the precursor were revealed.	throughput	well plate format, human or mouse	despite 96 well format.	

Frataxin ELISA Kits

FARA is grateful to the companies and academic groups that have developed systems for measurement of human and mouse frataxin and to the research community for characterization of these assays, which are mostly ELISA assays.

In addition to ELISA assays, Ian Blair's group at the Children's Hospital of Philadelphia has developed a mass spectroscopy method for frataxin measurement, as published in Guo et al (2018) "Liquid Chromatography-High Resolution Mass Spectrometry Analysis of Platelet Frataxin as a Protein Biomarker for the Rare Disease Friedreich's Ataxia." Anal Chem. 2018 Feb 6;90(3):2216-2223.

An overview of currently available human and mouse assay kits available commercially or referenced in the literature is detailed below:

Commercial Human Frataxin ELISA Kits				
Frataxin Assay	**Vendor**	**Catalogue #**	**References**	**Product Link**
ELISA	Abcam	ab176112	3	http://www.abcam.com/human-frataxin-elisa-kit-ab176112.html
Dipstick ELISA	Abcam	ab109881	16	http://www.abcam.com/frataxin-protein-quantity-dipstick-assay-kit-ab109881.html*
ELISA	Abcam	ab201121	0	http://www.abcam.com/human-frataxin-elisa-kit-10-x-96-well-plate-ab201121.html
ELISA	Millipore	EAMT002 (Discontinued)	0	http://www.emdmillipore.com/US/en/product/Frataxin-ELISA-Kit,EMD_BIO-EAMT002
ELISA	Biomatik	EKC33754	0	http://www.biomatik.com/products/elisa-kits/human-frataxin-mitochondrial-fxn-elisa-kit.html
ELISA	Antibodies online	ABIN4993459	0	https://www.antibodies-online.com/kit/4993459/Frataxin+FXN+ELISA+Kit/?utm_source=partner&utm_medium=biocompare&utm_campaign=non_sponsored&utm_content=kit&utm_term=ABIN4993459
ELISA	Antibodies online	ABIN2636770	0	https://www.antibodies-online.com/kit/2636770/Frataxin+FXN+ELISA+Kit/?utm_source=partner&utm_medium=biocompare&utm_campaign=non_sponsored&utm_content=kit&utm_term=ABIN2636770

ELISA	Aviva Systems Biology	OKCA01248	0	https://www.avivasysbio.com/en/fxn-elisa-kit-human-okca01248.html
ELISA	LifeSpan Biosciences	LS-F23370	0	https://www.lsbio.com/elisakits/human-fxn-frataxin-elisa-kit-sandwich-elisa-ls-f23370/23370?trid=247
ELISA	MyBioSource	MBS935917	1	https://www.mybiosource.com/prods/ELISA-Kit/Human/frataxin/FXN/datasheet.php?products_id=935917#QLREF
ELISA	MyBioSource	MBS2515805	1	https://www.mybiosource.com/prods/ELISA-Kit/Human/frataxin/FXN/datasheet.php?products_id=2515805#QLREF
ELISA	Biorbyt	orb404450	0	http://www.biorbyt.com/human-fxn-elisa-kit
AlphaLISA	PerkinElmer	AL322HV (100 assay); AL322C (500 assay); AL322F (5,000 assay)	0	http://www.perkinelmer.com/product/alphalisa-hfrataxin-kit-100pts-al322hv

*Check online comments on this kit.

Frataxin Assay	Frataxin Assay	Frataxin Assay
ELISA	developed by Koeppen group	Koeppen et al. (2011) "The neuropathy of late onset Friedreich's ataxia." *Cerebellum.* **10**:96-103.
ELISA	developed by Sturm group	Boehm et al. (2011) "Variations of frataxin protein levels in normal individuals." *Neurol Sci.* **32**:327-30.
ECLIA	developed by Sturm group	Steinkellner et al. (2010) "A high throughput electrochemiluminescence assay for the quantification of frataxin protein levels." *Anal Chim Acta.* **659(1-2):**129-32

Other ways to Measure Frataxin Assays in the Literature*:

	Western Blot	Immunofluorescence
Method	e.g. Campuzano et al, 1997	e.g. Campuzano et al, 1997
Detection	The strongest reacting antibody, mAb 1G2, was used to detect endogenous frataxin on Western blots of normal human muscle, heart, cerebellum and spinal cord extracts. They saw a smaller mature form as well as the larger products presumably representing intermediates of the maturation process. The same, though fainter, band was obtained with mAbs 1D4 (Fig. 2A) and 2F10. mAbs 1H1 and 2D4 failed to detect endogenous frataxin, indicating that the N-terminal epitope they recognize is removed during	The strongest reacting antibody, mAb 1G2, was used to detect endogenous frataxin on Western blots of normal human muscle, heart, cerebellum and spinal cord extracts. They saw a smaller mature form as well as the larger products presumably representing intermediates of the maturation process. The same, though fainter, band was obtained with mAbs 1D4 (Fig. 2A) and 2F10. mAbs 1H1 and 2D4 failed to detect endogenous frataxin, indicating that the N-terminal epitope they recognize is removed during maturation.

	maturation. Willis et al used: The two mAbs selected as dipstick immunocapture (clone ID# 17A11AC7) and detector (clone ID# 18A5DB1) mAbs recognize frataxin in each of these assay formats.	Willis et al used: The two mAbs selected as dipstick immunocapture (clone ID# 17A11AC7) and detector (clone ID# 18A5DB1) mAbs recognize frataxin in each of these assay formats.
Uses	Can detect which size of frataxin is being measured (e.g. pre-cursor, intermediate or mature), and relative amounts of each form	Can detect cellular location of frataxin.
Calibration	Needs to be repeated with each blot	Depends on antibody used
Reliability	Depends on antibodies and controls	Not quantitative
Sensitivity		Depends on antibody used
Tissues that can be assayed	Any tissues	Any tissues
Notes	Only marginally quantitative, time consuming.	Time consuming, non quantitative

*A summary of data supporting the use of different antibodies that can be used for these purposes will be added soon.

Examples of RT-PCR Primers and Controls Used in Literature

RT-PCR measures FXN mRNA which may or may not reflect levels of FXN protein or mature protein. However, it is a cheap, high throughput measure to look at FXN levels in most tissues.

Reference	Primers	Control Gene	Reliability / Sensitivity
Plasterer et al 2013	TaqMan Gene Expression Assay for frataxin (Hs00175940_m1) https://www.thermofisher.com/taqman-gene-expression/product/Hs00175940_m1?CID=&ICID=&subtype=	GAPDH	18.0% CV over a period of several weeks (intersample)
Chutake et al., 2014 and 2015	Ex1 AGCAGCATGTGGACTCTC TGGGCTGGGCTGGGTGACGCCAGG Ex1-In1 CCGACATCGATGCGACCTGC GTTCCCGGCGCGGATACTTACT Ex2-Ex4 CCGCGCAAGTTCGAACCAA TTCCTAGATCTCCACCCAGT	*Tbp* gene (forward primer: 5'-CCTTGTACCCTTCACCAATGAC-3', reverse primer: 5'-ACAGCCAAGATTCACGGTAGA-3') for experiments using fibroblasts OR RPS29 GCACTGCTGAGAGCAAGATG ATAGGCAGTGCCAAGGAAGA	
Chutake et al., 2014 and 2015	Ex1 AGCAGCATGTGGACTCTC TGGGCTGGGCTGGGTGACGCCAGG Ex1-In1 CCGACATCGATGCGACCTGC	Geometric mean of the control genes *Psmd4*(forward primer: 5'- TGGAGAGCACTATGGTTT	

	GTTCCCGGCGCGGATACTTACT Ex2-Ex4 CCGCGCAAGTTCGAACCAA TTCCTAGATCTCCACCCAGT	GTGT-3'; reverse primer: 5'-ACGTTATTCTCAGGGTTGCTTC-3') and *Eef2* (forward primer: 5'-TGTCAGTCATCGCCCATGTG-3'; reverse primer: 5'-CATCCTTGCGAGTGTCAGTGA-3') for mouse tissues	
Long et al., 2017	Repeat Sequences: GAA_F: 5′-GGCTTGAACTTCCCACACGTGTT and GAA_R: 5′-AGGACCATCATGGCCACACTT 5q23 locus 5q23F: 5′-GTTGCATAGATAAATCAAATTCAT and 5q23R: 5′-ACTCACAGAAAGTATTATTATTCC Intron 1- exon 2 In1Ex2F: 5′-AGCACTCGGTTACAGGCACT and In1Ex2R: 5′-GCCCAAAGTTCCAGATTTCC	Looking at size not expression level.	

This paper describes RNAseq analysis and appropriate genes to use as control genes for RT-PCR:

Dis Model Mech. 2017 Nov 1;10(11):1353-1369. doi: 10.1242/dmm.030536.
Comprehensive analysis of gene expression patterns in Friedreich's ataxia fibroblasts by RNA sequencing reveals altered levels of protein synthesis factors and solute carriers.
Napierala JS, Li Y, Lu Y, Lin K, Hauser LA, Lynch DR, Napierala M.

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