

# DEVELOPMENT OF A HIGH THROUGHPUT ASSAY FOR FABRY DISEASE IS EFFECTIVE FOR DETECTION OF AFFECTED VERSUS UNAFFECTED POPULATIONS

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## INTRODUCTION

Fabry Disease is an X-linked lysosomal disorder due to an enzyme deficiency of *alpha galactosidase A* (AGAL, EC 3.2.1.22) resulting in multi-systemic organ dysfunction, including major manifestations of renal, neurological, and cardiac disease. Fabry disease is an inherited X-linked disorder, caused by a defect of the *GLA* gene. Deficient AGAL causes a neutral glycosphingolipid with terminal  $\alpha$ -galactose moieties, predominantly globotriaosylceramide (Gb3, GL-3, or ceramide trihexoside), to accumulate progressively in lysosomes. The diagnosis of Fabry disease is generally made in males by documenting deficient *alpha-galactosidase A* enzyme activity in plasma, isolated leukocytes, and/or cultured cells which are labor intensive biochemical assays. Nearly 100% of affected males have total or near total enzyme deficiency. In females, however, measurement of AGAL enzyme activity is difficult to interpret, and some enzyme levels fall in the low-normal range. Although molecular testing is most reliable for diagnosis of females, it is not practical as a screening paradigm for large populations due to high cost. A rapid, cost-effective enzyme assay platform is needed to "tier" the indication for molecular testing.

## METHODS

Enzymatic and molecular testing methods on the same filter paper or EDTA collection tube was developed. 88 known Fabry patients (73% female) and 690 unaffected controls (41% female) were collected for validation following Clinical and Laboratory Standards Institute (CLSI) guidelines to create a Fabry Complete™ testing method. A complementary heteroduplex scanning method was developed for the *GLA* gene on a similar but smaller population to rapidly identify gene defects associated with Fabry disease when AGAL enzyme activity and clinical indications.

**Enzyme Assay:** The activity of lysosomal  $\alpha$ -galactosidase A was quantitatively measured by detecting the fluorescence of 4-Methylumbelliferone (4-MU) generated when the substrate 4-Methylumbelliferyl- $\alpha$ -D-glucopyranoside (4-MUGal) is cleaved by AGAL in an acidic pH within 20 hours. Mean AGAL activity with standard deviation and 95% CI of mean were reported for each group. The statistical analysis in between groups was performed using parametric techniques. Means were compared by two-tailed unpaired t-test or one-way analysis of variance (ANOVA) with Turkey's post test and 95% confidence interval.

**DNA Assay:** A heteroduplex scanning method was developed using LightScanner Technology (Idaho Technology) to detect mutations in the *GLA* gene.

## FABRY ADULTS (Affected) VS. CONTROL ADULTS (Unaffected)

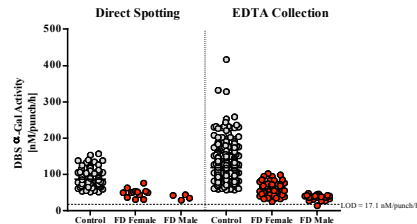


Figure 1. Comparison of Fabry Disease and Control Cohorts. Comparison of AGAL activity of 564 controls and 71 Fabry disease (FD) patients in DBS prepared from blood collected into EDTA tubes and AGAL activity of 126 controls and 17 Fabry disease (FD) patients in DBS prepared by direct blood spotting. Observed range AGAL activities for control and Fabry population are listed in Table 1. One male Fabry disease sample was below the LOD.

## Effect of Blood Collection Methods in Control Adults

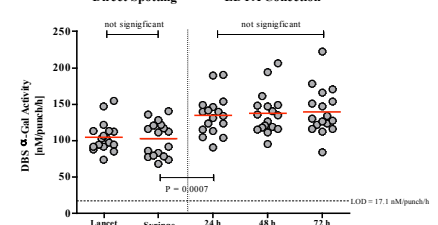


Figure 2. Effect of Blood Collection Methods on AGAL Activity in Control Adults. The mean AGAL activity in DBS prepared from blood collected into EDTA tube was significantly higher than AGAL activity in DBS prepared by direct blood spotting ( $p = 0.0007$ ) in all subjects. No difference was observed between direct blood spotting from venipuncture syringe draw or from lancet ( $p = 0.73$ ). The mean AGAL activity remained relatively stable when the blood was spotted after storage at 4 °C for 24, 48 and 72 h.

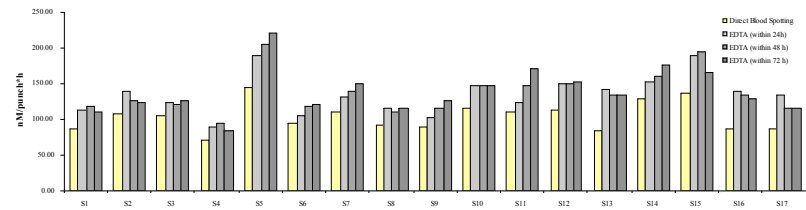


Figure 3. AGAL activity in Controls Over Serial Time Determinations. Time point sampling did not have a statistically significant effect on AGAL enzyme levels in the sample biologic patient.

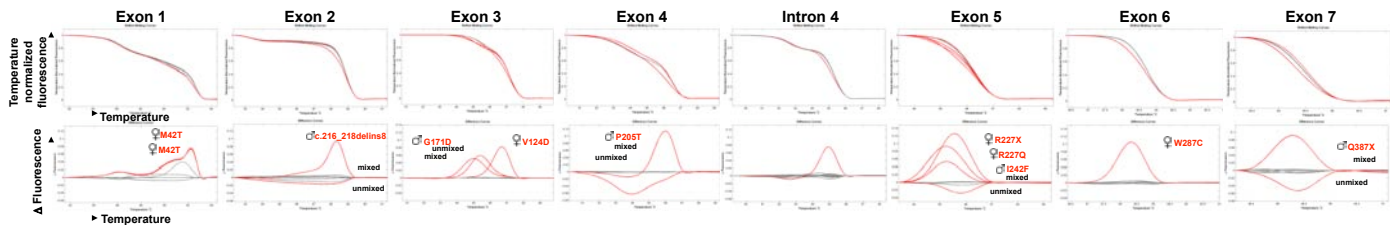


Figure 4. Fabry Validation Results from Hi-Res Mutational Screening. Shifted melting curves (TOP) and difference curves (BOTTOM) of PCR products from LightScanner Instrument and Analysis Software. Mutant positive control samples are indicated in red while negative samples are indicated in grey. Specificity of 95% (n=35) and sensitivity of 100% were determined.

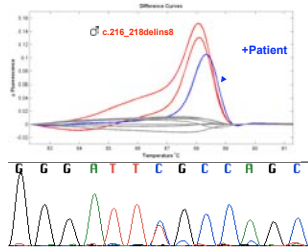


Figure 5. Successful identification of a symptomatic female carrier through our high-throughput method. A female "carrier" with reduced enzyme activity is identified through our reflex testing strategy. A heterozygous Exon 2 mutation c.353C>T (p.Arg118Cys) in a female. Symptoms include cardiac issues, renal stones and acroparathesias.

## RESULTS

Tier 1 was effective in differentiating females with FD and not (59.43 vs 102.18 nM/punch<sup>h</sup>,  $p < 0.01$ ) and males with FD and not (36.44 vs 120.47 nM/punch<sup>h</sup>,  $p < 0.01$ ). Assay precision was determined to be 17.4% of the CV and (LOD) for AGAL activity was determined to be 17.1 nM/punch<sup>h</sup>.

Validation of the mutational scanning technology revealed the molecular test could readily identify a variety of mutations in females as well as hemizygous males. These mutations included missense, nonsense, insertions, deletions, as well as deep intronic mutations associated with the cardiac phenotype associated with FD. Our mutational scanning method was determined to have a specificity of 95% and sensitivity of 100%.

## CONCLUSIONS

A high-throughput method that combines enzyme activity determination for AGAL and molecular mutation detection is possible in a robust manner.

## ACKNOWLEDGEMENTS

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