# Seeker™ 4-Plex Assay (IDUA|GAA|GBA|GLA)

Instructions for Use. Reagents for 1440 tests

Manufactured by:

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FOR *IN VITRO* DIAGNOSTIC USE

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**1. INTENDED USE ..... 5**

**2. SUMMARY AND EXPLANATION OF THE ASSAY ..... 6**

**3. PRINCIPLE OF TEST ..... 7**

3.1. IDUA (α-L-IDURONIDASE) ASSAY ..... 7
3.2. GAA (A-D-GLUCOSIDASE) ASSAY ..... 7
3.3. GBA (B-GLUCOCEREBROSIDASE) ASSAY ..... 7
3.4. GLA (A-D-GALACTOSIDASE A) ASSAY ..... 8
3.5. STOP REACTION ..... 8

**4. REAGENTS ..... 9**

4.1. SUBSTRATES ..... 10
4.2. CALIBRATORS ..... 11
4.3. QUALITY CONTROL SPOTS ..... 11

**5. WARNINGS AND PRECAUTIONS ..... 12**

**6. SEEKER™ ANALYZER ..... 13**

**7. SPECIMEN COLLECTION AND HANDLING ..... 14**

**8. PROCEDURE ..... 16**

8.1. SEEKER™ SYSTEM ..... 16
8.2. PROCEDURE ..... 17
8.3. PROCEDURAL NOTES ..... 18

**9. RESULTS AND REPORTING ..... 20**

9.1. CALIBRATION CURVE ..... 20
9.2. NON-ENZYMATIC HYDROLYSIS CHECK ..... 20
9.3. ENZYME ACTIVITY CALCULATIONS ..... 20
*9.3.1. Run Acceptance ..... 20*
*9.3.2. Individual Sample Acceptance ..... 20*
9.4. QUALITY CONTROL ..... 20

**10. SEEKER™ CLINICAL STUDY AND CUTOFF DETERMINATIONS ..... 22**

10.1. CLINICAL STUDY SUMMARY ..... 22
10.2. INTERPRETATION OF RESULTS ..... 23
*10.2.1. Applying Cutoffs and Retesting ..... 23*
10.3. PERFORMANCE ..... 24
*10.3.1. Pilot and Pivotal Combined, No Exclusions ..... 24*
*10.3.2. Pivotal Data Analysis ..... 25*
*10.3.3. False Negatives ..... 26*
*10.3.4. False Positives ..... 26*
*10.3.5. Retest Burden ..... 26*

**11. EXPECTED VALUES AND INTERPRETATION OF RESULTS ..... 28**

**12. LIMITATIONS OF THE PROCEDURE ..... 30**

**13. ANALYTICAL PERFORMANCE CHARACTERISTICS ..... 31**

13.1. PRECISION ..... 31
13.2. ANALYTICAL SENSITIVITY ..... 32
13.3. LINEARITY ..... 33
13.4. CARRY OVER ..... 33
13.5. INTERFERENCE ..... 33

**14. APPENDIX A ..... 35**
**15. REFERENCES ..... 36**

## SYMBOLS

| | |
|---|---|
|  | For *in vitro* diagnostic use |
|  | Reference item number |
|  | Expiration date |
|  | Storage temperature limits |
|  | Lot number |
|  | Do Not reuse |
|  | Serial number |
|  | Control |
|  | Caution |
|  | Biohazard |
|  | Number of tests |
|  | Manufacturer location |
|  | Instructions for use |

# 1. INTENDED USE

The Seeker™ System is intended for quantitative measurement of the activity of multiple lysosomal enzymes from newborn dried blood spot specimens. Reduced activity of these enzymes may be indicative of a lysosomal storage disorder. The enzymes measured using the Seeker™ Reagent Kit and their associated lysosomal storage disorder are listed in Table 1.

**Table 1:** Enzymes measured by the Seeker™ System and associated disorders

| Enzyme (abbreviation) | Disorder |
|---|---|
| α-L-iduronidase (IDUA) | Mucopolysaccharidosis Type I (MPS I) |
| α-D-glucosidase (GAA) | Pompe |
| β-glucocerebrosidase (GBA) | Gaucher |
| α-D-galactosidase A (GLA) | Fabry |

Reduced activity for any of the four enzymes must be confirmed by other confirmatory diagnostic methods.

## 2. SUMMARY AND EXPLANATION OF THE ASSAY

Lysosomal storage disorders (LSD) are a family of approximately 50 rare metabolic disorders resulting from a reduction or lack of enzymatic function[1]. Many of these disorders manifest with similar clinical outcomes, making them difficult to diagnose.

Alpha-L-Iduronidase (EC 3.2.1.76, *L-iduronidase*, *alpha-L-iduronidase*) participates in the degradation of heparan and dermatan sulfate, two glycosaminoglycans (GAGs) found in nearly all body tissues[2]. Consequently, α-L-iduronidase deficiency results in Mucopolysaccharidosis Type I (MPS I) that involves multiple organ systems resulting from the accumulation of undegradable GAG material throughout the body. The estimated incidence rate is between 1:54,000 and 1:185,000[2]. Three subtypes of MPS I exist: MPS IH (Hurler), MPS IS (Scheie) and MPS IH/S (Hurler-Scheie).

Acid alpha-glucosidase (EC 3.2.1.20 also known as acid maltase) is an enzyme that is active in lysosomes produced by the GAA gene. The enzyme normally breaks down glycogen into a simpler sugar called glucose, which is the main energy source for most cells. Mutations in the *GAA* gene prevent acid alpha-glucosidase from breaking down glycogen effectively, which allows this sugar to build up to toxic levels in lysosomes. This buildup damages organs and tissues throughout the body, particularly the muscles, leading to the progressive signs and symptoms of Pompe disorder. The overall estimated incidence rate for Pompe (either infantile or late-onset) is approximately 1:28,000[3] ; however, different ethnic populations have varying estimated incidence rates[4]. Pompe disorder presents as a continuum of disease severity – at one end of the spectrum is classic infantile Pompe disorder and the rest is classified as late-onset Pompe disorder.

β-Glucocerebrosidase (EC 3.2.1.45 also called acid β-glucosidase, D-glucosyl-N-acylsphingosine glucohydrolase) is an enzyme with glucosylceramidase activity that is needed to cleave, by hydrolysis, the beta-glucosidic linkage of the chemical glucocerebroside, an intermediate in glycolipid metabolism. A deficiency of this enzyme is associated with Gaucher's disorder. As a very rare variant, Gaucher disorder can also be caused by a deficiency of the non-enzymatic sphingolipid activator protein SAP C (or saposin C)[5]. The estimated incidence rate of Gaucher disorder is approximately 1:57,000[4]. Three types of Gaucher disorder have been identified: Type 1 (non-neuronopathic), Type 2 (acute neuronopathic) and Type 3 (sub-acute neuronopathic).

Alpha-galactosidase (EC 3.2.1.22) is a glycoside hydrolase enzyme that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. It is encoded by the *GLA* gene. Deficiency of this enzyme is associated with Fabry disorder, a rare genetic lysosomal storage disorder inherited in an X-linked manner. Population pilot studies have estimated that the incidence rate is between 1:1,500 and 1:13,000[4,6].

All of the aforementioned disorders result in irreversible organ damage, intellectual disability and eventually death if not treated in time. FDA-approved enzyme replacement therapies exist for all the conditions mentioned here. Since early detection of these disorders has the potential to positively impact health outcomes by initiating treatment before permanent damage occurs, several newborn screening programs mandated and implemented screening for these lysosomal storage disorders.

# 3. PRINCIPLE OF TEST

Enzymatic activity is measured in dried blood spot extracts by an end point method using synthetic fluorescent substrates. The fluorescent substrates use 4-methylumbelliferone as the fluorophore, which has an excitation peak at 365 nm and emission peak at 460 nm. The synthetic substrates are hydrolyzed by their corresponding enzyme at acidic pH conditions to release free 4-methylumbelliferone (4-MU). All enzymatic reactions are performed at 37°C. After a pre-specified incubation time, free 4-MU is measured using a UV fluorimeter on the analyzer after stopping the reactions using a high pH stop buffer.

The fluorescence value of the 4-MU product is converted to a 4-MU concentration using a 4-MU calibration curve. The amount of 4-MU generated, after correction for substrate background and non-enzymatic hydrolysis, is proportional to the enzyme concentration. Substrate background and non-enzymatic hydrolysis is estimated by substituting the dried blood spot extract in the reaction with extraction buffer. Enzymatic activity is reported as micromoles of 4-MU produced/ liter of blood / hour of incubation.

Samples of whole blood collected on standardized filter paper (e.g. Ahlstrom PerkinElmer® 226, Whatman® 903) are eluted using extraction solution in standard 96-well plates. The extracts are transferred to the digital microfluidic cartridge where the extract is mixed with enzyme-specific substrate reagent and incubated. End point fluorescence is measured after mixing the incubated reaction droplets with stop solution.

## 3.1. IDUA (α-L-iduronidase) Assay

The IDUA enzyme assay is based on a dried blood spot fluorimetric enzyme assay first developed by Chamoles *et al*[7]. The artificial substrate for IDUA is 4-methylumbelliferyl α-L-iduronide (4-MU-α-IDUA) prepared in an acetate buffer at pH 3.5. The chemical D-Saccharolactone is used to selectively inhibit endogenously present β-glucuronidase which is active for a stereoisomer of 4-MU-α-IDUA (impurity in chemical synthesis)[8].

$$\text{4-MU-}\propto\text{-L-iduronide} \xrightarrow{IDUA/pH\ 3.5} \text{4-MU} + \text{Iduronic acid}$$

## 3.2. GAA (α-D-glucosidase) Assay

The GAA enzyme assay is based on a dried blood spot fluorimetric enzyme assay first developed by Chamoles *et al* [9] and later refined by Zhang *et al*[10]. The artificial substrate for GAA is 4-methylumbelliferyl α-D-glucoside (4-MU-α-Gluc) prepared in an acetate buffer at pH 3.8. The chemical acarbose is used to selectively inhibit endogenously present maltose glucoamylase which is also active for 4-MU-α-Gluc [11,12,13,14].

$$\text{4-MU-}\propto\text{-D-glucoside} \xrightarrow{GAA/pH\ 3.8} \text{4-MU} + \text{D-Glucose}$$

## 3.3. GBA (β-glucocerebrosidase) Assay

The GBA enzyme assay is based on a dried blood spot fluorimetric enzyme assay first developed by Chamoles *et al*[15]. The artificial substrate for GBA is

4-methylumbelliferyl β-D-glucopyranoside (4-MU-β-Gluc) prepared in a citrate phosphate buffer at pH 5.2. The surfactant sodium taurocholate (NaTC) is present in the buffer and is required to activate the enzyme.

$$\text{4-MU-β-D-glucopyranoside} \xrightarrow{GBA/pH\ 5.2/NaTC} \text{4-MU} + \text{D-Glucose}$$

## 3.4. GLA (α-D-galactosidase A) assay

The GLA enzyme assay is based on a dried blood spot fluorimetric enzyme assay first developed by Chamoles *et al* [16]and later refined by Desnick *et al* [17]. The artificial substrate for GLA is 4-methylumbelliferyl α-D-galactopyranoside (4-MU-α-Gal) prepared in an acetate buffer at pH 4.6. The chemical N-acetyl-D-galacosamine is used to inhibit endogenously present α-N-acetylgalactosaminidase which is also active for 4-MU-α-Gal.

$$\text{4-MU-}\propto\text{-D-galactopyranoside} \xrightarrow{GLA/pH\ 4.6} \text{4-MU} + \text{D-Galactose}$$

## 3.5. Stop reaction

For all enzymatic assays, the reaction is stopped using a high pH stop buffer (sodium bicarbonate, pH 11). The high pH is not optimal for the enzymes and effectively stops the substrate turnover. 4-MU is also not fluorescent at the reaction pH (3.5-5.2) and is highly fluorescent at the stopped condition (pH 11) with an excitation at 365 nm and emission at 460 nm.

$$\text{4-MU (acid pH, non-fluorescent)} \xrightarrow{pH\ 11} \text{4-MU (fluorescent, Ex 365nm/Em 460nm)}$$

# 4. REAGENTS

Each Seeker™ 4-Plex Reagent Kit contains sufficient consumables for 1440 tests per analyte. The reagents in the kit along with the quantity and storage conditions are listed in Table 2.

**Table 2:** Consumables for Seeker™ System

| Component | Contents | Qty | Storage Conditions |
|---|---|---|---|
| Quality control dried blood spots | QC-Base Pool (QCBP) | 1 × 15 spots | -80°C to -70°C |
| | QC-Low (QCL) | 1 × 15 spots | -80°C to -70°C |
| | QC-Medium (QCM) | 1 × 15 spots | -80°C to -70°C |
| | QC-High (QCH) | 1 × 15 spots | -80°C to -70°C |
| Seeker™ 4-Plex Assay (IDUA\|GAA\|GBA\|GLA) Kit | EZ Reagent IDUA | 9 × 100 µL | -80°C to -70°C |
| | EZ Reagent GAA | 9 × 100 µL | -80°C to -70°C |
| | EZ Reagent GBA | 9 × 100 µL | -80°C to -70°C |
| | EZ Reagent GLA | 9 × 100 µL | -80°C to -70°C |
| | Calibrant A (CALA) | 9 × 50 µL | -80°C to -70°C |
| | Calibrant B (CALB) | 9 × 50 µL | -80°C to -70°C |
| | Calibrant C (CALC) | 9 × 50 µL | -80°C to -70°C |
| | Calibrant D (CALD) | 9 × 50 µL | -80°C to -70°C |
| | Stop Buffer (STB) | 9 × 500 µL | -80°C to -70°C |
| | Extraction Buffer (EXT) | 9 × 30 mL | 15°C to 25°C |
| | Filler Fluid | 9 × 10 mL | 15°C to 25°C |
| Seeker™ Cartridge | Cartridge | 1 × 36 | 15°C to 25°C |

A lot specific quality control certificate with enzymatic activity range for QC samples is provided with each kit.

## 4.1. Substrates

The composition of the enzyme substrate reagents and buffers are summarized in Table 3.

**Table 3:** Composition of enzyme substrate reagents and buffers for the enzymatic assays

| **Reagent (Description)** | **Composition** |
|---|---|
| EZ Reagent IDUA<br>(α-L-iduronidase substrate) | 2 mM 4-MU-α-L-iduronide sodium salt<br>3mM D-saccharolactone<br>0.04 M acetate buffer, pH 3.5<br>20 mM methyl β-cyclodextrin<br>0.01% Tween20 |
| EZ Reagent GAA<br>(α-D-glucosidase substrate) | 5 mM 4-MU-α-glucopyranoside<br>12 μM acarbose<br>0.04 M acetate buffer, pH 3.8<br>20 mM methyl β-cyclodextrin<br>0.01% Tween20 |
| EZ Reagent GBA<br>(β-glucocerebrosidase substrate) | 16 mM 4-MU-β-glucopyranoside<br>0.05 M/0.1M citrate phosphate buffer, pH 5.2<br>0.01% Tween20<br>1.5% sodium taurocholate |
| EZ Reagent GLA<br>(α-D-galactosidase A substrate) | 10 mM 4-MU-α-galactopyranoside<br>145 μM N-acetyl galactosamine<br>0.04 M acetate buffer, pH 4.6<br>20 mM methyl β-cyclodextrin<br>0.01% Tween20 |
| Stop Buffer STB<br>(reaction stopping buffer) | 0.6 M $NaHC0_3$, pH 11.0 in 0.04% Tween 20 |
| Extraction Buffer EXT<br>(dried blood spot extraction buffer) | 0.1% Tween 20 in water |
| Filler Fluid<br>(medium for droplet movement) | 0.1% Triton X-15 in 5cSt silicone oil |

## 4.2. Calibrators

The calibrators consist of 4 levels of aqueous preparation of 4-methylumbelliferone sodium salt (4-MU) in 0.6 M sodium bicarbonate buffer, pH 11.0 with 0.01% Tween 20. The concentration of 4-MU in each of the 4 calibrators is indicated in Table 4. The concentrations are chosen to provide a calibration curve around the cutoffs or decision making levels.

**Table 4:** Concentrations of 4-MU in each Calibrator

| Calibrator | Concentration of 4-MU |
|---|---|
| Calibrant A (CAL A) | 0.0375 μM |
| Calibrant B (CAL B) | 0.0750 μM |
| Calibrant C (CAL C) | 0.1500 μM |
| Calibrant D (CAL D) | 0.3000 μM |

## 4.3. Quality Control Spots

The quality control dried blood spots include 4 levels of control material: QC Low (QCL), QC Medium (QCM) and QC High (QCH). The composition of the 4 quality control specimens is summarized in Table 5. QCL and QCM are used as run controls. QCBP is used a filler sample to fill empty wells.

**Table 5:** Composition of Quality Control Dried Blood Spots

| Quality control specimen | Composition |
|---|---|
| QCBP | Heat inactivated human serum, adjusted to ~50% hematocrit using human red blood cells |
| QCL | 5% cord blood and 95% heat inactivated serum, adjusted to ~50% hematocrit using human red blood cells |
| QCM | 50% cord blood and 50% heat inactivated serum adjusted to ~50% hematocrit using human red blood cells |
| QCH | Human umbilical cord blood, adjusted to ~50% hematocrit using human red blood cells |

The quality control samples have been prepared by titrating cord blood and adult human blood with 50-55% hematocrit to obtain different levels of lysosomal enzyme activity. The enzymatic activity values for the quality control samples measured by the manufacturer are given on the lot specific quality control certificate included in each kit for all levels except QCBP. Each laboratory should establish its own mean and acceptable range using recommended sample layouts provided in Appendix A.

# 5. WARNINGS AND PRECAUTIONS

All the reagents provided with the kit have an established stability of 12 months from date of manufacture. The expiry date and the storage conditions are stated on the outer label of the reagent box.Warnings and Precautions

For *in vitro* diagnostic use only.

This kit should only be used by adequately trained personnel. This kit contains quality control samples that were produced using human blood specimens. All quality control blood samples were tested for HIV, Hepatitis B and Hepatitis C by FDA approved or equivalent methods and found to be negative for all infectious agents. However, all recommended precautions for handling of blood specimens as specified in the document provided by U.S. Department of Health and Human Services titled "Biosafety in Microbiological and Biomedical Laboratories"[18] should be followed. Disposal of all waste should be in accordance with the local regulations.

Handle all patient specimens as potentially infectious. Disposal of all waste should be in accordance with the local regulations. Ensure that all the cartridges from the Seeker™ device be incinerated after usage (and **NOT** autoclaved) and treated as Biohazard waste.

# 6. SEEKER™ ANALYZER

The Seeker™ Analyzer contains hardware and firmware required to provide the essential system functions such as droplet control, thermal control and fluorescence detection that are required to perform the assay. Installation procedures, principles of operation, performance characteristics, operating instructions, operational precautions and limitations, hazards and service and maintenance information are provided in detail in the Seeker™ Analyzer User Guide.

# 7. SPECIMEN COLLECTION AND HANDLING

Blood specimens should be taken directly from a heel prick onto the filter paper. Neonatal screening programs differ from one another in the type of specimen required. In the United States, the recommendation is that a blood spot, approximately 12.7 mm (0.5 inch) in diameter, is collected by heel prick and spotted onto filter paper. Blood from a newborn heel prick is usually collected 24-48 hours after birth. However, in some screening programs, the sample from the neonatal heel prick may be collected 2-6 days after birth. Consult local regulations for appropriate timing and screening specimen collection. A method based on dried blood specimens requires skillful collection, handling and transport of specimens. The manufacturer recommends following the procedures listed in the CLSI document number LA4-5A "Blood collection on filter paper for newborn screening programs; Approved standard"- Fifth edition (2008)[19] and some important points are mentioned below:

- Ensure that the expiration date of the blood collection card has not passed.
- Wipe the newborn skin with 70% isopropanol and allow the skin to dry.
- Puncture the infant's heel with a heel incision device or a sterile lancet by making a standardized incision of 1.0 mm deep. Ensure that the puncture does not exceed 2.0 mm in depth since a deep incision may cause bone damage in small infants.
- Wipe away the first drop of blood with a sterile gauze pad and allow a large drop of blood to form. Touch the filter paper against the large drop of blood and allow a sufficient quantity of blood to soak through and sufficiently fill the entire pre-printed circle. Examine both sides of the paper to ensure that the blood uniformly penetrated and saturated the filter paper.
    - Do not excessively squeeze the puncture since it may cause hemolysis of the specimen or result in a mixture of tissue fluids with the specimen and might adversely affect the test result.
    - Do not apply successive blood drops to the same printed circle or already partially dried spots can result in "caking".
- Allow the blood specimen to air dry on a horizontally level, non-absorbent, open surface for at least 3 hours at an ambient temperature of 18-25°C, away from direct sunlight. Ensure that the samples are not stacked to avoid cross-contamination.
- Ensure that the required information on the dried blood spot card is completed which includes:
    - Last name, First name, sex, Birth date, Birth weight and Patient identification number
    - First and last name of mother
    - Date of specimen collection
    - Name and address of the submitter
    - Name and phone number of the physician
    - Name of the newborn screening program and address
    - Each card should have a unique serial number
- Follow the basic triple packaging system i.e., blood absorbed into paper, a fold-over flap or inner envelope, and an outer envelope of high quality paper. Ensure that the local

regulation and institutional policies are followed when shipping dried blood spot specimens.

- Transport the dried blood spot specimens within 24 hours of collection unless otherwise directed by the newborn screening laboratory.

Lysosomal enzymes (IDUA, GAA, GBA and GLA) are stable for up to 5 days at 10°C at low humidity (20% RH) and high humidity (80% RH) levels. At elevated temperature, 45°C and low humidity (20% RH), there is significant loss in GBA and GLA enzymatic activity. At high temperature (45°C) and high humidity (80% RH) there is significant loss in activity for all enzymes. At 25°C and 50%RH, there is moderate loss in enzymatic activity for GLA after 5 days of exposure. Exposure to high temperature during shipping may result in increased rate of false positives. Hence, it is recommended that laboratories monitor the daily patient median to identify any systematic bias in activity due to seasonal weather changes and adjust the cutoff values to minimize the false positive rate.

The stability of GAA, GLA, and GBA in dried blood spots has also been previously evaluated at various storage temperatures (-20°C, 4°C, room temperature and 37°C) by Adam *et al*[20] from the Centers for Disease Control and Prevention (CDC). The study used an isochronous design and established 1 year stability at -20°C. Stability of IDUA, GAA, GBA and GLA in dried blood spot specimens have been shown to be stable for a period of 15 months when stored at -80°C.

# 8. PROCEDURE

## 8.1. Seeker™ System

The components in Table 6 comprise the Seeker™ System and are supplied by Baebies.

**Table 6:** Components provided by Baebies for the Seeker™ System

| **Component** | **Qty** |
|---|---|
| Seeker™ Analyzer (including USB and power cables) | As required |
| Desktop PC (Windows 7, Intel Celeron) with monitor, keyboard, mouse | 1 per set of 4 Analyzers |
| Spot Logic® software | 1 per Desktop PC |
| Seeker™ 4-Plex Assay Kit containing enzyme specific substrate reagents, dried blood spot extraction buffer, reaction stop buffer and 4 levels of calibrators | Sufficient for 1440 tests |
| Seeker™ Cartridge | Sufficient for 1440 tests |
| **Additional Components Provided:** | **Qty** |
| Finnipipette Novus 8-channel automatic pipette 1-10 µL (Thermo Scientific #46300000) | 1 |
| Finnipipette Novus 1-channel automatic pipette 10-100 µL (Thermo Scientific #46200400) | 1 |

**Other Materials Required (Not provided by Baebies)**

1. Finnpipette™ Novus 8-channel automatic pipette 100-1200 µL (Thermo Scientific #46300800)
2. Finnpipette™ Novus Flex tips 10µL, 200 µL (Thermo Scientific #94056980 and 94056520)
3. Fisherbrand pipette 1000 µL (Fisher Scientific #S98635A)
4. Fisherbrand Aerosol-barrier pipette tips 1000 µL (Fisher Scientific #0270751)
5. Fisherbrand 96-well round bottomed plates (Fisher Scientific #12565500 or equivalent)
6. Adhesive plate sealers for 96-well plate (Edge Bio #48461 or equivalent)
7. 96-well plate shaker (Thermo Scientific Titer Plate Shaker #4625 or equivalent)
8. Laboratory vortexer (Vortex Genie2 #G-560 or equivalent)
9. Mini Centrifuge (Southwest Science #545 or equivalent)
10. Isopropanol
11. Lint Free Cloth
12. Compressed air can
13. UPS battery for workstation

14. Automatic or Manual Puncher (capable of 3.2 mm punch) – Wallac DBS Puncher (#296-071) or Wallac MultiPuncher™ (#No. 1296-081) or a manual puncher to cut out filter paper disks
15. Specimen cards using an FDA approved filter paper such as Ahlstrom 226 (K062932)
16. External hard drive for database back up

## 8.2. Procedure

The 4-plex assay procedure is summarized in Table 7. A detailed description of each of these steps, including how to set up the assay run using Spot Logic® software, is provided in the Seeker™ Analyzer User Guide.

**Table 7:** Step by step description of 4-plex assay procedure

| Process | Steps | Description |
|---|---|---|
| Specimen Preparation | Specimen punching | Punch 3.2 mm diameter samples from DBS into extraction plate. |
| | Specimen extraction | Extract enzyme into 100 µL extraction buffer. |
| Run Preparation | Analyzer & software Preparation | Power up analyzer and thermal system, prepare instrument, start software. |
| | Reagent & Cartridge Preparation | Thaw reagents, vortex and spin. Load filler fluid into cartridges. |
| | Sample Setup | Obtain extracted samples. Transfer calibrators to extraction plate. |
| Run Execution | Load and check Cartridge | Insert cartridge into analyzer and engage with instrument. Check connectivity of cartridge to analyzer. |
| | Enter run info | Enter run information into Spot Logic®. |
| | Load samples/reagents | Load stop buffer, reagents and samples into cartridge. |
| | Start & Run Assay | Execute assay protocol. Results calculated by Spot Logic® Software. |
| Run Completion | Unload Cartridge | Remove cartridge from analyzer and dispose. |

Each Seeker™ Cartridge contains 48 wells for samples, calibrators and quality control samples. DBS samples are extracted in a 96-well plate and the extracts from one plate are transferred to two Seeker™ Cartridges as shown in Figure 1.

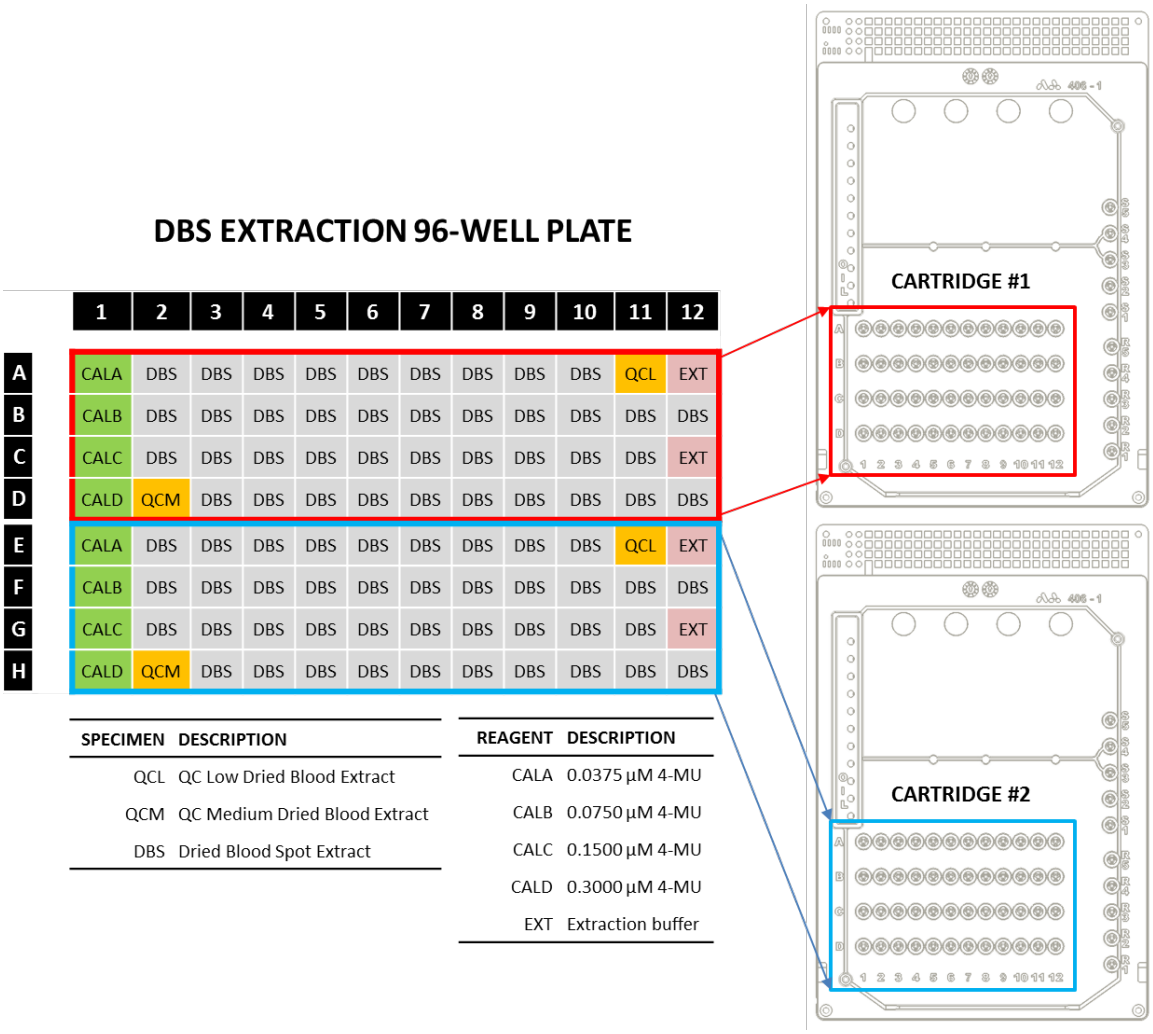



**Figure 1:** Layout of one 96 well plate which is transferred to 2 Seeker™ Cartridges

## 8.3. Procedural Notes

A thorough understanding of this package insert, Seeker™ Analyzer User Guide and Spot Logic® Software is necessary for successful use of the Seeker™ 4-plex reagent kit.

- Reagents are shipped in a box with dry ice in order for the reagents to be frozen and preserve the integrity of the formulations. A temperature monitoring device is included with each shipment. At any point of time during the transit, if the temperature is greater than -40°C (displayed by an alarm icon), the reagent lot should not be used and the manufacturer should be notified.
- The reagents should be used within 1.5 hours after thawing. It is recommended to place the reagents in a dark box while thawing.
- It is recommended to maintain a clean work area (dust-free) since dust or other particulates may potentially interfere with optical detection.
- The reagents supplied with this kit are intended for use as an integral unit. Do NOT mix identical reagents from different lots.
- Do NOT use reagents after the specified expiry date printed on the label.

- Each reagent aliquot will be used to load 4 cartridges. Do NOT use any left-over reagents for the next run. Discard any unused reagents.
- Do NOT reuse the Seeker™ Cartridge.
- Do NOT leave any wells in the cartridge empty. All empty wells should be filled with extract from base pool (QCBP).
- Any deviation from the assay procedure may affect the results in an adverse manner.
- Seeker™ 4-plex reagent kit can only be used with the Seeker™ Analyzer.

# 9. RESULTS AND REPORTING

## 9.1. Calibration Curve

Spot Logic® (device software) calculates a slope, intercept and $R^2$ by linear regression for every run. . If the calibration for a run is flagged as "failed" no activity values are reported for the entire run and the entire run must be repeated.

## 9.2. Non-Enzymatic Hydrolysis Check

The non-enzymatic hydrolysis value for each enzyme is checked against a pre-defined range in Spot Logic®. If the values are outside this range, the activity is not calculated and an "n/a" result is reported for the activity.

## 9.3. Enzyme Activity Calculations

If both calibration and non-enzymatic hydrolysis checks pass, enzymatic activity is calculated by the software and reported in µmol/hr/L.

### 9.3.1. Run Acceptance

No results are generated if the calibration routine fails on the cartridge. All the samples from the run should be repeated using a single re-punch from the same dried blood spot as the initial run.

### 9.3.2. Individual Sample Acceptance

An invalid data points triggers a single re-punch and retest from the same dried blood spot. Invalid data points fall into the following categories:

- Data point reported as "n/a"
- Negative enzymatic values for GAA and GBA
- IDUA enzymatic activity < -2 µmol/L/hr (Applies only for the first test from the specimen)
- GLA enzymatic activity < 1 µmol/L/hr
- At least one enzyme that is above the Upper threshold (highlighted ORANGE). The upper threshold corresponds to a high cutoff that is set by the laboratory at 3 SD above the normal median[1].

## 9.4. Quality Control

Each cartridge should include at least one punch of two levels of QC (QCL and QCM) to determine the validity of the run. These QC samples are subjected to the same protocol steps as the newborn sample and the enzymatic activity for all the 4 assays is determined. Median enzymatic activity values and standard deviation will be provided with each reagent kit by Baebies. Upper and lower specification limits for QCL and QCM samples can be set in Spot

[1] The standard deviation should be calculated as a geometric standard deviation since the distribution is lognormal.

Logic® software to accept or reject a run based on the enzymatic activity values obtained for each run. It is recommended that in any particular run, if both QCM and QCL are flagged to be below Median - 2 SD (lower specification limit) or above Median + 2 SD (upper specification limit), the newborn sample results should be reviewed and samples from that cartridge should be repeated based on their activity level. It is also recommended, if one of the QC samples is flagged to be outside of Median ±3SD, the newborn sample results be reviewed and samples from that cartridge repeated based on their activity level. It is recommended that each laboratory establishes its own acceptable range of enzymatic activity for QC samples.

The lab may also use two punches of each QC level (QCL and QCM) for run acceptance. If both QCMs or both QCLs are flagged to be outside of (Median ±3SD), the newborn sample results are reviewed and samples from that cartridge repeated based on their activity level.

The manufacturer also recommends participation in external quality assurance programs such as CDC's NSQAP (Centers for Disease Control Newborn Screening Quality Assurance Program). It is recommended that the laboratories should establish their own quality control samples representing different levels of enzymatic activity in addition to the ones provided in the kit. Baebies recommends that the user monitor the trends in the enzymatic activity values for the QC samples on a weekly basis and compare with the established limits. It is recommended to follow the CLSI guideline QMS12-A "Development and Use of Quality Indicators for process improvement and monitoring of laboratory quality; Approved Guideline" to monitor the trends in enzymatic activity of QC samples.

# 10. SEEKER™ CLINICAL STUDY AND CUTOFF DETERMINATIONS

## 10.1. Clinical Study Summary

A prospective clinical study was performed with the Seeker™ 4-plex reagent kit and Analyzer to screen the lysosomal enzymes IDUA, GAA, GBA and GLA.

Over the course of 24 months, 154,412 newborns were screened at the Missouri State Public Health Laboratory (MSPHL) in a pilot and pivotal study. All newborns were screened; there was no inclusion or exclusion criteria. To establish cutoff values MSPHL performed a preliminary study prior to the clinical trial by analyzing approximately 13,000 presumed normal de-identified specimens and 29 known affected specimens using the Seeker System. The cutoffs were selected to ensure that all known affected samples would be detected and to minimize the false positive rate, keeping in mind the expected incidence of the disorders. During the pilot study MSPHL adjusted cutoffs as needed to establish a satisfactory false positive rate while ensuring no false negatives.

The MSPHL utilized an LSD Task Force to evaluate cutoffs and required a review by the task force to adjust high risk cutoff values. The Task Force met quarterly or as needed and the cutoff values were adjusted during the course of the study in order to reduce the false negative and false positive rate and also to take into account seasonal changes that may affect the enzymatic activity. Additionally MSPHL instituted an additional set of cutoffs for samples based on the age of collection as it was observed that enzymatic activity values attenuated over time with the greatest attenuations after 14 days of age.

Final high risk and borderline cut off values used at the end of the clinical study period are shown in Tables 8-10.

**Table 8: Final MSPHL cutoffs (1-6 days of age)**

| Enzyme | High Risk Cutoff Value (µmol /L/h) | Borderline Cutoff Value (µmol/L/h) |
|---|---|---|
| IDUA | 1.5 | 5.0 |
| GAA | 7.2 | 10.0 |
| GBA | 5.5 | 7.0 |
| GLA | 7.0 | 9.0 |

**Table 9: Final MSPHL cutoffs (7-13 days of age)**

| Enzyme | High Risk Cutoff Value (µmol /L/h) | Borderline Cutoff Value (µmol/L/h) |
|---|---|---|
| IDUA | 1.5 | 5.0 |
| GAA | 4.5 | 10.0 |
| GBA | 4.0 | 7.0 |
| GLA | 5.0 | 5.0 |

**Table 10: Final MSPHL cutoffs (14+ days of age)**

| Enzyme | High Risk Cutoff Value (µmol /L/h) | Borderline Cutoff Value (µmol/L/h) |
|---|---|---|
| IDUA | 1.5 | 5.0 |
| GAA | 4.5 | 10.0 |
| GBA | 4.0 | 7.0 |
| GLA | 3.0 | 5.0 |

## 10.2. Interpretation of Results

### 10.2.1. Applying Cutoffs and Retesting

Samples with results above the borderline cutoff for all enzymes were considered low risk and were presumed normal. No additional action was taken.

Samples that fell below the borderline cutoff were retested in duplicate. The three test results were evaluated for outliers and if outliers were present additional tests were performed.

- If the average of all tests excluding outliers was above the high risk cutoff, the sample was presumed normal.
- If the average of all tests excluding outliers was below the high risk cutoff, risk level was assessed by reviewing other LSD enzyme results from the multiplex assay along with the infant's gestational age, age at specimen collection, and health status. All Missouri NBS specimens collected from premature infants, sick infants, or infants aged <24 hours automatically mandated a repeat screen because these circumstances can produce false-positive, false-negative, and unreliable NBS results. If none of the conditions applied to the positive screen, and if the quality of the specimens was not considered poor owing to multiple low enzyme levels, then the sample was considered high risk and referred to a contracted genetic referral center for evaluation and confirmatory testing and diagnosis.

## 10.3. Performance

### 10.3.1. Pilot and Pivotal Combined, No Exclusions

Of the 154,412 newborns born during the study, 48,813 were born during the pilot phase and 105,599 were born during the pivotal phase.

During the study period (both pilot and pivotal), 275 newborns were referred for confirmatory diagnosis based on the results of the screening. The results of the confirmatory testing are listed in Table 11. This table includes all samples that were referred during the pilot and pivotal phases with no exclusions.

**Table 11: Presumed affected individuals – entire study period**

| | IDUA | GAA | GBA | GLA |
|---|---|---|---|---|
| Presumed Affected | 73 | 79 | 19 | 104 |
| - Refused Follow Up | 1 | 0 | 1 | 4 |
| - Moved to Another State | 1 | 1 | | 1 |
| - Normal | 31 | 33 | 13 | 47 |
| - Carrier | 4 | 14 | 2 | 0 |
| - Pseudodeficient | 35 | 14 | 0 | 0 |
| **- Affected** | **1** | **17** | **3** | **52** |
| - Unclassified Onset | 1 | 0 | 1 | 45 |
| - Unknown Onset / Genotype of Unknown Significance | 0 | 3 | 2 | 4 |
| - Late Onset | 0 | 9 | 0 | 3 |
| **- Infantile Onset** | 0 | 5 | 0 | n/a |
| - Classical Infantile | 0 | 3 | 0 | n/a |
| - Non-Classical Infantile | 0 | 2 | 0 | n/a |
| False positive rate | 0.045% | 0.039% | 0.010% | 0.030% |

For the 154,412 newborn births during the two year period, the incidence of each of the disorders was calculated and is summarized along with previously published incidence.

**Table 12: Incidence Rate During Clinical Study**

| | **Incidence from MSPHL Study** | **Published Incidence** |
|---|---|---|
| MPS I (IDUA) | 1 : 154,412 | 1:54,000 – 1:185,000 |
| Pompe (GAA) | 1 : 9,083 | 1:28,000 |
| Gaucher (GBA) | 1 : 51,471 | 1:57,000 |
| Fabry (GLA) | 1 : 2,969 | 1:1,500 – 1:13,000 |

For Pompe disorder the MSPHL incidence was about 3 times higher than published rates of incidence. For MPS I, Gaucher, and Fabry the MSPHL incidence is comparable to the published rates of incidence.

### 10.3.2. Pivotal Data Analysis

#### 10.3.2.1. Samples Excluded from Analysis

When analyzing results, the following samples were excluded:

- Samples collected at < 24 h of life: Samples collected at less than 24 hours of life trigger a mandatory retest in Missouri. These samples were excluded from the analysis.
- Samples with no recorded age at collection: If no age at collection was recorded, the sample was excluded from the analysis because age-specific cutoff values could not be applied.
- Samples collected at > 30 days of life: Samples collected at > 30 days of life were excluded from the analysis because they do not fit the definition of "newborn" (from birth to 30 days).

**Table 13: Assay Performance During the Pivotal Phase**

| | | IDUA | GAA | GBA | GLA |
|---|---|---|---|---|---|
| | Newborns | 104,614 | 104,614 | 104,614 | 104,614 |
| 1st Test | All screens with first result above borderline | 103,809 | 103,406 | 103,915 | 103,347 |
| | At least one screen with first result below borderline | 805 | 1,208 | 699 | 1,267 |
| Average of all Tests | All screens with average above high risk | 757 | 1,102 | 642 | 1,070 |
| | At least one screen with average below high risk | 48 | 106 | 57 | 197 |
| | Referred | 32 | 44 | 7 | 60 |
| | Not Referred | 16 | 62 | 50 | 136 |
| | Referral Summary | IDUA | GAA | GBA | GLA |
| | True Positive | | 7 | 2 | 30 |
| Referred Sample Summary | Normal, False Positive | 8 | 23 | 2 | 26 |
| | Pseudodeficiency, False Positive | 20 | 7 | | |
| | Carrier, False Positive | 2 | 7 | 2 | |
| | Refused | 1 | | 1 | 3 |
| | Moved | 1 | | | 1 |
| Samples Not Referred Summary | Not Referred Summary | IDUA | GAA | GBA | GLA |
| | Prior Sample From Newborn Above Cutoff | 10 | 27 | 17 | 66 |
| | Other Assay Below Borderline | 2 | 12 | 10 | 24 |
| | Later Sample From Newborn Above Cutoff | 1 | 12 | 14 | 15 |
| | Outliers Excluded | 3 | 8 | 6 | 14 |
| | Transfused | | 2 | 2 | 6 |
| | Different Cutoff Applied | | | | 3 |
| | Spot Variability | | | | 3 |
| | Retrospectively Referred | | | | 2 |
| | Age Related Enzyme Decrease | | | | 1 |
| | Other Assay Low Normal | | | 1 | |
| | Contaminated Sample | | | | 1 |
| | Multiple Reasons | | 1 | | 1 |
| *Performance Summary | Total Presumed Normal | 104,582 | 104,570 | 104,607 | 106,017 |
| | Total Presumed Affected | 32 | 44 | 7 | 60 |
| | True Positives | - | 7 | 2 | 30 |
| | False Positives | 30 | 37 | 4 | 26 |
| | False Positive Rate (FPR) | 0.029% | 0.035% | 0.004% | 0.025% |

* Note that these calculations reflect the analysis completed with exclusions.

### 10.3.3. False Negatives

The Missouri Department of Health and Senior Services has an active surveillance program to track any reports of false negative results to the contracted metabolic centers they use for confirming diagnosis of any of these LSDs. Based on information from this surveillance program there were no known false negative results due to an incorrect screening result during the 2 year study. Newborns screened during the clinical study with early onset disorders would have been reported to one of these metabolic centers.

This false negative rate is based on limited data on late onset forms of the disorders since it would take several years to identify a missed late onset case.

Certain late onset forms for Pompe disorder may have GAA enzymatic activity in the normal range and result in a false negative[21].

For female Fabry patients GLA enzyme activity is highly variable and it could overlap with the normal range. Therefore, GLA enzyme activity for females should be interpreted with caution as some female carriers can have enzyme activity in the normal range and result in a false negative.

### 10.3.4. False Positives

During the clinical study, several patients with reduced enzymatic activity were confirmed by follow-up testing to be either carriers or pseudodeficient for the referred condition. Carriers were discovered for MPS I, Pompe and Gaucher disorders, while pseudodeficiencies were discovered for MPS I and Pompe disorders. Carriers and pseudodefiecient patients may exhibit enzymatic activity below the high risk cutoff while remaining asymptomatic for the disorder. Table 14 indicates the false positive rates during the pivotal phase for each assay.

**Table 14 - False Positive Rates During the Pivotal Phase**

| | IDUA | GAA | GBA | GLA |
|---|---|---|---|---|
| **Pivotal False Positive Rate (FPR)** | 0.029% | 0.035% | 0.004% | 0.025% |

### 10.3.5. Retest Burden

The dataset was also analyzed to determine the average number of tests required to arrive at a final result. The pivotal dataset was used for this analysis (which excludes samples collected at less than 24 hours of life, samples with no recorded age at collection, samples collected at greater than 30 days of life, and samples with no valid data point).

This analysis was performed on a “per sample” basis instead of a “per newborn” basis because additional screens collected from the same newborn were collected independently of the Seeker™ system performance (Seeker™ test results did not trigger a repeat screen). Additionally, repeat screen testing is performed identically to initial screen testing in regards to the retesting algorithm.

The vast majority (96.0%) of screens were only tested once. Including invalid test results, the average sample required 1.13 tests before a final result was determined. Therefore the retest

burden is 13%, of which only 5.5% is due to system performance (invalid tests). Remaining retests are due to the screening algorithm which requires results below borderline for any of the four assays to be repeated.

# 11. EXPECTED VALUES AND INTERPRETATION OF RESULTS

Please note that the values used in this section should be used only as a guideline, and each laboratory should establish their own reference range.

The measurement of enzymatic activity of IDUA, GAA, GBA and GLA from the dried blood spots specimens is performed by using a cutoff value that distinguishes between the affected and normal neonates. The common practice in newborn screening programs is to have two cutoffs – a high risk cutoff and a borderline cutoff. For disorders where the affected newborns have low activity (as is the case for LSDs), samples with enzymatic activity below the high risk cutoff (as the name suggests) have a high likelihood of having the disorder and are referred for diagnostic follow-up.

It is recommended that each laboratory run a study with approximately 10,000 newborn samples along with at least 5 samples from patients with known disorders (true positives). It is recommended to include as many true positive samples as possible to estimate the cutoff values with higher confidence levels. It is also recommended to evaluate multiple samples across various age ranges from 1-30 days to take into account for age related enzymatic reduction.

The cutoffs should be selected to ensure that all known affected samples will be detected and to minimize the false positive rate, keeping in mind the expected incidence of the disorders.

As larger numbers of samples are screened and presumed positive results are obtained, the high risk cutoff should be reviewed in consultation with metabolic disease specialists who can provide additional guidance based on incidence rates, disease severity and typical profiles of known positive patients. It is also recommended that the borderline cutoff values for each analyte to be around +2 SD above the high risk cutoff, where SD is the reproducibility of the assay at enzyme concentrations around the high risk cutoff.

- Samples with results above the borderline cutoff for all enzymes are considered low risk and should be presumed normal. Local regulations and guidelines should be followed for the handling and reporting of presumed normal results.
- Samples that fall below borderline cutoff should be retested in duplicate. The three test results should be evaluated for outliers and if outliers are present additional tests should be performed.
    - If the average of all tests excluding outliers is above the high risk cutoff the sample should be presumed normal. Local regulations and guidelines should be followed for the handling and reporting of presumed normal results.
    - If the average of all tests excluding outliers is below the high risk cutoff, the risk level should be assessed by reviewing other LSD enzyme results from the multiplex assay along with the infant's gestational age, age at specimen collection, and health status. All specimens collected from premature infants, sick infants, or infants aged <24 hours should be considered for a repeat screen because these circumstances can produce false-positive, false-negative, and unreliable NBS results. If none of the conditions above apply to a positive screen, and if the quality of the specimens is not considered poor owing to multiple low enzyme levels, then the sample may be considered presumed affected. Local regulations

and guidelines should be followed for the handling and reporting of presumed affected results.

- As applicable, for a positive screen, all newborn screening results for all prior screening results should be considered when determining a need for referral.

## 12. LIMITATIONS OF THE PROCEDURE

As with any other *in vitro* screening test, the data obtained using the Seeker™ System should be used as an aid to other medically established procedures and interpreted in conjunction with other clinical data available to the clinician.

Conditions that are known to cause anomalous results are[19]:

- Sample spot not uniformly saturated with blood.
- Sample spot punched too close to the edge of the blood spot.
- Poorly collected and improperly dried specimens.
- Non-eluting blood spot due to deterioration of sample caused by exposure to heat and humidity.

Also, the Seeker™ Analyzer may result in:

- False negatives for Fabry disorder in females[22].
- False positives by identifying pseudo deficiencies and carriers as affected for MPS I, Gaucher and Pompe disorders[2,23].
- False negative by not detecting certain late onset forms for Pompe disorder[24]

Please also refer to the Section titled “Procedural Notes”.

# 13. ANALYTICAL PERFORMANCE CHARACTERISTICS

## 13.1. Precision

A precision and reproducibility study was designed based on CLSI-EP05-A3. Dried blood spot (DBS) specimens representing 5 enzymatic activity levels for each enzyme were included in the precision study. DBS specimens were prepared by titrating human umbilical cord blood with heat inactivated serum and hematocrit adjusted to 50%. The study was performed using 4 Seeker™ Analyzers over 21 non-consecutive days, with 2 runs per day and 2 dried blood spot punches of each specimen per run generating a total of 336 replicates per enzymatic activity level. The study was conducted using 3 lots of Seeker™ 4-Plex Reagent Kit with each lot balanced between all the runs i.e. 56 cartridges were performed with each lot. The study was performed at the Baebies' facility.

Repeatability (within-run precision), between-day precision, between-instrument precision, between reagent lot and overall reproducibility were evaluated for the four lysosomal enzymes (IDUA, GAA, GBA and GLA). Invalid data points were removed from the analysis. Precision estimates were calculated by using three-way nested Analysis of Variance technique and are summarized in Table 9 (IDUA), Table 10 (GAA), Table 11 (GBA) and Table 12 (GLA). Precision estimates are reported either as a standard deviation or a %CV depending on the mean activity level for each enzyme.

**Table 8:** Summary of precision data for IDUA

| Sample | N | Mean (µmol/ L/hr) | Repeatability | Between Reagent Lot | Between Instrument | Between Day | Reproducibility |
|---|---|---|---|---|---|---|---|
| S02[a] | 331 | 2.40 | 1.79 | 0.55 | 0.00 | 0.83 | 1.97 |
| S03[a] | 334 | 3.53 | 0.80 | 0.55 | 0.00 | 0.26 | 0.96 |
| S05[a] | 333 | 6.12 | 0.95 | 0.71 | 0.00 | 0.24 | 1.21 |
| S10 | 333 | 12.03 | 12.4% | 10.5% | 0.0% | 0.0% | 16.6% |
| S15 | 335 | 24.06 | 9.0% | 9.8% | 0.0% | 0.0% | 14.2% |

**Table 9:** Summary of precision data for GAA

| Sample | N | Mean (µmol/ L/hr) | Repeatability | Between Reagent Lot | Between Instrument | Between Day | Reproducibility |
|---|---|---|---|---|---|---|---|
| S02[a] | 331 | 4.29 | 0.67 | 0.25 | 0.00 | 0.14 | 0.73 |
| S03[a] | 334 | 6.27 | 0.95 | 0.26 | 0.00 | 0.00 | 0.99 |
| S05 | 335 | 9.59 | 9.9% | 7.0% | 0.0% | 0.0% | 12.0% |
| S10 | 334 | 18.06 | 13.6% | 5.9% | 0.0% | 0.0% | 14.8% |
| S15 | 335 | 27.37 | 9.8% | 6.3% | 1.6% | 0.0% | 12.9% |

**Table 10:** Summary of precision data for GBA

| Sample | N | Mean (µmol/ L/hr) | Repeatability | Between Reagent Lot | Between Instrument | Between Day | Reproducibility |
|---|---|---|---|---|---|---|---|
| S02[a] | 331 | 2.84 | 0.99 | 0.36 | 0.05 | 0.07 | 1.08 |
| S03[a] | 334 | 3.47 | 0.47 | 0.37 | 0.08 | 0.00 | 0.64 |
| S05[a] | 335 | 5.07 | 0.56 | 0.57 | 0.00 | 0.00 | 0.84 |
| S10 | 334 | 8.55 | 11.6% | 10.4% | 1.4% | 0.0% | 15.8% |
| S15 | 335 | 15.00 | 11.3% | 11.4% | 2.1% | 1.2% | 15.7% |

**Table 11:** Summary of precision data for GLA

| Sample | N | Mean (µmol/ L/hr) | Repeatability | Between Reagent Lot | Between Instrument | Between Day | Reproducibility |
|---|---|---|---|---|---|---|---|
| S02 | 330 | 6.92 | 14.5% | 0.35[a] | 0.00[a] | 0.08[a] | 1.01[a] |
| S03 | 334 | 9.80 | 10.4% | 6.4% | 0.0% | 2.9% | 13.6% |
| S05 | 335 | 15.32 | 7.7% | 7.2% | 0.0% | 3.1% | 11.5% |
| S10 | 334 | 28.76 | 7.8% | 4.8% | 0.0% | 0.0% | 9.4% |
| S15 | 335 | 52.66 | 8.8% | 4.1% | 1.3% | 2.7% | 10.6% |

[a] Precision estimate reported as standard deviation in µmol/L/hr

## 13.2. Analytical Sensitivity

Limit of Blank (LoB) and Limit of Detection (LoD) were determined as per CLSI EP17 A- "Protocols for determination of limits of detection and limits of quantitation-Approved guideline." Limit of Quantitation (LoQ) was defined using a functional sensitivity study since there is no recognized reference method.

The analytical sensitivity of the 4-plex assays was determined using 240 replicates of for the blank sample (blank extract was used as surrogate blank sample) and 192 replicates for a low sample (QC Low was used as surrogate low sample). The study was performed using 3 lots of the Seeker 4-Plex Reagent Kit and the worst of the three lots is reported. Table 13 summarizes the LoB and LoD for each enzymatic assay.

**Table 12:** Determination of Limit of Blank and Limit of Detection

| Detection Capability (µmol/L/hr) | N | IDUA | GAA | GBA | GLA |
|---|---|---|---|---|---|
| Limit of Blank (LoB) | 240 | 1.78 | 0.50 | 0.72 | 1.96 |
| Limit of Detection (LoD) | 192 | 2.77 | 2.18 | 1.07 | 3.18 |

Functional sensitivity was estimated by determining the precision profile of each assay and calculating the concentration at which the total imprecision was ≤ 20% CV or ≤1.5µmol/L/h whichever is greater. If the imprecision criteria was met for all concentrations tested, the LoQ was set to be equal to the LoD. Table 14 summarizes the LoQ for each enzymatic assay.

**Table 13:** Determination of Limit of Quantitation

| Detection Capability (µmol/L/hr) | IDUA | GAA | GBA | GLA |
|---|---|---|---|---|
| Limit of Quantitation (LOQ) | 2.77 | 2.18 | 1.85 | 4.88 |

It is recommended that cutoffs should be at or above the LoQ.

## 13.3. Linearity

Linearity of the system was evaluated in accordance with CLSI EP06-A "Evaluation of Linearity of Quantitative Measurement Procedures."

The linearity interval is reported in Table 15. It is recommended to retest the samples with activity above the upper end of the range to ensure that the elevated activity is not due to other causes such as sample contamination.

**Table 14:** Linear range

| Enzyme | Linear range (µmol/L/hr) |
|---|---|
| IDUA | 2.77-50.75 |
| GAA | 2.18-94.66 |
| GBA | 2.14-73.24 |
| GLA | 4.88-153.74 |

## 13.4. Carry Over

Carry over was evaluated by two sample layouts for each assay per high sample: one with carry over and another with no carry over. Carryover was estimated as the percent bias between the average values of representative affected samples obtained between the carry over and no carry over layout. There is no clinically significant carry-over for IDUA, GAA and GBA. For GLA there is a positive bias of 21% when a sample with activity 120.79µmol/L/hr is in the same column as a low sample around the cutoff. This represents a carryover of 1.95µmol/L/hr. A sample with GLA activity within +2µmol/L/hr of the borderline cutoff should be retested if a sample with GLA activity ≥120µmol/L/hr is present in the same sample column.

## 13.5. Interference

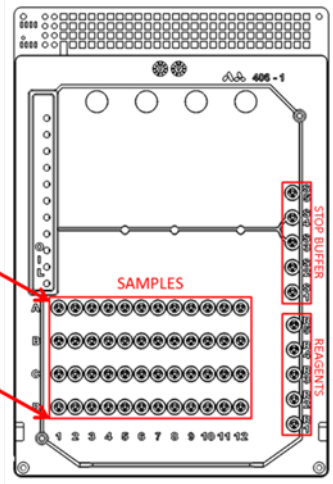
An interfering substances study was performed to determine and characterize the effects of potential endogenous and exogenous interfering substances on the performance of the 4-plex assay. A total of 9 different substances were tested for interference on the system at the highest recommended concentration as per CLSI EP07-A2. Interfering substances that were tested

include Hemoglobin, D-Glucose, D-Galactose, EDTA, Heparin, Bilirubin (conjugated and unconjugated), total protein and triglycerides.

Total Protein was found to have a positive bias of 1.41µmol/L/h for IDUA at a test concentration of 120mg/mL. Bias decreased with reducing concentration of total protein and was found not to interfere at ≤75mg/mL.

# 14. APPENDIX A

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | CALA | QCBP | QCL | QCM | QCH | QCBP | QCL | QCM | QCH | QCBP | QCL | EXT |
| B | CALB | QCH | QCM | QCBP | QCL | QCH | QCM | QCBP | QCL | QCH | QCM | EXT |
| C | CALC | QCL | QCBP | QCH | QCM | QCL | QCBP | QCH | QCM | QCL | QCBP | EXT |
| D | CALD | QCM | QCH | QCL | QCBP | QCM | QCH | QCL | QCBP | QCM | QCH | EXT |



# 15. REFERENCES

[1] Millington DS. Newborn screening for lysosomal storage disorders (Editorial). *Clinical Chemistry*. 2005; 51:808-9.
[2] Kemper AR, Brosco J, Green NS, Ojodu J, Jones E, Comeau AM, *et al*. Newborn screening for Mucopolysaccharidosis Type I (MPS I): a systematic review of evidence. Report for the United States Secretary of Health and Human Services Advisory Committee on Heritable Disorders in Newborns and Children. March 16, 2015. Available at: http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/nominatecondition/reviews/mps1finalreport.pdf.
[3] Kemper AR, Comeau AM, Green NS, et al. Evidence report: newborn screening for Pompe disease. Report for the United States Secretary of Health and Human Services Advisory Committee on Heritable Disorders in Newborns and Children. June 3, 2013. Available at: http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/nominatecondition/reviews/pompereport2013.pdf.
[4] Matern D, Gavrilov D, Oglesbee D, Raymond K, Rinaldo P, Tortorelli S. Newborn screening for lysosomal storage disorders. *Seminars in Perinatology*. 2015; 39:206-216.
[5] Tylki-Szymanska A, Czartoryska B, Vanier MT, Poorthuis BJ, Groener JA, Lugowska A, *et al*. Non-neuronopathic Gaucher disease due to saposin C deficiency. *Clinical Genetics*. 2007; 72:538–542.
[6] Daitx VV, Mezzalira J, Moraes VDC, Breier AC, Cé J, Coelho JC. Comparing the alpha-galactosidase A biochemical properties from healthy individuals and Fabry disease patients. *Clinical Chimica Acta*. 2015; 445:60-4.
[7] Chamoles NA, Blanco D, Gaggioli D. Diagnosis of α-L-iduronidase deficiency in dried bloodspots on filter paper: the possibility of newborn diagnosis. *Clinical Chemistry*. 2001; 47:780-781.
[8] Chamoles N, Blanco, M, Gaggioli D. Diagnosis of α-L-iduronidase deficiency in dried blood spots on filter paper: the possibility of newborn diagnosis. *Clinical Chemistry*. 2001; 47:780-781.
[9] Chamoles NA, Niizawa G, Blanco M, Gaggioli D, Casentini C. Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper. *Clinica Chimica Acta*. 2004; 347:97-102.
[10] Zhang H, Kallwass H, Young SP, Carr C, Dai J, Kishnani PS, et al. A comparison of maltose and acarbose as inhibitors of maltase-glucoamylase activity in assaying acid α-glucosidase activity in dried blood spots for the diagnosis of infantile Pompe disease. *Genetics in Medicine*. 2006; 8:302-6.
[11] Winchester B, Bali D, Bodamer OA, Caillaud C, Christiensen E, Cooper A., *et al*. Methods for a prompt and reliable laboratory diagnosis of Pompe disease: Report from an international consensus meeting. *Molecular Genetics and Metabolism*.2008; 93(3):275-281.
[12] Goldstein JL, Young SP, Changela M, et al. Screening for Pompe disease using a rapid dried blood spot method: experience of a clinical diagnostic laboratory. *Muscle Nerve*. 2009;40(1):32-36.
[13] Li Y, Scott CR, Chamoles NA, Chavami A, Pinto BM, Turecek F, *et al*. Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. *Clinical Chemistry*. 2004; 50(10):1785-1796.
[14] Kallwass H, Carr C, Gerrein J, Titlow M, Pomponio R, Bali D, *et al*. Rapid diagnosis of late-onset Pompe disease by fluorometric assay of alpha-glucosidase activities in dried blood spots. *Molecular Genetics and Metabolism*. 2007; 90(4):449-452.
[15] Chamoles NA, Blanco M, Gaggioli D, Casentini C. Gaucher and Niemann-Pick diseases—enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn-screening cards. *Clinica Chimica Acta*. 2002; 317:191-197.
[16] Chamoles NA, Blanco M, Gaggioli D. Fabry disease: enzymatic diagnosis in dried blood spots on filter paper. *Clinica Chimica Acta*. 2001; 308:195-196.
[17] Desnick RJ, Ioannou YA, Eng CM. α-galactosidase A deficiency: Fabry disease. In: Wonsiewicz M, Noujaim S, Boyle P, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill; 2001:3733-74.
[18] U.S. Department of Health and Human Services. Biosafety in Microbiological and Biomedical Laboratories. 5th edition. 2009. Available at: http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf.
[19] Clinical and Laboratory Standards Institute (CLSI). Blood Collection on Filter Paper for Newborn Screening Programs: Approved Standard – Sixth Edition. CLSI document NBS01-A6 (ISBN 1-56238-883-5 [Print]; ISBN 1-

56238-884-3 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA. 2013.

[20] Adam BW, Orsini JJ, Martin M, Hall EM, Zobel SD, Caggana M. Hannon WH. The preparation and storage of dried-blood spot quality control materials for lysosomal storage disease screening tests. *Clinical Biochemistry*. 2011; 44(8-9): 704-10.

[21] Chiang S-C, Hwu W-L, Lee N-C, Hsu L-W, Chien Y-H. Algorithm for Pompe disease newborn screening: Results from the Taiwan screening program. Mol Genet Metab. 2012;106 (3):281-286.

[22] Linthorst GE, Poorthuis BJHM, Hollak CEM. Enzyme activity for determination of presence of Fabry disease in women results in 40% false-negative results. *Journal of the American College of Cardiology*. 2008; 51(21):2082.

[23] Hopkins PV, Campbell C, Klug T, Rogers S, Raburn-Miller J, Kiesling J. Lysosomal storage disorder screening implementation: findings from the first six months of full population pilot testing in Missouri. Journal of Pediatrics. 2015; 166(1): 172-177.

[24] Chiang S-C, Hwu W-L, Lee N-C, Hsu L-W, Chien Y-H. Algorithm for Pompe disease newborn screening: Results from the Taiwan screening program. Mol Genet Metab. 2012;106(3):281-286.