Standard Operating Procedure:

Galactose-1-phosphate uridylyltransferase (GALT) and Biotinidase (BTD) Duplex Enzyme Activity Assay Kit Cat. No. CD3

SOP ID:	SOPPCD3
Version Number:	V4.0
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Product Name:	GALT/BTD Duplex Enzyme Activity Assay Kit
Matrices:	Dried Blood Spot

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Section 1. Materials and Reagents

Table 1.1: Reagents and Standards

Material	Vendor	Vendor Reference
GALT/BTD Duplex Enzyme Activity Assay Kit	Enfanos	CD3
MilliQ Purified Water (18.2 MΩ-cm resistivity), or Equivalent	Various	n/a
LC-MS Grade Ethyl Acetate* (CAS: 141-78-6)	JT Baker	9828-03
HPLC Grade Methanol (CAS: 67-56-1)	Fisher	A452
HPLC Grade Acetonitrile (CAS: 75-05-8)	Fisher	A998
Sodium Chloride (ACS Reagent Grade) (CAS: 7647-14-5)	Sigma Aldrich	S9888

** Note: Trace amounts of oxidizers (e.g., peracetic acid) are present in some grades of Ethyl Acetate due to the manufacturing process. We suggest using the LC-MS grade of Ethyl Acetate available from J.T. Baker (cat. no. 9828-03), which has been found to be free of this issue. At this point, we cannot guarantee that Ethyl Acetate from this source will always be free of this issue. If the Ethyl Acetate quality is poor, the signal intensity and response factor could be impacted.*

Table 1.2: Equipment and Consumables

Material	Vendor	Vendor Reference
MilliQ Water Purification System	MilliQ	Any System
3.2 mm (1/8") paper punch	Any	Any
Parafilm	Any	Any
Deep-well 96-well plate (polypropylene)	Costar	3959
Silicone mat for 96-well plate	Costar	3080
(optional) 1.5 mL tube with snap cap (polypropylene)	VWR	89000-028
96-well microplate (polypropylene)*	Greiner Bio-One	651201
Zone-Free Sealing Film for 96-well microplate	Sigma	Z721646
Incubator with orbital shaker	Any	Any
Pipette, Single Channel, 2-20 µL (or equivalent)	Any	Any
Pipette, Single Channel, 100-1000 µL (or equivalent)	Any	Any
Pipette, Multichannel, 20-200 µL (or equivalent)	Any	Any
P10, P200, and P1000 Pipette tips	Any	Any
Calibrated Hamilton Syringes for accurate small volume measurement	Hamilton	Any
Centrifuge for 96-well plate (swinging bucket rotor)	Any	Any
96-Well plate nitrogen jet manifold (for use at ambient temperature)	Any	Any
2-8°C Refrigerator (Explosion Proof Recommended)	Any	Any
≤ -10°C Freezer	Any	Any
Fume Hood	Any	Any
Mass Spectrometer (see Table 5.1)	Any	Any

** Note: Choose autosampler vials or 96-well autosampler plates that are compatible with the UPLC autosampler that will be used for analysis.*

Section 2. Preparation of the Assay Cocktail with LgtC

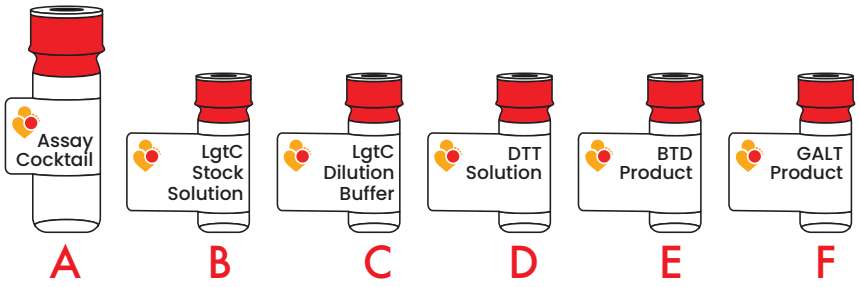

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Figure 2.1 Components of the GALT/BTD Duplex Enzyme Activity Assay Kit

2.1 Instructions for Preparation of the Assay Cocktail

The frozen Assay Cocktail (A), LgtC Stock Solution (B), LgtC Dilution Buffer (C), and DTT Solution (D) are provided by Enfanos as a part of the GALT/BTD Duplex Enzyme Activity Assay Kit (Cat. No. CD3-25). Enzymatic Products (E) and (F) are provided as needed.

Step 1. Allow the Assay Cocktail (A), LgtC Stock Solution (B), LgtC Dilution Buffer (C), and DTT Solution (D) to warm to room temperature.

Step 2. Next, prepare the **LgtC Working Solution** fresh as needed. You need 10 µL of LgtC Working Solution per well. For 25 Assays, dilute 25 µL of the LgtC Stock Solution (B) with 225 µL of LgtC Dilution Buffer (C) (for a 10-fold dilution) using a calibrated Pipette. To this solution, add 2.5 µL of DTT Solution (D) (or add 1 µL for every 100 µL of LgtC Working Solution) using a calibrated Pipette. Mix gently by pipetting up and down 10 times. *For other vial sizes, scale the above recipe as appropriate.*

Mix gently by pipetting up and down 10 times.

Step 3. On the same day as use, freshly prepare 40 µL of **Assay Cocktail with LgtC** for each Duplex Assay by mixing 3 volumes of thawed Assay Cocktail (A) with 1 volume of the **LgtC Working Solution** using a calibrated Pipette. Mix gently by pipetting up and down 10 times.

The resulting **Assay Cocktail** contains 1.0 mM GALT-S, 20 µM GALT-IS, 200 µM BTD-S, and 1.0 µM BTD-IS. If you have question about the composition of the resulting **Assay Cocktail**, please contact us at contact@enfanos.com. Additional components (detergent, inhibitors, activators, buffer salt, etc.) are non-hazardous and are not listed.

Step 4. To prepare a 200 µM methanolic solution of the BTD Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 5 µL of methanol per 1 nmole of BTD Enzymatic Product (E) in the respective Product vial using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions with 1:1 (v:v) Water:Acetonitrile (Reconstitution Solvent, see Section 3.3) as needed. The molecular weight of the BTD Enzymatic Product is 282.39 g/mol.

Step 5. To prepare a 200 µM methanolic solution of the GALT Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 5 µL of methanol per 1 nmole of GALT Enzymatic Product (F) in the respective Product vial using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions with 1:1 (v:v) Water:Acetonitrile (Reconstitution Solvent, see Section 3.3) as needed. The molecular weight of the GALT Enzymatic Product is 924.5 g/mol.

2.2 Storage and Stability of Reagents

The Assay Cocktail (A), LgtC Dilution Buffer (C), and DTT Solution (D) must be stored below −10 °C. The Assay Cocktail (A) is stable frozen at ≤ −10 °C for at least one year. It can be frozen and thawed before use multiple times. The LgtC Stock Solution (B) must be stored between 2-8°C.

Section 3. Recipes for Other Stocks and Buffers

3.1 Quench Solvent (1:1 (v:v) Methanol:Ethyl Acetate)

To prepare 500 mL of Quench Solvent, combine 250 mL of Methanol (HPLC Grade) and 250 mL of Ethyl Acetate (LC-MS Grade, see note in Table 1.1) in a borosilicate glass solvent bottle and mix well. Store at room temperature for up to 6 months.

3.2 NaCl Solution (0.5 M Sodium Chloride in Water)

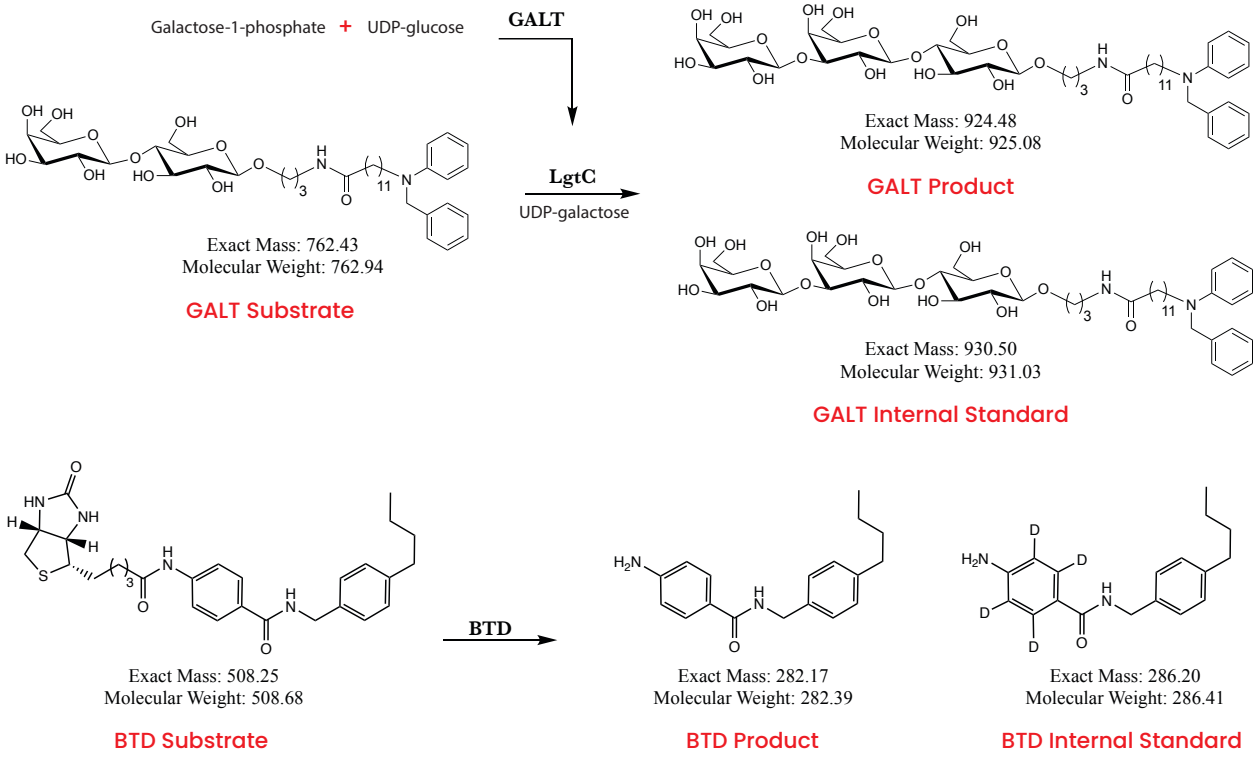
To prepare 500 mL of NaCl Solution, weigh 14.6 g of Sodium Chloride (ACS Reagent Grade) and transfer to a borosilicate glass or polypropylene solvent bottle with 500 mL of MilliQ Purified Water (or equivalent). Mix well until all solids have dissolved. Store between 2-8°C for up to 12 months.

3.3 Reconstitution Solvent (1:1 (v:v) Water:Acetonitrile)

To prepare 500 mL of Reconstitution Solvent, combine 250 mL of Acetonitrile (HPLC Grade) and 250 mL of MilliQ Purified Water (or equivalent) in a borosilicate glass solvent bottle and mix well. Store at room temperature for up to 6 months.

Section 4. Step-by-Step Method

Figure 4.1 Enzyme Assay Scheme



Step 1. Add one 3.2 mm DBS punch to a well in a 96-well deep-well plate (alternatively, use a 1.5 mL polypropylene microcentrifuge tube) and add 40 µL of **Assay Cocktail with LgtC** (prepared in Section 2.1, Step 3)

Seal the plate with a silicone 96-well plate mat (or cap the tube). Spin the plate (or tube) in a centrifuge at 200*g* for 10-20 seconds (sec) to bring all the liquid to the bottom, then shake on an orbital shaker set to shake appropriately for 3 hours at 37°C. Record the time when the plate is put on the incubator. This is the start time for the enzyme activity assay incubation.

** Note: The exact shaking setting will vary depending on the equipment used. The laboratory should determine the optimal shaking speed for their incubator/shaker as part of method implementation.*

Step 2. After incubation, spin the plate (or tube) in a centrifuge at 200g for 10-20 seconds to bring all the liquid to the bottom, then quench the reaction by addition of 100 µL of Quench Solvent (see Section 3.1), 400 µL of LC-MS Grade Ethyl Acetate (see note in Table 1.1), and 200 µL of NaCl Solution (see Section 3.2). Pipet the mixture up and down 10 times to mix well.

Note: only polypropylene or glass materials should be used to dispense Ethyl Acetate, as the solvent dissolves most plastics. Work with Ethyl Acetate should be done in a fume hood.

Record the time when the Quench Solvent is added: this is the end time for the enzyme activity assay incubation.

Cover the plate with the silicone 96-well plate mat (or cap the tube) and spin in a centrifuge at 3000*g* for 5 minutes at ambient temperature to completely separate the aqueous (bottom) and Ethyl Acetate (top) layers.

Step 3. After centrifugation, transfer 150 µL of the top Ethyl Acetate layer to a 96-well polypropylene microplate appropriate for the autosampler you will use.

Evaporate the solvent from each well at room temperature under a nitrogen jet 96-well manifold evaporator.

** Note: If the plate must be stored overnight prior to LC-MS/MS analysis, cover with zone-free sealing film and store at ≤ -10°C. On the day of LC-MS/MS analysis, allow the plate to warm to room temperature and proceed to Step 4.*

Step 4. On the same day as LC-MS/MS analysis, add 200 µL of Reconstitution Solvent (see Section 3.3) to each well. Mix with a multichannel Pipette by pipetting up and down 10x.

Seal the plate using zone-free sealing film, or as appropriate for your autosampler, and submit to LC-MS/MS analysis (see Section 5 for recommended method conditions).

Section 5. Recommended Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method

Table 5.1: Materials and Reagents

Material	Vendor	Vendor Reference
Optima Water (LC-MS) (CAS: 7732-18-5)	Fisher	W6500
Optima Acetonitrile (LC-MS) (CAS: 75-05-8)	Fisher	A955
Optima Isopropanol (LC-MS) (CAS: 67-63-0)	Fisher	A461
Optima Formic Acid (LC-MS) (CAS: 64-18-6)	Fisher	A117
CSH C18 Column, 1.7 µm, 2.1 X 50 mm*	Waters	186005296
CSH C18 Pre-column, 1.7 µm, 2.1 X 5 mm*	Waters	186005303
Acquity 2D UPLC System*	Waters	Or Equivalent
Triple Quad Mass Spectrometer	Any	Any

** Note: While Enfanos recommends use of Waters equipment for LC-MS/MS analysis, equivalent instrumentation may be used for analysis of our enzyme activity assay products and internal standards.*

5.1 LC Method

- **Solvent A.** 0.1% Formic Acid in 70:30 (v:v) Water:Acetonitrile
- **Solvent B.** 0.1% Formic Acid in 65:35 (v:v) Isopropanol:Acetonitrile
- **Weak Needle Wash.** 0.1% Formic Acid in 90:10 (v:v) Water: Acetonitrile
- **Strong Needle Wash.** 0.1% Formic Acid in Acetonitrile
- **Column Temperature.** 55°C

Table 5.2: Gradient Program

Time (min)	Flow (mL/min)	Solvent B (%)
initial	0.8	0.5
0.75	0.8	22
1.00	0.8	28
1.50	0.8	40
1.80	0.8	100
2.15	0.8	100
2.20	0.8	0.5

** Note: To minimize contamination of the ESI source, we advise to divert the LC flow to waste except in the retention time region (see Table 5.3) where the products and internal standards elute (~1.20 to ~1.70 min).*

*** Note: In-source breakdown of the BTD Substrate may result in an interfering peak in the BTD Product MRM trace chromatogram. To prevent this interference, special attention must be paid to ensure baseline separation between the substrate and the product/internal standard peaks. If baseline separation is not achieved on the first try, modify the LC gradient. The BTD-S MRM is given in Table 5.3 for this purpose. Reach out to us with any questions (contact@enfanos.com).*

5.2 MRM Method

MRM Transitions (see Table 5.3) are measured in ESI positive mode.

** Note: Method developed for analysis on Waters XEVO TQD Instrument (or instrument with equivalent sensitivity). If a higher sensitivity instrument, such as a Waters XEVO TQ-S Micro is being used we recommend adjusting sample preparation so the injected solution contains less moles of analyte or detuning the collision energy of your analytes so the internal standard peak areas are approximately 10,000-100,000 AUC. This will ensure linearity throughout the analytical range*

Table 5.3: MRM Method

Analyte	Parent (m/z)	Product (m/z)	Retention Time (min)
BTD-Product	283.3	91.0	1.20
BTD-Internal Standard	287.2	91.0	1.20
BTD-Substrate	509.2	120.1	1.30
GALT-Product	925.6	439.3	1.70
GALT-Internal Standard	931.8	439.3	1.70

** Note: Transitions, cone voltage, collision energy, and retention times need to be confirmed or determined by tuning on your LC-MS/MS instrument in the usual way.*

5.3 Formula for Specific Activity

$$\boldsymbol{BTD\ Specific\ Activity\ (\mu mol/h/L) = \frac{P}{IS} \times \frac{0.00003\ \mu mole}{3\ h \times 3.2 \times 10^{-6} L}}$$

$$\boldsymbol{GALT\ Specific\ Activity\ (\mu mol/h/L) = \frac{P}{IS} \times \frac{0.0006\ \mu mole}{3\ h \times 3.2 \times 10^{-6} L}}$$

To obtain the enzymatic activity, multiply the product-to-internal standard peak area ratio by the micromoles of internal standard in the assay (0.00003 µmole BTD or 0.0006 µmole GALT). This number is then divided by the incubation time (3 hours) and the liters of blood in a 3.2 mm DBS punch (3.2 x 10^{-6} L).

** Note: To calculate the incubation time, subtract the enzyme activity assay end time (see Section 4, Step 2) from the enzyme activity assay start time (see Section 4, Step 1).*

*** Note: This procedure describes the preparation and handling of research-use-only materials. It does not establish or support clinical testing, diagnostic use, or patient result interpretation. Any use of this product in laboratory developed tests or clinical applications is the sole responsibility of the user. Enfanos does not validate or support clinical or diagnostic use.*

Section 6. References

Hong, X., Kumar, A.B., Scott, C.R. and Gelb, M.H., 2018. Multiplex tandem mass spectrometry assay for newborn screening of X-linked adrenoleukodystrophy, biotinidase deficiency, and galactosemia with flexibility to assay other enzyme assays and biomarkers. Molecular genetics and metabolism, 124(2), pp.101-108.

Hong, X., Sadilek, M. and Gelb, M.H., 2020. A highly multiplexed biochemical assay for analytes in dried blood spots: application to newborn screening and diagnosis of lysosomal storage disorders and other inborn errors of metabolism. Genetics in Medicine, 22(7), pp.1262-1268.

Section 7. Document Audit Trail

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