



**SOP: 6-Plex Enzyme Activity Assay
Kit for IDUA, GAA, GLA, GALC, GBA,
& ASM Cat. No. CM2
LC-MS/MS Method**

**ID: SOPPCM2LC
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Standard Operating Procedure:

6-Plex Enzyme Activity Assay Kit IDUA, GAA, GLA, GALC, GBA, & ASM Cat. No. CM2

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method

SOP ID: SOPPCM2LC
Version Number: V7.0
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Product Name: 6-Plex 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
Enfanos 6-Plex Enzyme Activity Assay Kit	Enfanos	CM2
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.

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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

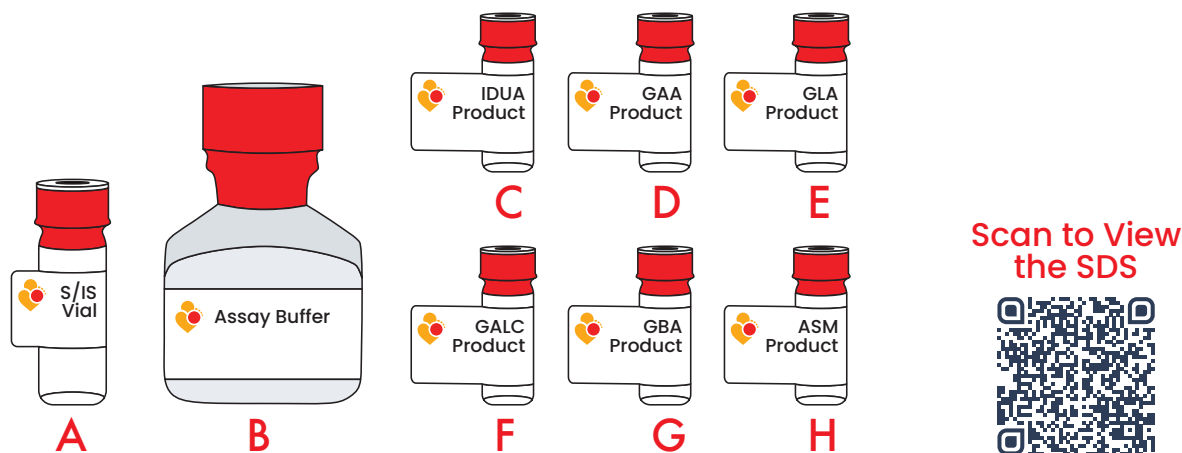


Figure 2.1 Components of the 6-Plex Enzyme Activity Assay Kit

2.1 Instructions for Preparation of the Assay Cocktail

The S/IS Vial(s) (**A**) and Assay Buffer (**B**) are provided by Enfanos as a part of the 6-Plex Enzyme Activity Assay Kit (Cat. No. CM2). Enzymatic Products (**C - H**) are provided as needed.

Step 1. Allow the Assay Buffer (**B**) and S/IS Mixture vial (**A**) to warm to room temperature.

Step 2. Next, add 1.50 mL of the Assay Buffer (**B**) to the S/IS Mixture vial (for 50 assays) (**A**) using a calibrated Pipette. *For other vial sizes, reconstitution volume is printed on the S/IS Mixture Vial.*

Vortex the vial and allow to sit at room temperature for at least 30 minutes. After 30 minutes, vortex vial again and visually inspect with light behind it to ensure all particulates and/or film on the glass is dissolved and a clear solution is formed. If a clear solution is not obtained after vortexing, the vial should be sonicated at room temperature. Avoid prolonged sonication (complete dissolution usually takes 10-20 seconds of sonication). The vial should be visually inspected with light behind it to ensure all particulates and /or film on the glass is dissolved and a clear solution is formed. If a clear solution is not obtained after sonication, heat the cocktail vial in a water bath set to 55°C for 1-3 minutes until a crystal clear solution is obtained. **A crystal clear solution must be obtained before you proceed.**



The resulting **Assay Cocktail** contains the components given in Table 2.1.

Table 2.1: Assay Cocktail Component Concentrations

Enzyme	Substrate	Internal Standard
IDUA	250 μ M	5 μ M
GAA	350 μ M	10 μ M
GLA	1200 μ M	10 μ M
GALC	850 μ M	5 μ M
GBA	500 μ M	5 μ M
ASM	750 μ M	15 μ M

If you have questions 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 3. To prepare a 200 μ M methanolic solution of the IDUA Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 5 μ L of methanol per 1 nmole of Enzymatic Product in the IDUA Enzymatic Product vial (**C**) using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions using 70:30 (v:v) Acetonitrile:Water (Reconstitution Solvent, see Section 3.3).

To prepare a 100 μ M methanolic solution of the GAA, GLA, GALC, GBA, or ASM Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 10 μ L of methanol per 1 nmole of Enzymatic Product in the GAA (**D**), GLA (**E**), GALC (**F**), GBA (**G**), or ASM (**H**) Enzymatic Product vial using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions using 70:30 (v:v) Acetonitrile:Water (Reconstitution Solvent, see Section 3.3) as needed. The molecular weights of the Enzymatic Products are provided in Table 2.2.

Table 2.2: Molecular Weights of the Enzymatic Products

Enzymatic Product	Molecular Weight, (g/mol)
IDUA-P	232.28
GAA-P	497.64
GLA-P	483.61
GALC-P	425.70
GBA-P	481.81
ASM-P	397.64

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2.2 Storage and Stability of Reagents

Formal stability data for long-term storage of S/IS Vials (**A**) and Enzymatic Product Vials (**C - H**) are not available.

The Assay Buffer (**B**) should be stored at 2-8°C. The Assay Buffer (**B**) is good for 1 year from date of manufacture.

The Assay Cocktail (prepared in Section 2.1, Step 2) should be prepared fresh before use. Formal stability data for the Assay Cocktail are not available.

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 14.61 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 at 2-8°C for up to 12 months.

3.3 Reconstitution Solvent (70:30 (v:v) Acetonitrile:Water)

To prepare 500 mL of Reconstitution Solvent, combine 350 mL of HPLC Grade Acetonitrile and 150 mL of MilliQ purified water (or equivalent) in a borosilicate glass or polypropylene solvent bottle and mix well. Store at room temperature for up to 6 months.



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 30 μ L of the Assay Cocktail (prepared in Section 2.1, Step 2).

Seal the plate with a silicone 96-well plate mat (or cap the tube). Spin the plate (or tube) in a centrifuge at 200g for 10-20 seconds to bring all the liquid to the bottom, then incubate on an orbital shaker set to shake appropriately for 16 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 μ L of Quench Solvent (see Section 3.1), 400 μ L of LC-MS Grade Ethyl Acetate (see note in Table 1.1), and 200 μ L 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 3000g for 5 minutes at ambient temperature to completely separate the aqueous (bottom) and Ethyl Acetate (top) layers.

Step 3. After centrifugation, transfer 200 μ L 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.

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** Note: If the plate must be stored overnight prior to LC-MS/MS analysis, cover with zone-free sealing film and store at $\leq -10^{\circ}\text{C}$. 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 150 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 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	Waters	Or Equivalent

** 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 Water
- **Solvent B.** 0.1% Formic Acid in 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.** 40°C

Table 5.2: Gradient Program

Time (min)	Flow (mL/min)	Solvent B (%)
initial	0.7	30
0.80	0.7	100
1.90	0.7	100
2.00	0.7	30

* 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 (~0.69 min, ~0.65 min, ~1.34 min, ~1.77 min, and ~1.21 min).

* Note: In-source breakdown of the enzymatic substrate may result in an interfering peak in the enzymatic product MRM trace chromatograms. This interfering peak will not be present in the internal standard MRM trace chromatogram. Such interference can be prevented by modifying the LC Gradient so that baseline separation is achieved between the interfering substrate peak and the enzymatic product peak. 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)
IDUA Product	233.20	163.20	0.75
IDUA Internal Standard	237.20	166.20	0.75
GAA Product	498.53	398.38	0.69
GAA Internal Standard	503.53	403.38	0.69
GLA Product	484.51	384.46	0.65
GLA Internal Standard	489.51	389.46	0.65
GALC Product	426.38	264.40	1.34
GALC Internal Standard	431.38	264.40	1.34
GBA Product	482.50	264.40	1.77
GBA Internal Standard	487.50	264.40	1.77
ASM Product	398.50	264.40	1.21
ASM Internal Standard	405.50	264.40	1.21

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

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5.3 Formula for Specific Activity

$$IDUA \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00015 \mu\text{moles IDUA IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

$$GAA \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00030 \mu\text{moles GAA IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

$$GLA \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00030 \mu\text{moles GLA IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

$$GALC \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00015 \mu\text{moles GALC IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

$$GBA \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00015 \mu\text{moles GBA IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

$$ASM \text{ Specific Activity } (\mu\text{mol/h/L}) = \frac{P}{IS} \times \frac{0.00045 \mu\text{moles ASM IS}}{16 \text{ h} \times 3.2 \times 10^{-6} \text{ L}}$$

To obtain the enzymatic activity, multiply the product-to-internal standard peak area ratio by the micromoles of internal standard in the assay (see formula above for each enzyme). This number is then divided by the incubation time (16 hours) the liters of blood in a 3.2 mm DBS punch (3.2×10^{-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.*

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Section 6. References

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Section 7. Document Audit Trail

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