# Standard Operating Procedure:

# α-L-Fucosidase 1 (FUCA1) Enzyme Activity Assay Kit Cat. No. CS1

**SOP ID:** SOPPCS1
**Version Number:** V1.0
**Version Date:** 05 February 2026
**Product Name:** FUCA1 Enzyme Activity Assay Kit
**Matrices:** Dried Blood Spot

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|---|---|---|
| **Authored By:**<br>Olive Lee White<br>Bioanalytical Technician<br>Enfanos, LLC. | Signed by Olive Lee White<br>Olive Lee White<br>I am the author of this document<br>06-Feb-26 \| 11:47:49 AM PST<br>F8940F4C7FA34049A98114DD1A9A89B2 | **Date:** 06-Feb-26 |
| **Reviewed By:**<br>Zackary M. Herbst<br>Chief Science Officer<br>Enfanos, LLC. | Signed by Zackary M. Herbst<br>I have reviewed this document<br>06-Feb-26 \| 12:36:07 PM PST<br>95BFF6AAC0DB48458BA48EAB3EB25F25 | **Date:** 06-Feb-26 |
| **Approved By:**<br>Hamid Khaledi<br>Chief Executive Officer<br>Enfanos, LLC. | Signed by Hamid Khaledi<br>I approve this document<br>06-Feb-26 \| 4:00:43 PM PST<br>61319B2C6B7745E88E51504BBC9F42F9 | **Date:** 06-Feb-26 |

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# Section 1. Materials and Reagents

## Table 1.1: Reagents and Standards

| Material | Vendor | Vendor Reference |
|---|---|---|
| FUCA1 Enzyme Activity Assay Kit | Enfanos | CS1 |
| 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

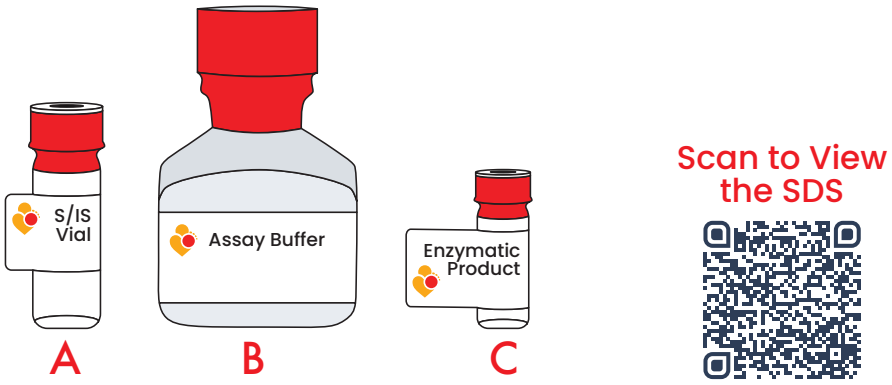



Figure 2.1 Components of the FUCA1 Enzyme Activity Assay Kit

### 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 FUCA1 Enzyme Activity Assay Kit (Cat. No. CS1). Enzymatic Product (C) is provided as needed.

Step 1. Allow the Assay Buffer (B) and S/IS 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 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. **A crystal clear solution must be obtained before you proceed**.

The resulting **Assay Cocktail** contains 1.0 mM FUCA1-S and 20 µM FUCA1-IS. If you have question about the composition of the resulting **Assay Cocktail**, please contact us at contact@enfanos.com. Assay cocktail components (detergent, inhibitors, activators, buffer salt, etc.) are non-hazardous or are at non-hazardous levels and are not listed.

**Step 3.** To prepare a 200 µM methanolic solution of the Enzymatic Product for LC-MS/MS tuning or standard curve preparation, add 5 µL of methanol per 1 nmole of Enzymatic Product in the Enzymatic Product vial (**C**) using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions as needed in 1:1 (v:v) Water:Methanol. The molecular weight of Enzymatic Product is 369.2 g/mol.

## 2.2 Storage and Stability of Reagents

Formal stability data for long-term storage of S/IS Vials (**A**) and Enzymatic Product Vials (**C**) are not available. We prefer to refrain from giving artificially short expiration dates, which would lead to wasteful discarding of reagents. However, our knowledge of chemical stability leads us to suggest that these solids are expected to be indefinitely stable when stored at ≤-10°C in a jar containing desiccant (desiccants should contain a colored moisture indicator and should be replaced when spent).

The Assay Buffer (**B**) should be stored at 2-8°C.

The **Assay Cocktail** (prepared in Section 2.1, Step 2) should be prepared fresh before use. Freeze-thawing the **Assay Cocktail** results in precipitation of reagents and errors in the assay. Storage of the **Assay Cocktail** at 2-8°C results in inaccurate measurements of the enzyme's specific activity.

# 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)

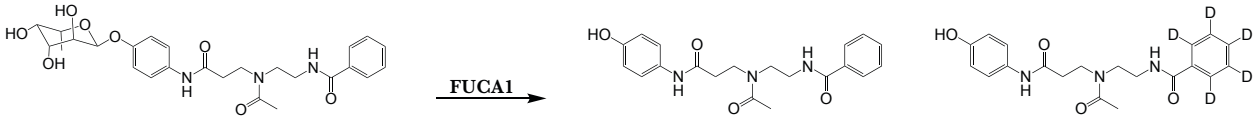
To prepare 500 mL of NaCl Solution, weigh 14.61 g of Sodium Chloride (ACS Reagent Grade) and transfer to a borosilicate glass or polypropylene solvent bottle with 500 mL of MilliQ Purified Water (or equivalent). Mix well until all solids have dissolved. Store at 2-8°C for up to 12 months.

## 3.3 Reconstitution Solvent (3:1 (v:v) Water:Acetonitrile)

To prepare 500 mL of Reconstitution Solvent, combine 125 mL of HPLC Grade Acetonitrile and 375 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.

# Section 4. Step-by-Step Method

## Figure 4.1 Enzyme Assay Scheme




Exact Mass: 515.23
Molecular Weight: 515.56

FUCA1 Substrate

Exact Mass: 369.17
Molecular Weight: 369.42

FUCA1 Product

Exact Mass: 374.20
Molecular Weight: 374.45

FUCA1 Internal Standard

**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 µL 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 200*g* 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 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 100 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 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.** 10% Acetonitrile in Water
- **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 | 0.8 | 15 |
| 1.15 | 0.8 | 20 |
| 1.26 | 0.8 | 99 |
| 1.60 | 0.8 | 99 |
| 1.80 | 0.8 | 15 |
| 2.00 | 0.8 | 15 |

** 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 product and internal standard elute (~1.03 min).*

** Note: To prevent interference due to substrate-to-product conversion in the ESI source, special attention must be paid to ensure baseline separation between the substrate and the product/internal standard peaks (the substrate peak will be visible in the FUCA1 Product MRM channel, but not in the FUCA1 Internal Standard MRM channel). If baseline separation is not achieved on the first try, modify the LC gradient. The FUCA1-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) |
|---|---|---|---|
| FUCA1-Product | 370.4 | 261.1 | 1.03 |
| FUCA1-Internal Standard | 375.3 | 266.1 | 1.03 |
| FUCA1-Substrate | 516.5 | 261.1 | 0.90 |

** 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

$$\boldsymbol{FUCA1\ Specific\ Activity\ (\mu mol/h/L)} = \frac{\boldsymbol{P}}{\boldsymbol{IS}} \times \frac{\boldsymbol{0.0006\ \mu mole}}{\boldsymbol{16\ h\ \times 3.2 \times 10^{-6} L}}$$

To obtain the enzymatic activity, multiply the product-to-internal standard peak area ratio by the micromoles of internal standard in the assay (0.0006 µmol). This number is then divided by the incubation time (16 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

Kumar, A.B., Hong, X., Yi, F., Wood, T. and Gelb, M.H., 2019. Tandem mass spectrometry-based multiplex assays for α-mannosidosis and fucosidosis. Molecular Genetics and Metabolism, 127(3), pp.207-211.

## Section 7. Document Audit Trail

*Previous versions of this SOP are stored internally by Enfanos, LLC. If you require a copy of a previous version for comparison to this most recent version, please contact us at contact@enfanos.com.*