Standard Operating Procedure:

Palmitoyl Protein Thioesterase-1 (PPT1) & Tripeptidyl Peptidase-1(TPP1) Duplex Assay Kit Cat. No. CD1

SOP ID:	SOPPCD1
Version Number:	V2.0
Version Date:	31 March 2025
Product Name:	PPT1/TPP1 Duplex Assay Kit
Matrices:	Dried Blood Spot

Authored & Approved By: Zackary Herbst Chief Science Officer Enfanos, LLC.	Signed by Zackary Herbst I approve this document 31-Mar-25 | 3:23:19 PM PDT 95BFF6AAC0DB48458BA48EAB3EB25F25	**Date:** 31-Mar-25
Reviewed & Approved By: Hamid Khaledi Chief Executive Officer Enfanos, LLC.	Signed by Hamid Khaledi I approve this document 31-Mar-25 | 3:12:20 PM PDT 61319B2C6B7745E88E51504BBC9F42F9	**Date:** 31-Mar-25

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Section 1. Materials and Reagents

Table 1.1: Reagents and Standards

Material	Vendor	Vendor Reference
PPT1/TPP1 Duplex Assay Kit	Enfanos	CD1
MilliQ Purified Water, or Equivalent	Various	n/a
S-Methylmethanethiosulfonate (MMTS)	Sigma Aldrich	64306
HPLC Grade Methanol	Fisher	A452
HPLC Grade Acetonitrile	Fisher	A998
Optima (LC-MS) Grade Formic Acid	Fisher	A117

Table 1.2: Equipment and Consumables

Material	Vendor	Vendor Reference
MilliQ Water Purification System	MilliQ	Any System
3.0 mm paper punch	Any	Any
Parafilm	Any	Any
Deep-well 96-well plate (polypropylene)	Costar	3959
(optional) 1.5 mL tube with snap cap (polypropylene)	VWR	89000-028
96-well microplate (polypropylene)*	Greiner Bio-One	651201
Silicone mat for 96-well plate	Sigma	Z374938
Aluminum foil or Sealing film for LC plate	Any	Any
Incubator with orbital shaker	Any	Any
Pipetman, Single Channel, 2-20 µL (or equivalent)	Any	Any
Pipetman, Single Channel, 100-1000 µL (or equivalent)	Any	Any
Pipetman, Multichannel, 20-200 µL (or equivalent)	Any	Any
P10, P200, and P1000 Pipetman tips	Any	Any
Calibrated Hamilton Syringes for accurate small volume measurement	Hamilton	Any
Centrifuge for 96-well plate (swinging bucket rotor)	Any	Any
2-8°C Refrigerator (Explosion Proof Recommended)	Any	Any
-20°C Freezer	Any	Any

* 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

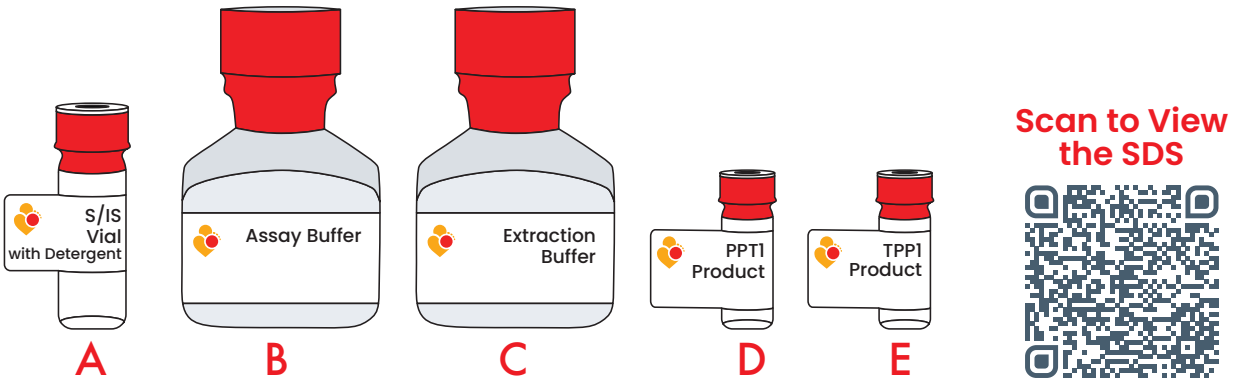


Figure 1. Components of the PPT1/TPP1 Duplex Assay Kit

2.1 Step-by-Step Instructions

The Assay Buffer (**B**) (50 mM Sodium Acetate, pH 4.5) and DBS Extraction Buffer (**B**) (200 mM Citrate Phosphate, pH 4.5) are provided by Enfanos as a part of the PPT1/TPP1 Duplex Assay Kit (Cat. No. CD1).

Step 1. Add 1000 µL of the Assay Buffer (**B**) to the S/IS Mixture vial for 50 Duplex Assays (**A**) using a calibrated Pipetman. Vortex until all material is dissolved and a clear solution is obtained. The vial should be visually inspected with light behind it to ensure all particulates and/or film on the glass is dissolved. The resulting **Assay Cocktail** contains 0.6 mM of PPT1 substrate, 45 µM of PPT1 internal standard, 0.6 mM of TPP1 substrate, and 45 µM TPP1 internal standard, and 70 mM octyl ß-glucoside detergent.

Step 2. For LC-MS/MS tuning or standard curve preparation of the PPT1 Product, add 5 µL of Product Diluent (see Section 3.2) per 1 nmole of PPT1 Product (**D**) in the respective Product vial using a Hamilton Syringe to prepare a 200 µM stock. Vortex to mix. Prepare additional dilutions with 1:1 (v/v) Water: Acetonitrile as needed.

Step 3. To prepare a 200 µM methanolic solution of the TPP1 Product for LC-MS/MS tuning or standard curve preparation, add 5 µL of methanol per 1 nmole of TPP1 Product (**E**) in the respective Product vial using a Hamilton Syringe. Vortex to mix. Prepare additional dilutions with 1:1 (v/v) Water:Acetonitrile as needed.

2.2 Storage and Stability of Reagents

Long term storage data on reagents provided as solids 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 below –10°C in a jar containing desiccant (desiccants should contain a colored moisture indicator and should be replaced when spent).

The Assay Cocktail (prepared in Section 2.1, Step 1) is stable frozen below –10°C for at least one month. Before storing the Assay Cocktail, wrap the cap tightly with ParaFilm. Upon thawing, allow the Assay Cocktail to warm to room temperature without heating. It may take up to 15 minutes to get a clear solution. The Assay Cocktail can be frozen and thawed before use at least two times but freeze-thaw cycles should be minimized if possible.

Section 3. Recipes for Other Stocks and Buffers

3.1 MMTS Aqueous Solution (10 mM S-methylmethanethiosulfonate in water)

To prepare 100 mL of MMTS Aqueous Solution, add 94 µL (126.2 mg) of MMTS (126.20 g/mol) into a borosilicate glass or polypropylene solvent bottle using a Pipetman. To this, add 100 mL of MilliQ Purified Water (or equivalent) and mix well by vortexing. Store between 2-8°C with cap wrapped tightly in ParaFilm for up to 1 year.

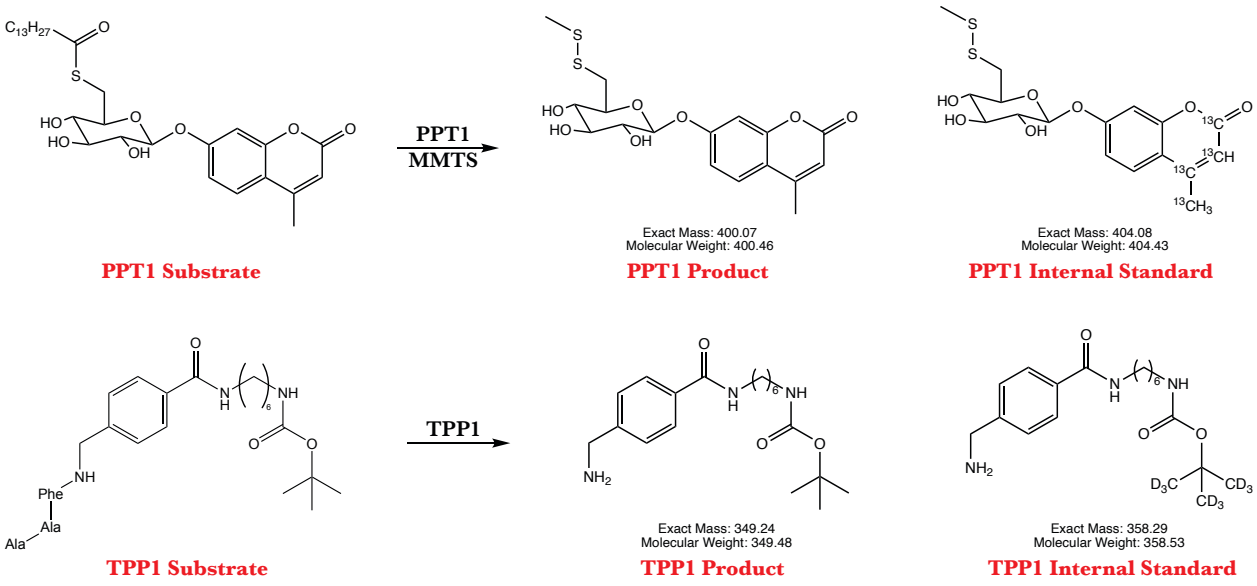
3.2 Product Diluent (1 mM MMTS in 9:1 (v/v) Methanol:Water)

To prepare 1 mL of Product Diluent, add 900 µL of Methanol (HPLC Grade) and 100 µL of 10 mM MMTS Aqueous Solution (as prepared in Section 3.1) to a borosilicate glass or polypropylene vial. Mix well. Prepare fresh before use and discard extra Product Diluent that is not used on the same day as preparation. *Note that the Product Diluent is only needed for Section 2.1, Step 3. It is not needed for the Step-by-Step method (Section 4).*

3.3 LC-MS Diluent (0.1% (v/v) formic acid in water)

To prepare 500 mL of LC-MS Diluent, add 500 µL of Optima Grade Formic Acid to 499.5 mL of MilliQ Purified Water (or equivalent) in a borosilicate glass or polypropylene solvent bottle. Mix well. Store between 2-8°C for up to 1 year.

Section 4. Step-by-Step Method



Step 1. Add one 3 mm DBS punch and 50 µL of Extraction Buffer (**C**) (provided by Enfanos, see Section 2) to a well in a 96-well deep-well plate (alternatively, use a 1.5 mL polypropylene microcentrifuge tube).

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 seconds (sec) to bring all the liquid to the bottom, then shake on an orbital shaker set to 400 rpm for 90 minutes at room temperature.

Step 2. Next, pipet the DBS extracts up and down 5 times, then transfer 30 µL of the liquid to a new deep-well 96-well plate.

Add 5 µL of 10 mM MMTS Aqueous Solution (see Section 3.1) and mix by pipetting up and down 10 times with a Pipetman. Next, add 20 µL of **Assay Cocktail** (prepared in Section 2.1, Step 1) to each well.

Cover the plate with the silicone 96-well plate mat (or cap the tube) and spin in a centrifuge at 200*g* for 10 sec to bring the liquid to the bottom of the well (or tube). Incubate at 37°C while shaking at 250 rpm for 16 hours. *Note: If you require an alternative incubation time, contact us at contact@enfanos.com.*

Step 3. After incubation, quench each well by adding 200 µL of Acetonitrile (HPLC grade or better). Mix each assay thoroughly with a Pipetman (use multichannel for 96-well plates and single channel for single tubes) by pipetting up and down 10x.

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 pellet precipitates and other solid material.

Step 4. After centrifugation, transfer 50 µL of the supernatant liquid (avoiding the pellet) to a 96-well polypropylene microplate appropriate for the autosampler you will use. Add 150 µL of LC-MS Diluent (see Section 3.3) to each well. Mix with a multichannel Pipetman by pipetting up and down 10x.

Wrap the plate with aluminum foil or seal the plate as appropriate for your autosampler and submit to LC-MS/MS analysis (see Section 5 for recommended method conditions).

Note: If you prefer the method which uses an ethyl acetate extraction instead of acetonitrile quench, please contact us at contact@enfanos.com.

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)	Fisher	W6500
Optima Acetonitrile (LC-MS)	Fisher	A955
Optima Formic Acid (LC-MS)	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
Xevo TQ (ESI Probe)*	Waters	Or Equivalent

** While Enfanos recommends use of Waters equipment for LC-MS/MS analysis, equivalent instrumentation may be used for analysis of our enzyme 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:** 55°C

Table 5.2: Gradient Program

Time (min)	Flow (mL/min)	Solvent B (%)
initial	0.8	22
1.00	0.8	30
1.05	0.8	99
1.20	0.8	100
1.25	0.8	22
1.35	0.8	22

NOTE: Due to the presence of large amounts of octyl glucoside detergent in the samples, it is advised to divert the LC flow to waste except in the retention time region where the products and internal standards elute (~0.35 to ~0.89 min). This will prevent contamination of the ESI source. When developing the method, make sure the PPT1-Product has eluted prior to increasing the % Solvent B to 99%. It is important to ensure that baseline separation is achieved between the PPT1-Product and octyl ß-glucoside peaks. The gradient given above may therefore need to be adjusted for your instrument.

5.2 MRM Method.

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

Table 5.3: MRM Method

Analyte	Parent (m/z)	Product (m/z)	Retention Time
Octyl ß-glucoside	293.0	84.9	1.1 min
PPT1-Product	401.3	177.2	0.89 min
PPT1-Internal Standard	405.2	181.2	0.89 min
TPP1-Product	350.2	250.2	0.35 min
TPP1-Internal Standard	359.3	251.2	0.35 min

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

$$\boldsymbol{PPT1\ Enzymatic\ Specific\ Activity}\ (\mu\boldsymbol{mol/h/L}) = \frac{\boldsymbol{P}}{\boldsymbol{IS}} \times \frac{\mathbf{0.0009}\ \mu\boldsymbol{mole}}{\mathbf{16}\ \boldsymbol{h} \times \mathbf{0.6} \times \mathbf{3.2} \times \mathbf{10^{-6}}\boldsymbol{L}}$$

$$\boldsymbol{TPP1\ Enzymatic\ Specific\ Activity}\ (\mu\boldsymbol{mol/h/L}) = \frac{\boldsymbol{P}}{\boldsymbol{IS}} \times \frac{\mathbf{0.0009}\ \mu\boldsymbol{mole}}{\mathbf{16}\ \boldsymbol{h} \times \mathbf{0.6} \times \mathbf{3.2} \times \mathbf{10^{-6}}\boldsymbol{L}}$$

To obtain the enzymatic activity, multiply the product-to-internal standard peak area ratio by the micromoles of internal standard in the assay (0.0009 µmol for PPT1 and TPP1). This number is then divided by the incubation time (16 hours) and 3/5th of the microliters of blood in a 3 mm DBS punch (0.6 X 3.2 µL).

Section 6. References

Khaledi, H., Liu, Y., Masi, S. and Gelb, M.H., 2018. Detection of infantile batten disease by tandem mass spectrometry assay of PPT1 enzyme activity in dried blood spots. Analytical Chemistry, 90(20), pp.12168-12171.

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

Date	Version	Recorded Revisions
17MAR23	*DRAFT*	*First draft created*
17MAR23	*V1.0*	*No previous version, no revisions.*
31MAR25	*V2.0*	• *Updated SOP title and branding from GelbChem to Enfanos throughout;* • *Updated links throughout;* • *Updated email references to contact@enfanos.com throughout;* • *Updated formatting of cover page for new SOP template;* • *Added Section 7, Document Audit Trail;* • *Updated Table 1.1;* ○ *Added MilliQ water;* • *All 4°C storage conditions updated to between 2-8°C;* • *Removed recommended instrumentation on page 4;* • *Figure 1 updated with new branding and SDS Link;* • *TPP1-S Structure Updated;* • *Step 3. Specify HPLC grade or better Acetonitrile;* • *Section 5.3 formula updated for clarity;* • *Add statement about measuring in ESI positive mode in Section 5.2.*