

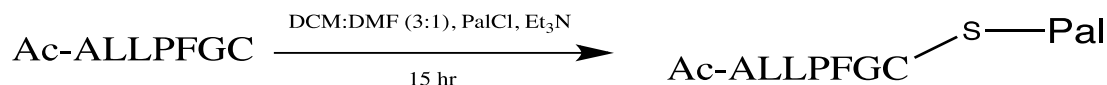
Supporting Information for

Tandem Mass Spectrometry Assays of Palmitoyl Protein Thioesterase and Tripeptidyl Peptidase Activity in Dried Blood Spots for the Detection of Neuronal Ceroid Lipofuscinoses in Newborns

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PPT1 Substrate Synthesis. The substrate for PPT1 was prepared by *S*-acylation of peptide ALLPFGC following a procedure reported by Rijkers et al.^{s1} and summarized in Scheme S1. *N*-Acetylated peptide ALLPFGC (20 mg, 26.3 μmol, Lifetein, Hillsborough, NJ) was dissolved in a 3:1 mixture of dichloromethane/dimethylformamide and mixed with triethylamine (73 μL, 523 μmol). After stirring for 5 minutes, 0.1 M solution of palmitoyl chloride (162 μL, 525 μmol) in dichloromethane was added slowly. The mixture was allowed to react for 15 hours at room temperature. Upon reaction completion the solvent was removed under reduced pressure. The product was redissolved in acetonitrile and filtered. The filtered solution was purified by column chromatography on silica gel, elution with a 5:1 dichloromethane/methanol mixture containing 0.5% acetic acid (*R*_f 0.76). The substrate was isolated as a white solid in 69% yield (18.1 mg, 18.1 μmol). Mass spectrometry data confirmed the identity of the substrate.

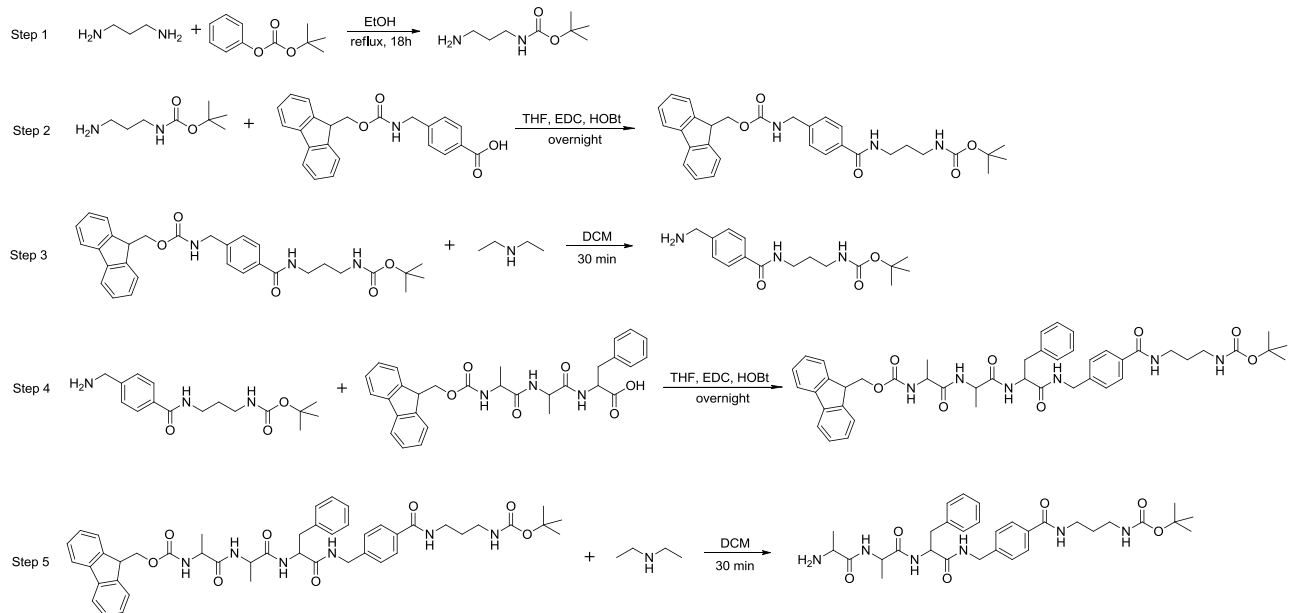


Scheme S1. Synthesis of the palmitoylated substrate for PPT1.

(s1) Rijkers, D., Kruijtzter, J., Killian, J.A., Liskamp, R.M.J. A convenient solid phase synthesis of *S*-palmitoyl transmembrane peptides. *Tetrahedron Lett.* **2005**, *46*, 3341-3345.

TPP1 Substrate Synthesis. The synthetic steps are shown in [Scheme S2](#) and described below.

Step1: *tert*-Butyl phenyl carbonate (9.7 mL, 50 mmol) was added to a solution of 1,3-diaminopropane (3.7 mL, 50 mmol) in 35 mL ethanol in a 200-mL, single-necked, round-bottomed flask equipped with a stirring bar and a reflux condenser.^{s2} The reaction mixture was heated gently to reflux overnight ensuring that the temperature was in the range of 80-85 °C, resulting in a yellow solution. The reaction mixture was cooled to room temperature and the solution was concentrated to approximately 7-8 mL using a rotary evaporator, which left a yellow solution. Water (50 mL) was added and pH was adjusted to approximately pH 3 by careful addition of aqueous HCl, followed by extraction with CH₂Cl₂ (3 x 80 mL). The aqueous layer was adjusted to pH 13 by the addition of 2M NaOH and extracted with CH₂Cl₂ (5 x 100 mL). The combined organic extracts were concentrated using a rotary evaporator to afford 2.8 g of a yellow oil which crystallized within ~1 h. Yield: 33% MW: 174.2 MS: *m/z* 175.0 [M+H]⁺



Scheme S2.

Step 2: A solution of Fmoc-4-aminomethylbenzoic acid (74.7 mg, 0.2 mmol, 1 eq) in 10 mL of anhydrous tetrahydrofuran was cooled to 0 °C on ice. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (42.2 mg, 0.22 mmol, 1.1 eq) and 1-hydroxybenzotriazole (27.1 mg, 0.22 mmol, 1.1 eq) were added, and the suspension was stirred for 30 min at 0 °C. A solution of *N*-Boc-1,3-diaminopropane (34.8 mg, 0.2 mmol, 1 eq) in 2 mL of *N,N*-dimethylformamide was added dropwise to the suspension.^{s3} The reaction mixture was allowed to warm to room temperature and then was stirred overnight. The reaction mixture was concentrated using a rotary evaporator. The residue was taken up in ethyl acetate (100 mL), and then washed with 1M HCl (80 mL) and water (3 x 80 mL). The organic solvent was evaporated using a rotary evaporator. The solid product was purified by crystallization from isopropyl alcohol to give the pure compound. MW = 529.4 MS: m/z 552.4 [M+Na]⁺.

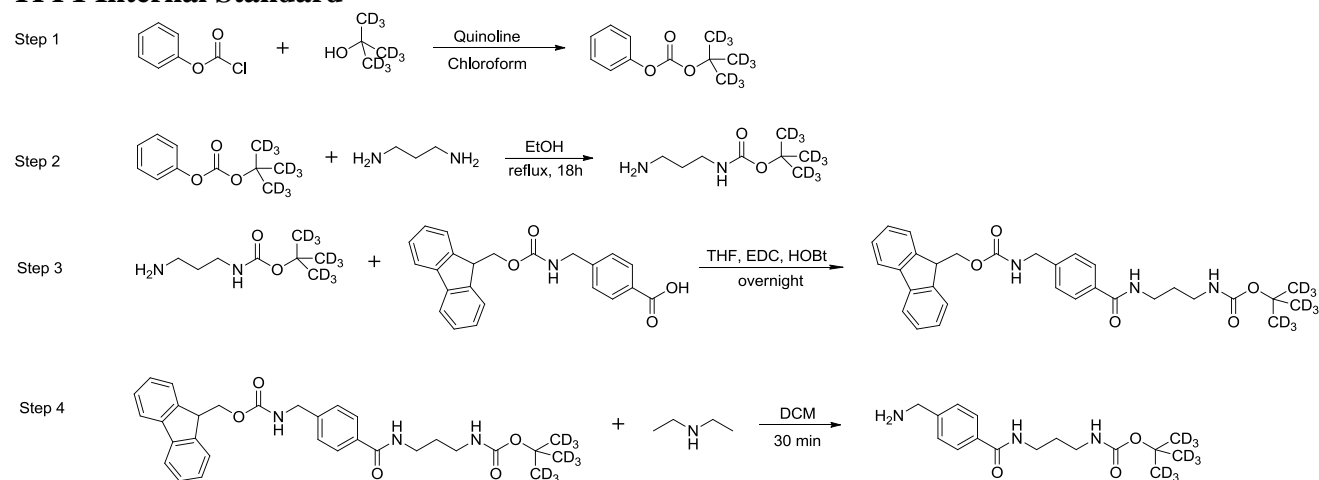
Step 3: A mixture of the Fmoc-protected product synthesized in Step 2 (80 mg, 0.15 mmol) was dissolved in 6 mL of a diethylamine (DEA)/dichloromethane mixture (v/v = 1:1). The mixture was magnetically stirred until the white colored solution turned yellow (approximately 30 min). The organic solvent was evaporated using a rotary evaporator and the residual mass (yellow oil) was purified by column chromatography (SiO₂, particle size 40-63 μm) using stepped gradient of ethyl acetate: methanol(100 → 80:20), 1% triethylamine. The 80:20 fraction was evaporated on a rotary evaporator. The sample was left in a desiccator for a day or two to afford crystals that had a yellow tint. Yield: 82% MW: 307.3 MS: m/z 308.3 [M+H]⁺; m/z 615.3 [2M+H]⁺; ¹H-NMR(CDCl₃): 1.38 (s, 9H), 1.65 (m, 2H), 3.06 (t, 2H), 3.25 (t, 2H), 4.07 (s, 2H), 7.58 (d, 2H), 7.89 (d, 2H).

Step 4: A solution of Fmoc-AAF (4 mg, 0.008 mmol, 1 eq) in 2 mL of anhydrous tetrahydrofuran was cooled to 0 °C on ice. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide

hydrochloride (1.6 mg, 0.0085 mmol, 1.1 eq) and 1-hydroxybenzotriazole (1.15 mg, 0.0085 mmol, 1.1 eq) were added, and the suspension was stirred for 30 min at 0 °C. A solution of the product from step 3 (2.5 mg, 0.008 mmol, 1 eq) in 200 µL of *N,N*-dimethylformamide was added drop-wise to the suspension. The reaction mixture was allowed to warm to room temperature and then stirred overnight. Then it was concentrated using a rotary evaporator. The residue was taken up in ethyl acetate (5 mL) and washed with 1M HCl (5 mL), then water (3 x 5 mL). The organic solvent was evaporated using a rotary evaporator. The solid product was washed with hot 2-propanol, filtered, and dried. The product was a white solid. Yield: 65% MW: 818.9

Step 5: A mixture of Fmoc-protected product synthesized in Step 4 (4.1 mg, 0.005 mmol) was dissolved in 1 mL of diethylamine (DEA)/dichloromethane (v/v = 1:1) mixture. The mixture was magnetically stirred until the white colored solution turned yellow (approximately 30 min). The organic solvent was evaporated using rotary evaporator and the residual mass (yellow oil) was purified by column chromatography (SiO₂, particle size 40-63 µm) using dichloromethane : ethyl acetate (v/v = 1:1), with 1% triethylamine followed by dichloromethane : ethyl acetate : methanol (v/v/v = 1:1:0.5) with 1% triethylamine to elute the substrate. Yield: 79% MW: 596.6. MS: *m/z* 597.7 (M+H⁺).

TPP1 Internal Standard



Scheme S3.

Step 1: *tert*-Butyl-*d*₉ alcohol (0.128 mL, 1.20 mmol, 1 eq) and quinoline (0.141 mL, 1.20 mmol, 1eq) were combined in a dry screw-cap vial with 1 mL of anhydrous dichloromethane. Phenyl chloroformate (0.155 mL, 1.2 mmol, 1eq) was then added drop-wise. The reaction was allowed to proceed overnight. The resulting *d*₉-*tert*-butyl phenyl carbonate was purified on silica using a hexane/ethyl acetate gradient up to hexane: ethyl acetate (V:V = 1:1). ¹H NMR 400 MHz (CDCl₃) δ 1.54 (9H, s), 6.97-7.50 (5H, m) MS *m/z* 226.1 (M+Na)⁺.

Step 2: The compound synthesized in the previous step 1 (9.3 mL, 50 mmol) was added to a solution of 1,3-diaminopropane (3.7 mL, 50 mmol) in 35 mL EtOH in a 200-mL, single-necked, round-bottomed flask equipped with a stirring bar and a reflux condenser. The reaction mixture was heated gently to reflux overnight ensuring that the temperature was in the range of 80-85 °C, resulting in a yellow solution. The reaction mixture was cooled to room temperature and the

solution was concentrated to approximately 7-8 mL using a rotary evaporator, which left a yellow solution. Water (50 mL) was added and pH was adjusted to approximately pH 3 by careful addition of aqueous HCl followed by extraction with CH₂Cl₂ (3 x 80 mL). The aqueous layer was adjusted to pH 13 by the addition of 2M NaOH and extracted with CH₂Cl₂ (5 x 100 mL). The combined organic extracts were concentrated using a rotary evaporator to afford 2.8 g of a yellow oil which crystallized within ~1 h. Yield: 33% MW: 183.2 MS: *m/z* 184.1 (M+H)⁺

Step 3: A solution of Fmoc-4-aminomethyl benzoic acid (0.1494 g, 0.4 mmol, 1 eq) in a 20 mL of anhydrous tetrahydrofuran was cooled to 0 °C on ice. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.0844 g, 0.44 mmol, 1.1 eq) and 1-hydroxybenzotriazole (0.0542 g, 0.44 mmol, 1.1 eq) were added, and the suspension was stirred for 30 min at 0 °C. A solution of *N*-d₉-Boc-1,3-diaminopropane (0.0732 g, 0.4 mmol, 1 eq) in 4 mL of *N,N*-dimethylformamide was added drop-wise to the suspension. The reaction mixture was allowed to warm to room temperature and stirred overnight. Then, it was concentrated using a rotary evaporator. The residue was taken up in 200 mL of ethyl acetate and washed with 1M HCl (160 mL), then water (3 x 160 mL). The organic solvent was evaporated using a rotary evaporator. The solid product was purified by crystallization from isopropyl alcohol to give 0.1513 g compound. Yield: 70% MW = 538.3.

Step 4: A mixture of the Fmoc-protected compound synthesized in Step 3 (0.0807 g, 0.15 mmol) was dissolved in 6 mL of diethylamine (DEA)/dichloromethane mixture (v/v = 1:1). The mixture was magnetically stirred until the white colored solution turned yellow (approximately 30 min). The organic solvent was evaporated using a rotary evaporator and the residual mass (yellow oil) was purified by column chromatography (SiO₂, particle size 40-63 μm) using stepped gradient of ethyl acetate: methanol (100 → 80:20), 1% triethylamine. The pertinent fraction was evaporated on the rotary evaporator to give 0.0250 g of the internal standard. Yield: 53% MW: 316.3. MS: *m/z* 317.3 (M+H)⁺; ¹H-NMR(CDCl₃): 1.62 (m, 2H), 3.00 (t, 2H), 3.26 (t, 2H), 3.83 (s, 2H), 7.45 (d, 2H), 7.81 (d, 2H).

(s2) Pitterkow, M.; Lewinsky R.; Christensen, J. B. Selective Synthesis of Carbamate Protected Polyamines Using Alkyl Phenyl Carbonates, *Synthesis*, **2002**, *15*, 2195-2202.

(s3) Blanchard, S.; Sadilek, M.; Scott, C.R.; Turecek, F.; Gelb, M.H. Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: application to screening newborns for mucopolysaccharidosis I. *Clin Chem.* **2008**, *54*, 2067-2070.

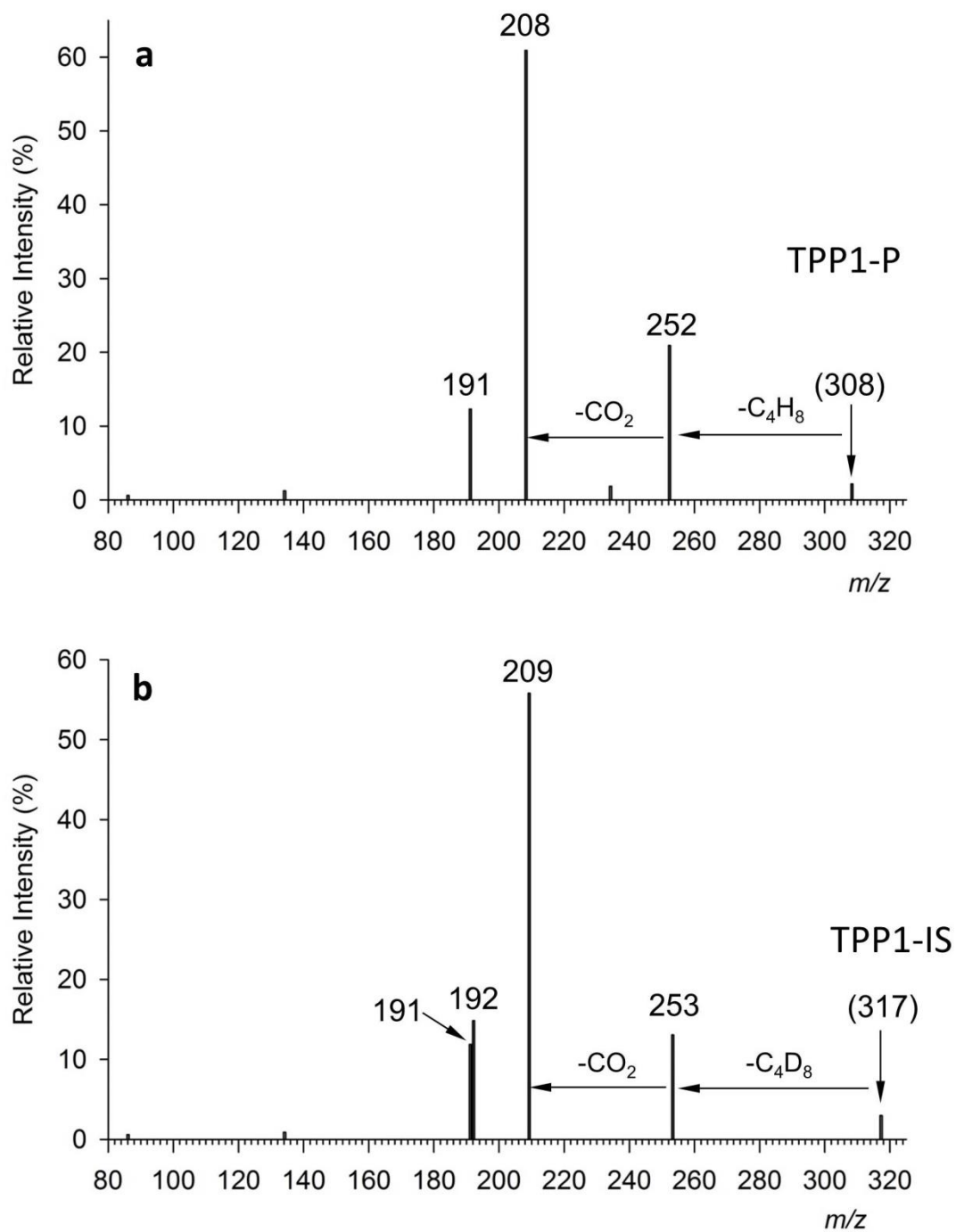


Figure S1. CID-MS/MS spectra of (a) $(M + H)^+$ ion m/z 308 from TPP1-P, (b) $(M + H)^+$ ion m/z 308 from TPP1-IS.

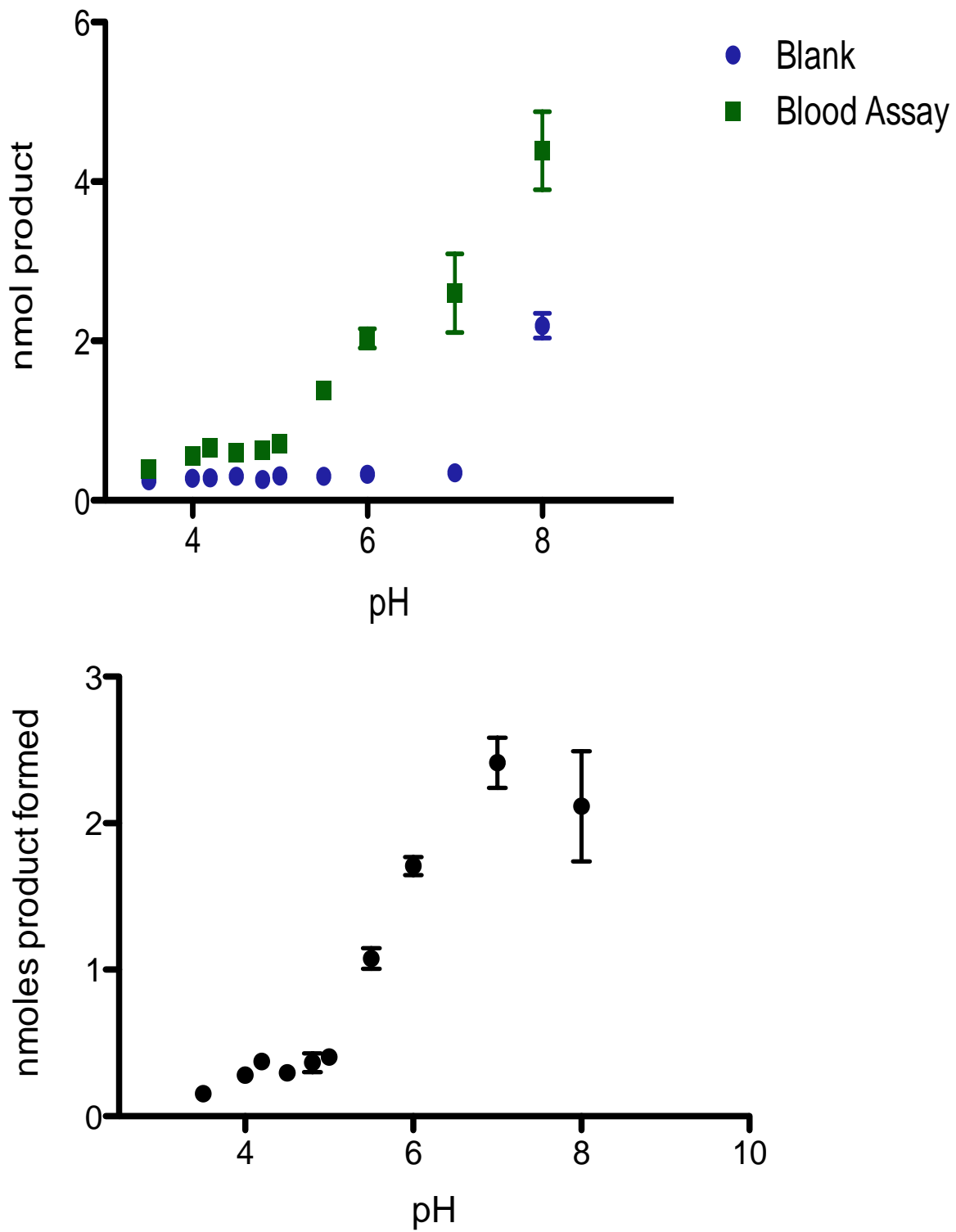


Figure S2. Dependence on pH of PPT1 product formation. Top panel: Total product formed in the presence of DBS (green squares) and product formed in the absence of DBS (blue circles). Bottom panel: Product formed by enzyme action after correction for blank.

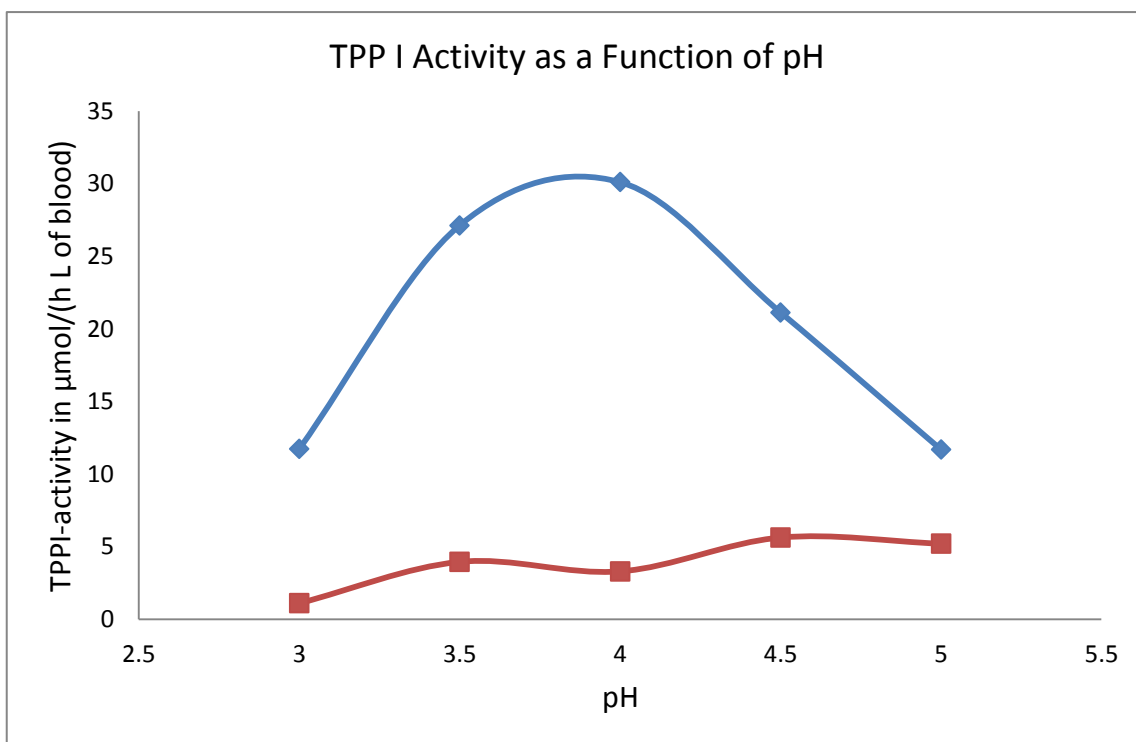


Figure S3. Dependence of TPP1 activity on pH.

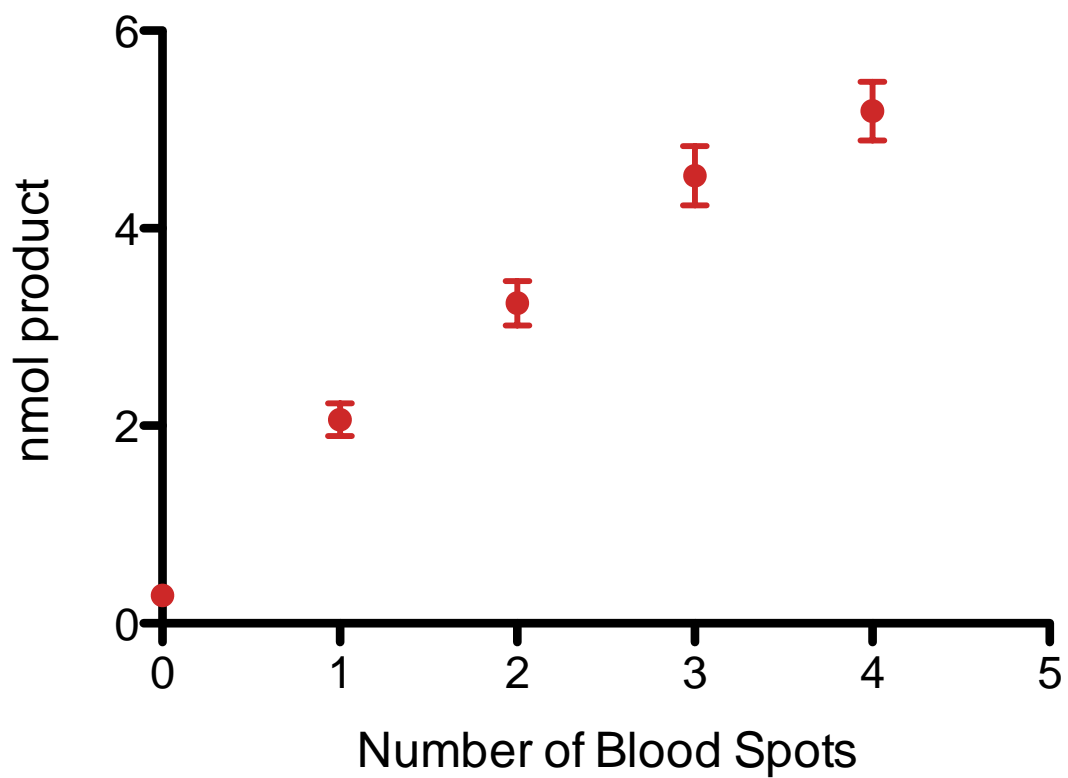


Figure S4. PPT1 activity as a function of the number of DBS.

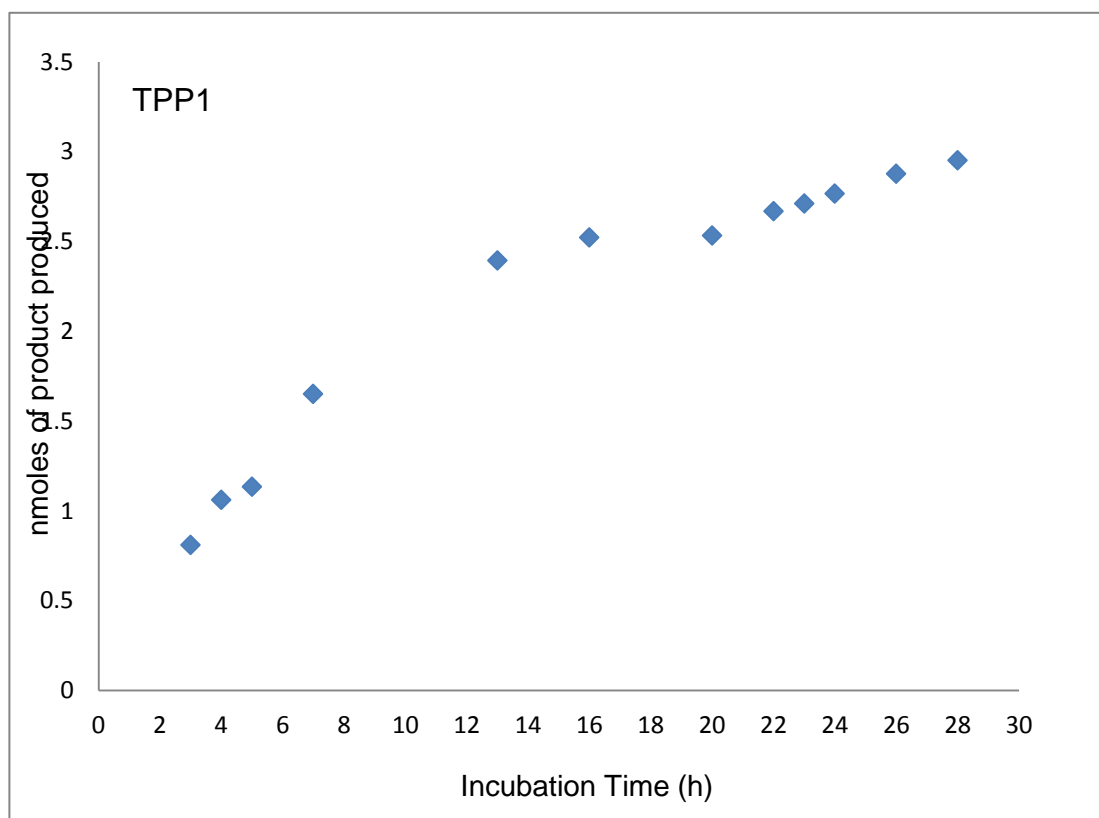
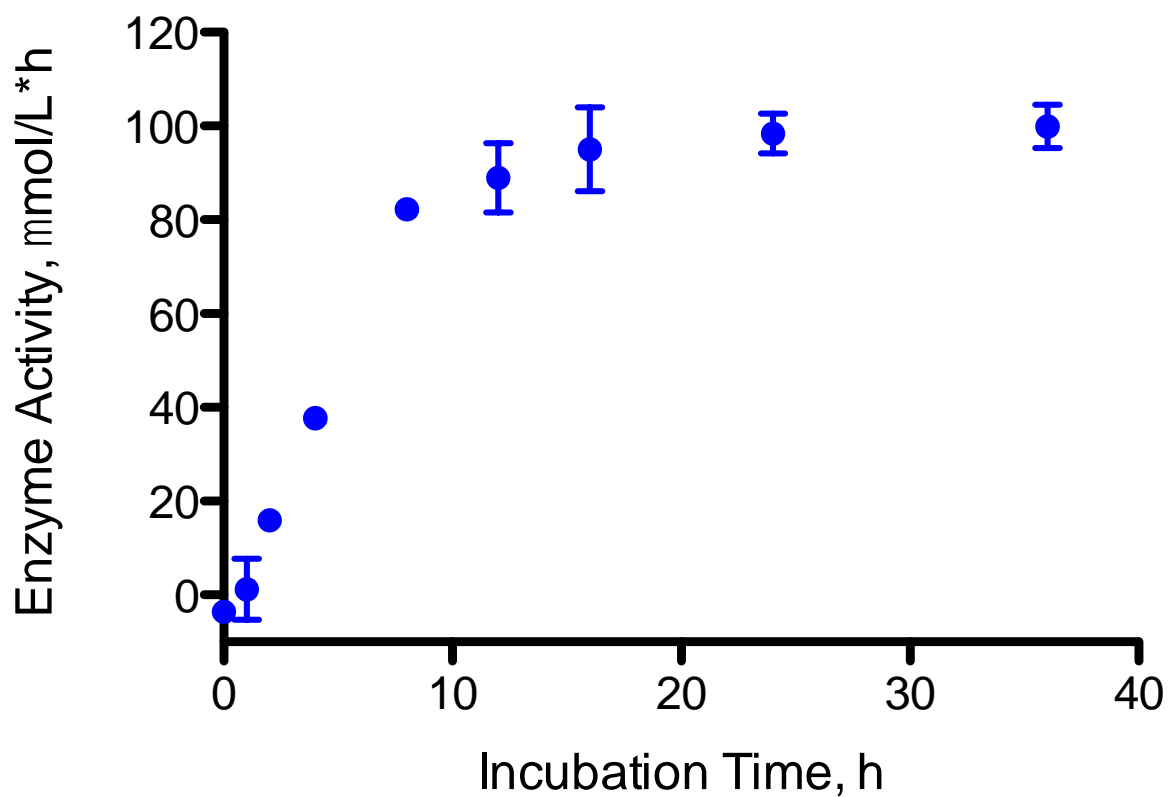


Figure S5. PPT1 (top panel) and TPP1 (bottom panel) product formation as a function of incubation time.

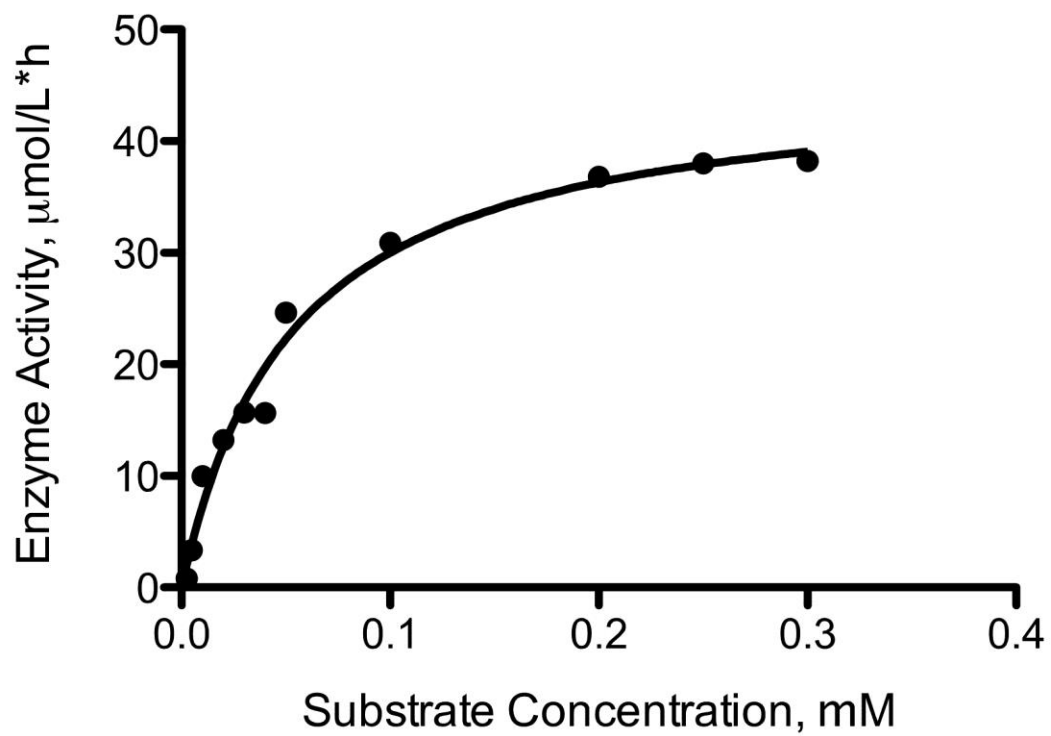
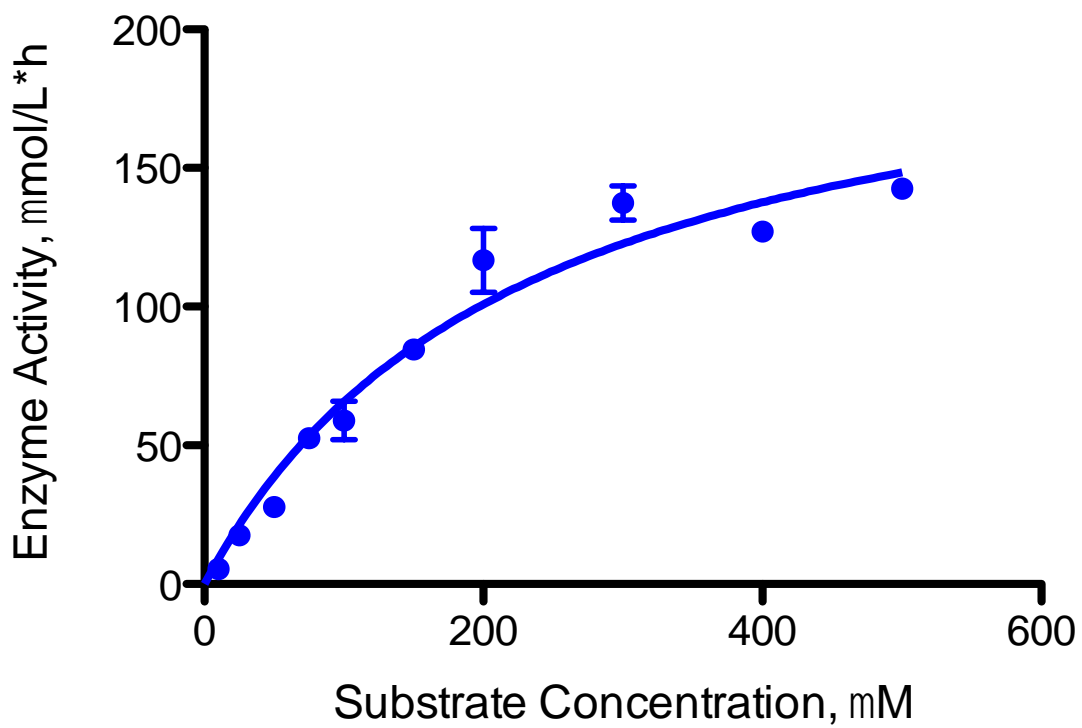


Figure S6. Michaelis-Menten plots for (top) PPT1 and (bottom) TPP1.

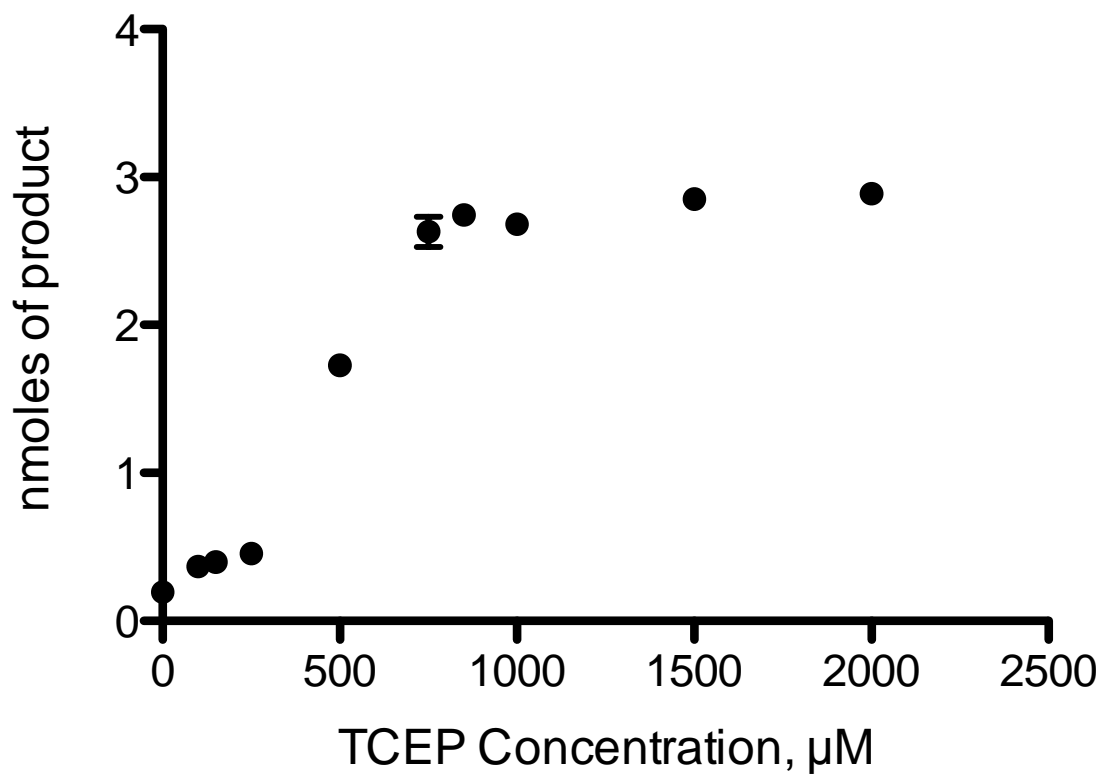


Figure S7. PPT1 product amount detected by SRM as a function of the concentration of tris-(2-carboxyethyl)phosphine reducing reagent.

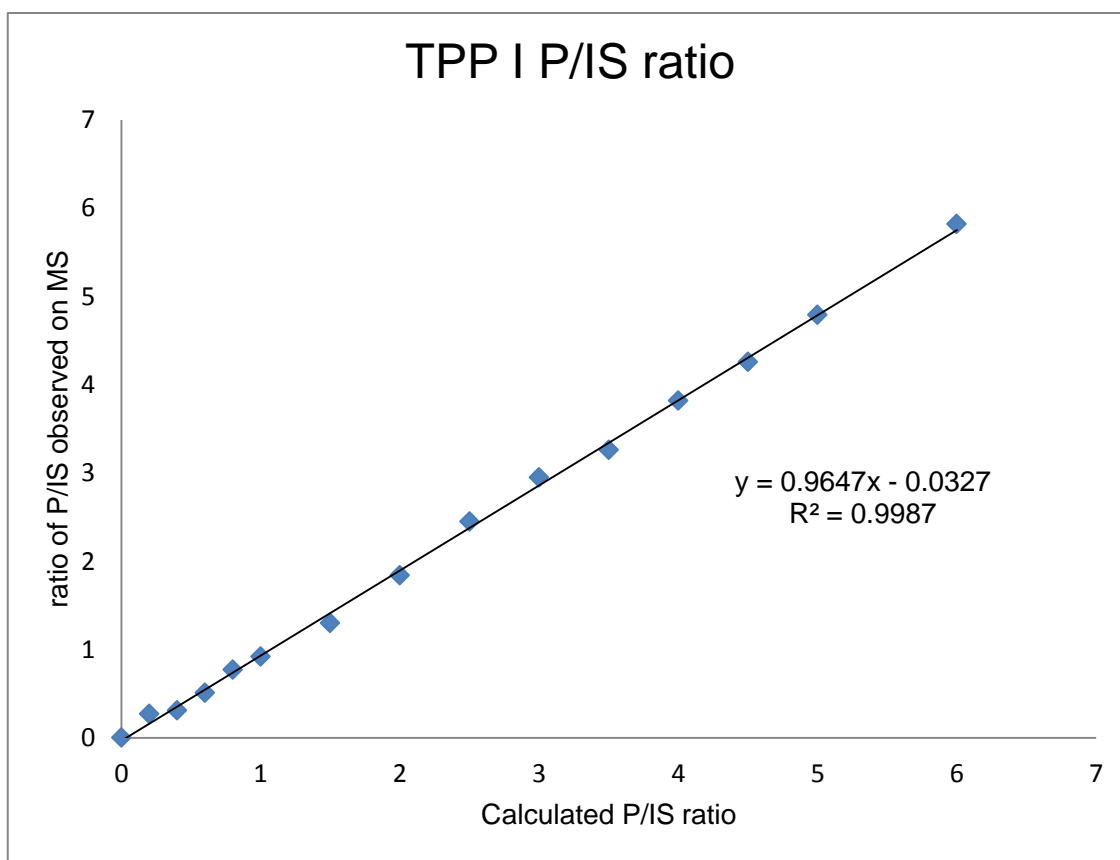
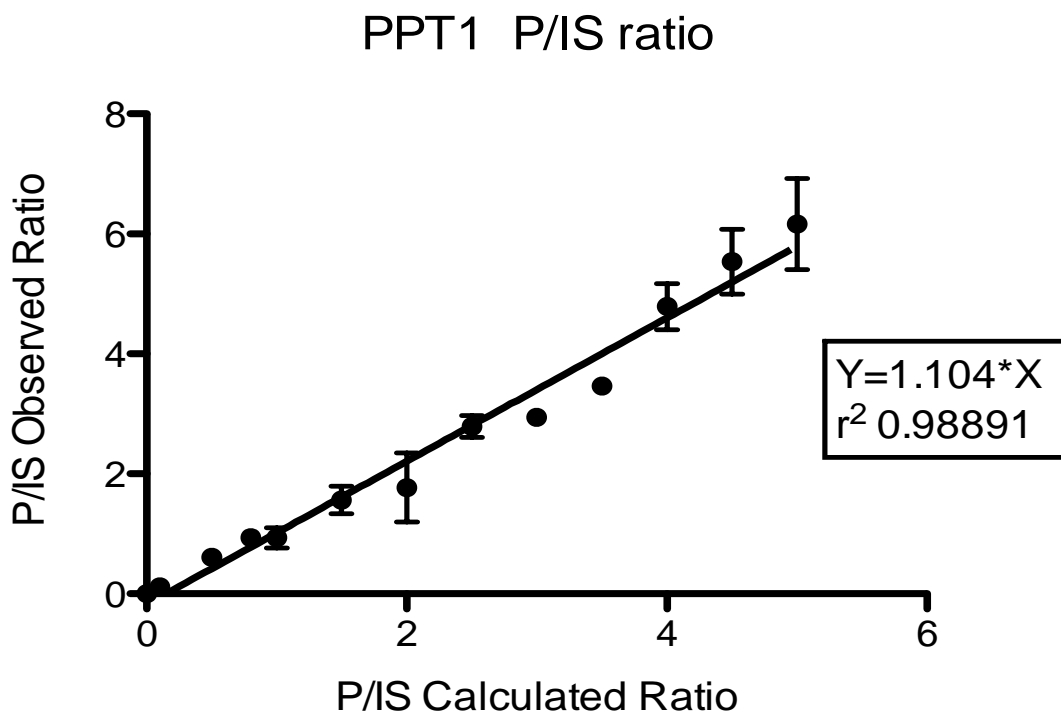


Figure S8. Response plots of observed SRM signal (peak areas) ratio for product and internal standard versus the theoretical concentration ratio. Top panel: PPT1, bottom panel TPP1.

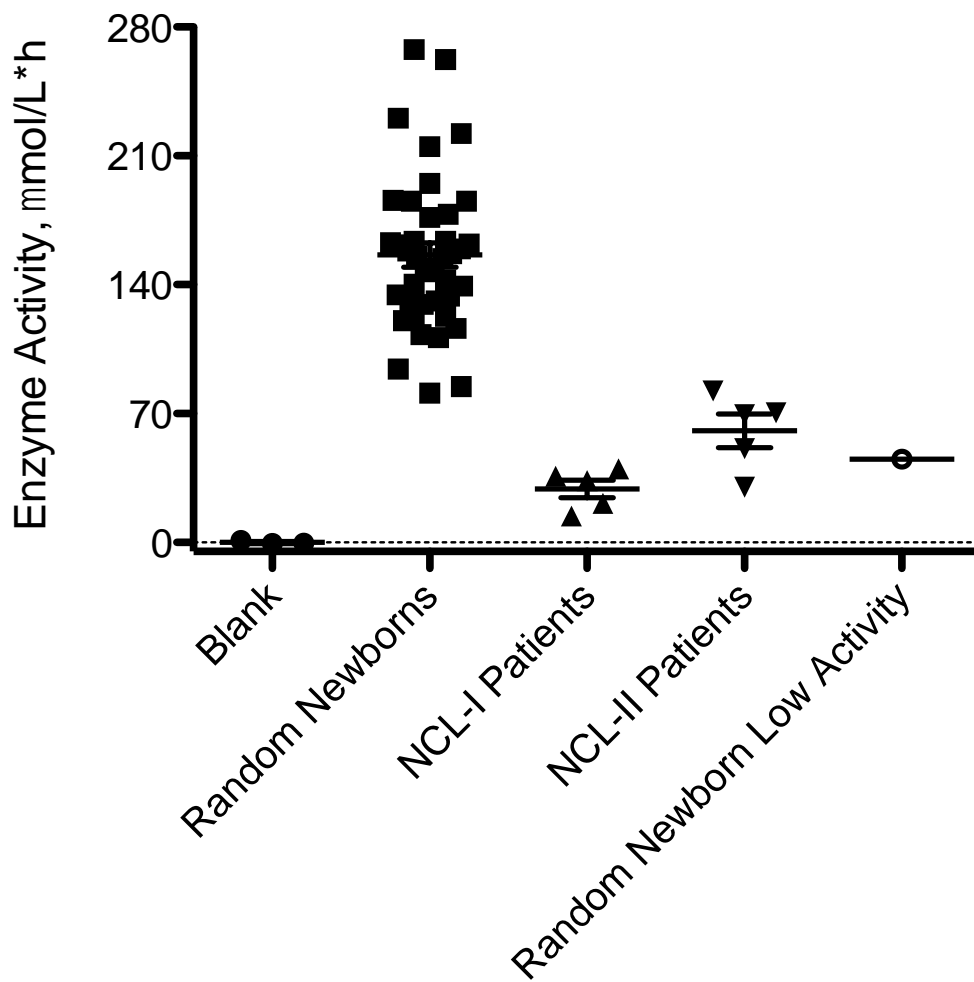


Figure S9. Graphical representation of PPT1 and TPP1 activities in DBS from duplex assays.

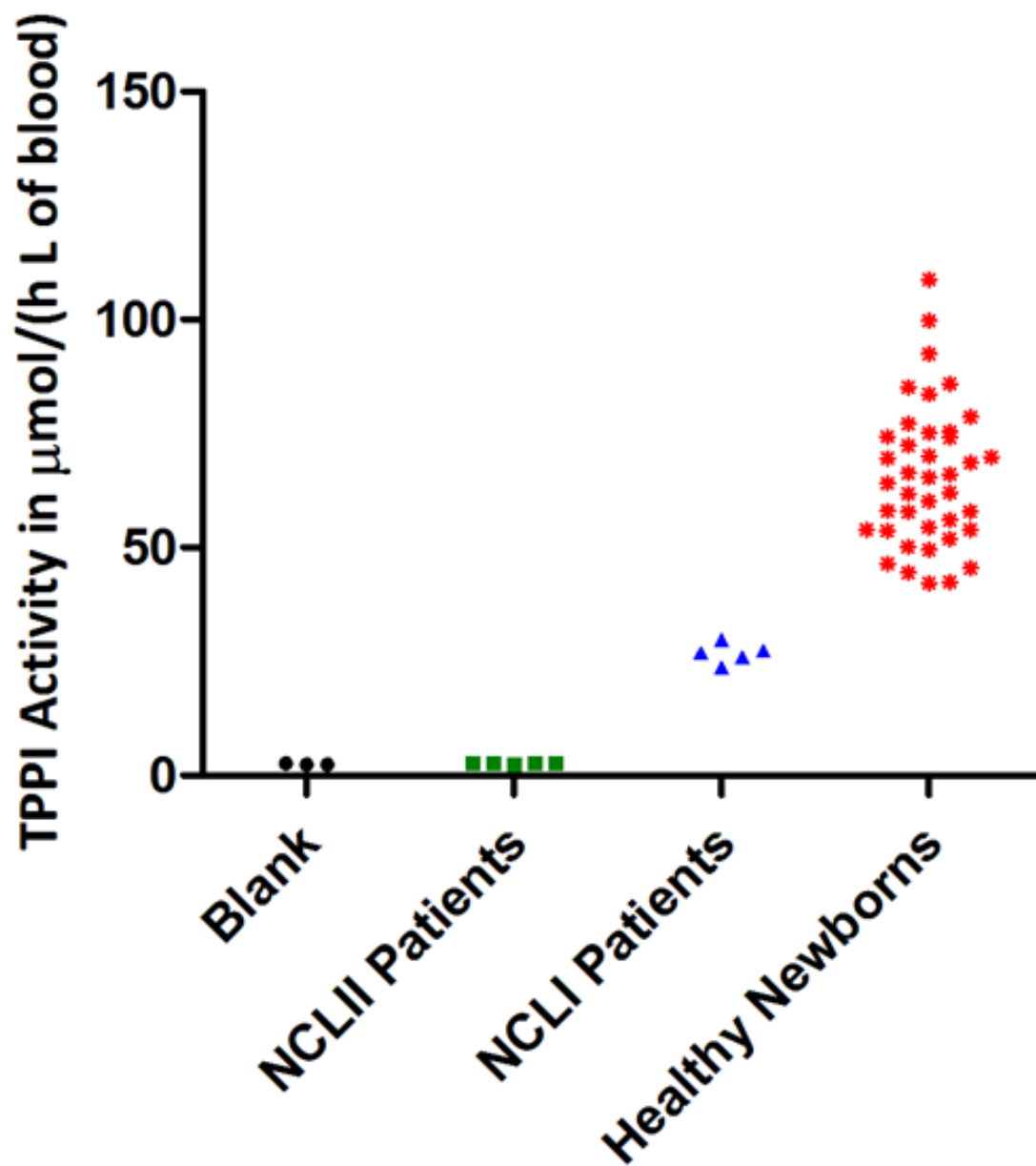


Figure S10. Graphical representation of TPP1 and PPT1 activities in DBS from duplex assays.

Table S1. Tandem Mass spectrometry Data for Simplex Assay of PPT1 in DBS.

	Ion counts		Enzyme Activity	Blank Corrected	
	Product	Internal Std	($\mu\text{mol/h}\cdot\text{L}$)		
Blank	4.65E+02	3.38E+03	10.2		Blank Mean 9.0
Blank	2.10E+02	1.50E+03	10.3		Blank St. Dev. 2.6
Blank	4.46E+02	2.50E+03	13.1		
Blank	4.88E+02	5.05E+03	7.1		
Blank	4.82E+02	2.63E+03	13.4		
Blank	4.72E+02	4.86E+03	7.1		
Blank	4.09E+02	4.62E+03	6.5		
Blank	5.26E+02	5.41E+03	7.1		
Blank	2.59E+02	1.63E+03	11.7		
Blank	5.65E+02	5.71E+03	7.3		
Blank	5.80E+02	6.09E+03	7.0		
Blank	3.61E+02	3.56E+03	7.4		
Random newborn 1	5.23E+03	1.96E+03	187.8	178.7	Random Mean 146.7
Random newborn 2	5.27E+03	2.08E+03	177.7	168.7	
Random newborn 3	4.51E+03	1.99E+03	158.2	149.1	
Random newborn 4	4.08E+03	1.98E+03	142.1	133.1	Random Max. 212.9
Random newborn 5	3.91E+03	1.77E+03	153.4	144.4	
Random newborn 6	4.58E+03	1.75E+03	183.8	174.8	
Random newborn 7	3.29E+03	1.46E+03	156.4	147.4	Random Min. 71.4
Random newborn 8	3.45E+03	1.38E+03	174.6	165.6	
Random newborn 9	3.70E+03	1.75E+03	146.7	137.7	
Random newborn 10	3.56E+03	1.43E+03	174.4	165.4	
Random newborn 11	2.74E+03	1.24E+03	153.9	144.9	
Random newborn 12	3.57E+03	1.45E+03	172.4	163.3	
Random newborn 13	3.14E+03	1.51E+03	144.8	135.7	
Random newborn 14	2.46E+03	1.17E+03	146.6	137.6	
Random newborn 15	3.18E+03	1.24E+03	179.1	170.1	
Random newborn 16	3.48E+03	1.41E+03	172.2	163.2	
Random newborn 17	3.61E+03	1.49E+03	169.7	160.7	
Random newborn 18	3.15E+03	1.37E+03	160.2	151.2	

Random newborn 19	3.76E+03	1.49E+03	177.0	168.0
Random newborn 20	3.83E+03	1.46E+03	184.0	175.0
Random newborn 21	3.25E+03	1.37E+03	165.6	156.6
Random newborn 22	2.75E+03	1.23E+03	156.0	147.0
Random newborn 23	4.02E+03	1.66E+03	169.4	160.4
Random newborn 24	3.69E+03	1.36E+03	190.6	181.5
Random newborn 25	2.65E+03	1.15E+03	161.3	152.3
Random newborn 26	2.60E+03	1.06E+03	172.2	163.2
Random newborn 27	3.06E+03	1.48E+03	142.9	133.8
Random newborn 28	2.52E+03	1.05E+03	168.1	159.1
Random newborn 29	3.74E+03	1.41E+03	185.5	176.4
Random newborn 30	3.23E+03	1.22E+03	185.8	176.8
Random newborn 31	3.54E+03	1.58E+03	155.6	146.6
Random newborn 32	3.59E+03	1.49E+03	168.5	159.5
Random newborn 33	3.38E+03	1.36E+03	174.6	165.6
Random newborn 34	3.71E+03	1.18E+03	221.9	212.9
Random newborn 35	2.56E+03	1.08E+03	165.7	156.7
Random newborn 36	2.93E+03	1.24E+03	165.1	156.1
Random newborn 37	3.64E+03	1.33E+03	192.8	183.8
Random newborn 38	2.57E+03	1.03E+03	174.6	165.6
Random newborn 39	5.70E+03	2.25E+03	177.2	168.2
Random newborn 40	3.88E+03	2.24E+03	118.4	109.4
Random newborn 41	3.41E+03	1.75E+03	134.7	125.7
Random newborn 42	5.22E+03	1.78E+03	207.1	198.0
Random newborn 43	3.48E+03	1.86E+03	128.8	119.8
Random newborn 44	3.70E+03	2.18E+03	116.2	107.2
Random newborn 45	3.64E+03	1.46E+03	174.6	165.6
Random newborn 46	3.43E+03	1.64E+03	145.1	136.1
Random newborn 47	2.66E+03	1.27E+03	145.0	136.0
Random newborn 48	2.79E+03	1.13E+03	172.2	163.1
Random newborn 49	2.66E+03	1.10E+03	168.2	159.1
Random newborn 50	1.79E+03	1.21E+03	99.3	90.3
Random newborn 51	1.80E+03	1.06E+03	115.6	106.6
Random newborn 52	3.17E+03	1.10E+03	202.7	193.7
Random newborn 53	1.57E+03	1.20E+03	87.4	78.4
Random newborn 54	1.23E+03	1.01E+03	80.5	71.4
Random newborn 55	1.86E+03	1.30E+03	96.3	87.3
Random newborn 56	2.17E+03	1.36E+03	107.9	98.9
Random newborn 57	1.49E+03	1.13E+03	87.6	78.5
Random newborn 58	2.45E+03	1.05E+03	163.0	153.9

Random newborn 59	2.11E+03	1.59E+03	88.5	79.4	
Random newborn 60	7.28E+03	3.58E+03	140.8	131.7	
Random newborn 61	1.91E+03	1.54E+03	82.3	73.3	
Random newborn 62	4.56E+03	1.73E+03	184.5	175.5	
NCL1 Patient 1	8.19E+02	1.99E+03	21.2	12.2	NCL1 Mean
NCL1 Patient 2	1.52E+03	3.53E+03	22.8	13.8	14.9
NCL1 Patient 3	5.95E+02	1.36E+03	23.0	14.0	
NCL1 Patient 4	1.18E+03	2.53E+03	25.3	16.2	
NCL1 Patient 5	7.08E+02	1.43E+03	27.3	18.3	

Table S2. Tandem Mass spectrometry Data for Simplex Assay of TPP1 in DBS.

	peak area		Enzyme Activity	Blank Corrected	Blank Mean
	Product	Internal Std	($\mu\text{mol/h}\cdot\text{L}$)		
Blank	3.36E+02	5.28E+04	2.1		2.0
Blank	3.31E+02	5.29E+04	1.9		Blank St. Dev.
Blank	3.48E+02	4.96E+04	2.0		0.1
Blank	3.19E+02	4.89E+04	2.0		
Blank	3.42E+02	4.86E+04	2.1		
Blank	3.25E+02	5.27E+04	1.9		
Random newborn 1	1.17E+04	5.09E+04	67.19	65.2	Random Mean
Random newborn 2	1.09E+04	5.35E+04	59.09	57.1	58.4
Random newborn 3	1.31E+04	5.39E+04	70.89	68.9	
Random newborn 4	1.04E+04	4.80E+04	63.09	61.1	Random Max.
Random newborn 5	1.02E+04	5.18E+04	57.29	55.3	87.5
Random newborn 6	1.07E+04	5.45E+04	56.89	54.9	Random Min.
Random newborn 7	1.03E+04	5.19E+04	57.79	55.8	34.4
Random newborn 8	1.30E+04	5.48E+04	69.19	67.2	
Random newborn 9	1.09E+04	5.37E+04	59.19	57.2	
Random newborn 10	1.10E+04	5.24E+04	60.99	59	
Random newborn 11	1.25E+04	5.56E+04	65.69	63.7	
Random newborn 12	1.13E+04	4.96E+04	66.09	64.1	
Random newborn 13	1.16E+04	4.89E+04	68.79	66.8	
Random newborn 14	1.22E+04	4.86E+04	73.19	71.2	
Random newborn 15	6.74E+03	5.28E+04	36.39	34.4	
Random newborn 16	1.43E+04	5.31E+04	79.09	77.1	
Random newborn 17	1.04E+04	5.27E+04	57.19	55.2	
Random newborn 18	9.50E+03	5.29E+04	51.99	50	
Random newborn 19	1.25E+04	5.60E+04	64.89	62.9	
Random newborn 20	1.00E+04	5.66E+04	51.29	49.3	
Random newborn 21	1.14E+04	5.63E+04	58.99	57	
Random newborn 22	1.10E+04	5.18E+04	61.79	59.8	
Random newborn 23	7.75E+03	5.18E+04	42.99	41	
Random newborn 24	1.31E+04	5.35E+04	71.49	69.5	
Random newborn 25	1.12E+04	5.49E+04	59.29	57.3	

Random newborn 26	1.08E+04	5.32E+04	58.99	57	
Random newborn 27	9.48E+03	5.39E+04	50.89	48.9	
Random newborn 28	1.19E+04	4.84E+04	71.59	69.6	
Random newborn 29	1.20E+04	4.79E+04	73.19	71.2	
Random newborn 30	1.02E+04	4.77E+04	61.99	60	
Random newborn 31	1.12E+04	5.17E+04	62.89	60.9	
Random newborn 32	1.59E+04	5.21E+04	89.49	87.5	
Random newborn 33	1.09E+04	5.18E+04	61.29	59.3	
Random newborn 34	1.30E+04	5.27E+04	71.99	70	
Random newborn 35	1.19E+04	5.26E+04	66.09	64.1	
Random newborn 36	8.38E+03	5.19E+04	46.49	44.5	
Random newborn 37	9.10E+03	5.24E+04	50.19	48.2	
Random newborn 38	9.90E+03	5.49E+04	52.29	50.3	
Random newborn 39	1.30E+04	5.63E+04	67.69	65.7	
Random newborn 40	8.95E+03	5.55E+04	46.59	44.6	
Random newborn 41	9.22E+03	5.32E+04	50.09	48.1	
Random newborn 42	9.29E+03	5.17E+04	51.99	50	
Random newborn 43	9.70E+03	5.18E+04	54.29	52.3	
Random newborn 44	1.13E+04	4.77E+04	68.69	66.7	
Random newborn 45	1.28E+04	5.66E+04	66.19	64.2	
Random newborn 46	1.14E+04	5.24E+04	63.09	61.1	
Random newborn 47	1.03E+04	5.33E+04	55.89	53.9	
Random newborn 48	1.06E+04	5.10E+04	60.09	58.1	
Random newborn 49	1.12E+04	5.43E+04	59.79	57.8	
Random newborn 50	8.71E+03	5.00E+04	50.29	48.3	
Random newborn 51	9.45E+03	5.05E+04	54.19	52.2	
Random newborn 52	9.99E+03	5.19E+04	55.79	53.8	
Random newborn 53	9.61E+03	5.20E+04	53.49	51.5	
Random newborn 54	9.44E+03	5.18E+04	52.69	50.7	
NCL2 Patient 1	6.86E+02	5.17E+04	2.04	0.05	NCL2 Mean
NCL2 Patient 2	6.95E+02	5.18E+04	2.09	0.1	0.33
NCL2 Patient 3	7.30E+02	5.18E+04	2.29	0.3	
NCL2 Patient 4	7.54E+02	5.27E+04	2.39	0.4	
NCL2 Patient 5	8.33E+02	5.36E+04	2.79	0.8	

Table S3. Tandem Mass spectrometry Data for Duplex Assay of PPT1 in DBS.

	Ion Counts		Enzyme Activity	Blank Corrected	Blank Mean
	Product	Int Std			
Blank	4.47E+02	2.44E+03	13.5		13.9
Blank	3.94E+02	1.95E+03	14.9		Blank St. Dev.
Blank	4.60E+02	2.55E+03	13.3		0.9
Random newborn 1	4.42E+03	2.00E+03	148.4	134.5	
Random newborn 2	4.20E+03	2.22E+03	125.2	111.3	
Random newborn 3	4.80E+03	2.19E+03	147.7	133.8	Random Mean
Random newborn 4	4.23E+03	2.16E+03	130.1	116.2	156.2
Random newborn 5	4.49E+03	1.73E+03	177.5	163.6	
Random newborn 6	3.28E+03	1.27E+03	176.6	162.7	Random Max.
Random newborn 7	4.20E+03	1.69E+03	169.5	155.6	267.9
Random newborn 8	4.47E+03	2.09E+03	143.2	129.3	
Random newborn 9	6.28E+03	2.61E+03	163.0	149.1	Random Min.
Random newborn 10	4.54E+03	1.99E+03	154.2	140.3	81.1
Random newborn 11	5.32E+03	1.52E+03	244.4	230.5	
Random newborn 12	5.20E+03	2.25E+03	156.2	142.3	
Random newborn 13	3.84E+03	2.32E+03	108.1	94.2	
Random newborn 14	4.13E+03	2.05E+03	134.5	120.6	
Random newborn 15	3.35E+03	2.27E+03	95.0	81.1	
Random newborn 16	5.27E+03	2.32E+03	153.2	139.2	
Random newborn 17	4.75E+03	1.92E+03	168.2	154.3	
Random newborn 18	5.70E+03	2.64E+03	145.0	131.1	
Random newborn 19	5.52E+03	1.99E+03	190.5	176.6	
Random newborn 20	6.14E+03	2.41E+03	173.3	159.4	
Random newborn 21	4.68E+03	1.61E+03	199.7	185.8	
Random newborn 22	4.62E+03	2.14E+03	145.0	131.1	
Random newborn 23	6.23E+03	2.15E+03	199.2	185.2	
Random newborn 24	6.50E+03	2.52E+03	175.6	161.7	
Random newborn 25	6.36E+03	2.67E+03	161.1	147.2	
Random newborn 26	5.14E+03	3.36E+03	98.5	84.6	
Random newborn 27	6.31E+03	1.91E+03	228.9	215.0	
Random newborn 28	6.17E+03	3.23E+03	126.8	112.8	
Random newborn 29	3.65E+03	1.78E+03	136.7	122.8	
Random newborn 30	4.87E+03	1.93E+03	172.2	158.3	

Random newborn 31	4.82E+03	1.85E+03	177.4	163.5	
Random newborn 32	5.09E+03	1.76E+03	199.3	185.4	
Random newborn 33	5.24E+03	2.09E+03	170.9	157.0	
Random newborn 34	5.34E+03	2.60E+03	137.4	123.5	
Random newborn 35	5.91E+03	1.74E+03	236.2	222.3	
Random newborn 36	3.94E+03	1.30E+03	208.9	195.0	
Random newborn 37	3.31E+03	1.18E+03	192.2	178.3	
Random newborn 38	7.31E+03	1.82E+03	281.8	267.9	
Random newborn 39	7.17E+03	1.82E+03	276.3	262.4	
Random newborn 40	4.46E+03	1.73E+03	176.0	162.1	
random newborn low activity	1.68E+03	2.09E+03	45.3		
NCL1 Patient 1	1.36E+03	1.63E+03	47.4	33.5	NCL1 Mean
NCL1 Patient 2	9.12E+02	1.59E+03	28.3	14.4	29.1
NCL1 Patient 3	1.45E+03	1.57E+03	53.9	40.0	NCL1 St. Dev.
NCL1 Patient 4	1.22E+03	1.41E+03	49.9	36.0	10.8
NCL1 Patient 5	8.89E+02	1.33E+03	35.2	21.3	
NCL2 Patient 1	1.52E+03	1.93E+03	43.8	29.9	NCL2 Mean
NCL2 Patient 2	1.62E+03	1.23E+03	83.4	69.5	60.6
NCL2 Patient 3	1.89E+03	1.42E+03	84.3	70.4	
NCL2 Patient 4	1.96E+03	1.31E+03	96.1	82.2	
NCL2 Patient 5	9.94E+02	9.31E+02	64.8	50.9	

Table S4. Tandem Mass spectrometry Data for Duplex Assay of TPP1 in DBS.

	Peak area		Enzyme	Blank Corrected	Blank Mean 2.51 Blank St. Dev. 0.2
	Product	Int Std	Activity		
Blank	5.67E+02	3.90E+04	2.4		
Blank	5.34E+02	3.79E+04	2.4		
Blank	6.99E+02	3.45E+04	2.7		
Random newborn 1	3.83E+04	3.70E+04	52.0	49.5	Random Mean
Random newborn 2	4.33E+04	3.74E+04	58.0	55.5	63.2
Random newborn 3	3.07E+04	3.33E+04	46.5	44.0	Random Max.
Random newborn 4	3.64E+04	3.70E+04	49.6	47.0	106.3
Random newborn 5	5.97E+04	3.19E+04	92.6	90.1	Random Min.
Random newborn 6	5.19E+04	2.99E+04	86.0	83.5	39.7
Random newborn 7	3.85E+04	3.58E+04	53.9	51.4	
Random newborn 8	3.48E+04	3.24E+04	53.7	51.2	
Random newborn 9	4.32E+04	3.24E+04	66.4	63.9	
Random newborn 10	4.75E+04	3.44E+04	68.7	66.2	
Random newborn 11	4.37E+04	3.40E+04	64.2	61.7	
Random newborn 12	4.60E+04	3.46E+04	66.1	63.6	
Random newborn 13	2.14E+04	2.56E+04	42.2	39.7	
Random newborn 14	3.98E+04	2.84E+04	69.8	67.3	
Random newborn 15	3.18E+04	2.92E+04	54.5	52.0	
Random newborn 16	3.81E+04	3.30E+04	57.8	55.3	
Random newborn 17	3.87E+04	3.13E+04	61.8	59.2	
Random newborn 18	3.54E+04	2.52E+04	70.1	67.6	
Random newborn 19	4.19E+04	2.81E+04	74.2	71.6	
Random newborn 20	3.15E+04	3.57E+04	44.6	42.0	
Random newborn 21	3.19E+04	2.96E+04	53.9	51.4	
Random newborn 22	3.88E+04	3.12E+04	62.0	59.5	
Random newborn 23	2.64E+04	2.93E+04	45.6	43.1	
Random newborn 24	4.55E+04	2.92E+04	77.2	74.7	
Random newborn 25	4.15E+04	2.74E+04	75.1	72.6	
Random newborn 26	3.83E+04	2.92E+04	65.4	62.9	
Random newborn 27	4.76E+04	2.77E+04	85.2	82.7	
Random newborn 28	2.71E+04	2.42E+04	56.1	53.6	
Random newborn 29	2.69E+04	2.70E+04	50.2	47.7	
Random newborn 30	3.98E+04	2.84E+04	69.6	67.1	

Random newborn 31	4.15E+04	2.74E+04	75.4	72.9	
Random newborn 32	4.61E+04	2.73E+04	83.7	81.2	
Random newborn 33	3.40E+04	2.27E+04	74.3	71.8	
Random newborn 34	4.01E+04	2.76E+04	72.4	69.9	
Random newborn 35	1.61E+04	7.97E+03	99.8	97.3	
Random newborn 36	1.70E+04	7.68E+03	108.8	106.3	
Random newborn 37	7.56E+03	6.27E+03	60.2	57.7	
Random newborn 38	4.09E+04	2.58E+04	78.7	76.2	
Random newborn 39	3.97E+04	3.42E+04	58.1	55.6	
Random newborn 40	2.25E+04	2.68E+04	42.5	40.0	
random newborn low activity	2.12E+04	2.60E+04	41.3		
NCL1 Patient 1	1.62E+04	3.25E+04	26.0	23.5	Mean NCL1
NCL1 Patient 2	1.53E+04	3.42E+04	23.6	21.1	26.8
NCL1 Patient 3	1.42E+04	2.70E+04	27.4	24.9	
NCL1 Patient 4	1.59E+04	2.74E+04	29.8	27.3	
NCL1 Patient 5	1.22E+04	2.35E+04	26.9	24.4	
NCL2 Patient 1	6.02E+02	3.02E+04	2.7	0.2	Mean NCL2
NCL2 Patient 2	6.65E+02	2.85E+04	2.9	0.3	0.2
NCL2 Patient 3	4.85E+02	2.42E+04	2.7	0.2	
NCL2 Patient 4	4.43E+02	2.46E+04	2.6	0.1	
NCL2 Patient 5	5.58E+02	2.55E+04	2.8	0.3	