

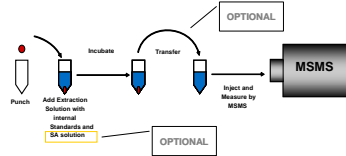
Non-derivatized Newborn Screening MS/MS Assay Analytical and Clinical Performance

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1 Introduction

Succinylacetone (SA) is the primary marker for Tyrosinemia Type 1. Seminal work on SA detection includes measurement using secondary extraction of a residual dried blood spot (DBS) [1,2]. More recent work describes a derivatized method for the simultaneous detection of SA, amino acids, free carnitine, and acylcarnitines [3]. We have developed an MS/MS assay for the simultaneous extraction and measurement of 11 amino acids, free carnitine, 30 acylcarnitines, and succinylacetone that does not require sample derivatization. The assay can also be performed omitting the extraction of SA without any significant changes in analytical and clinical performance. Here we share our findings regarding the analytical and clinical performance of the non-derivatized MS/MS assay across multiple newborn screening facilities as well as clinical comparisons to our widely used derivatized method (NeoGram AAAC MSMS kit).

2 Non-derivatized Assay Procedure



3 Analytical Performance – Linearity

Assay performance characteristics were determined using DBS enriched with amino acids, succinylacetone, free carnitine and acylcarnitines at concentrations covering significant clinical ranges (endogenous level to above cutoffs). Linearity was evaluated based on CLSI EP6A guidelines. Table 1 shows the linearity range ($R^2 > 0.99$) for all analytes for the NeoGram and non-derivatized assays.

Table 1: Linearity Ranges

Analyte	Non-derivatized Assay Linearity Range (μM)		NeoGram Assay Linearity Range (μM)		Reference Cutoff Ranges, (μM)
	Lower (Endogenous level)	Upper	Lower (Endogenous level)	Upper	
ALA	387	4109	426	4233	975-1625
ARG	24	3754	29	4488	180-300
CIT	27	1711	27	1488	115-188
GLY	337	4794	363	4442	975-1625
LEU	219	2568	182	2410	263-438
MET	30	1182	31	1142	120-200
ORN	111	3825	114	3903	360-600
PHE	72	2395	68	1912	225-375
PRO	251	3659	NA	450-750	
SA	0.4	158	NA	NA	4-7
TYR	73	2867	78	2875	578-963
VAL	207	2388	180	1988	300-500
C0	41	2268	43	2467	90-150
C2	35	732	49	751	128-213
C3	3	88	3.65	99.55	9.75-16.25
C4	0.14	69.81	0.20	64.68	2.25-3.75
C5	0.17	58.73	0.22	67.25	1.88-3.13
C5DC	0.12	28.88	0.08	35.18	0.6-1
C6	0.02	81.50	0.03	55.95	0.98-1.63
C8	0.04	35.42	0.05	43.00	1.2-2
C10	0.06	28.69	0.07	25.88	1.35-2.25
C12	0.04	42.99	0.08	41.42	1.88-3.13
C14	0.09	41.88	0.13	38.32	1.5-2.5
C16	2.71	107.40	2.89	98.87	11.25-18.75
C18	2.03	32.19	2.20	31.97	3-5.0

4 Analytical Performance – Precision

Assay precision was evaluated based on CLSI EP5A2 guidelines. Table 2 shows the total imprecision (%CV) for all spiked analytes for the NeoGram and non-derivatized assays.

Table 2: Total Imprecision (%CV)

	ALA	ARG	CIT	GLY	LEU	MET	ORN	PHE	PRO	SA	TYR	VAL
Non-derivatized	8	7	8	9	7	7	8	8	8	10	7	8
NeoGram	6	14	11	7	12	8	9	8	NA	NA	9	7

	C0	C2	C3	C4	C5	C5DC	C6	C8	C10	C12	C14	C16	C18
Non-derivatized	8	8	8	8	8	8	8	8	8	8	8	8	8
NeoGram	9	7	7	8	8	8	10	8	7	8	9	7	8

5 Analytical Performance – Recovery

Assay recovery was evaluated over five runs. Table 3 shows the range (95% upper and lower confidence limits) of analyte recovery for all spiked analytes for the NeoGram and non-derivatized assays.

Table 3: Percent Recovery

	ALA	ARG	CIT	GLY	LEU	MET	ORN	PHE	PRO	SA	TYR	VAL
Non-derivatized	84-118	80-108	88-114	81-119	78-115	77-110	86-113	76-108	51-75	89-114	71-109	
NeoGram	65-100	75-109	69-103	71-103	49-93	72-93	78-91	53-91	NA	NA	74-107	84

	C0	C2	C3	C4	C5	C5DC	C6	C8	C10	C12	C14	C16	C18
Non-derivatized	79-106	86-108	90-109	85-103	87-109	87-111	87-104	91-107	81-111	86-108	82-103	82-105	
NeoGram	80-117	78-104	82-110	87-105	74-97	101-99	105-112	97-98	84-102	84-102	74-95	75-96	

6 Inter-site Analytical Performance at NBS Laboratories

The performance of the non-derivatized assay was evaluated in parallel across four independent sites – three newborn screening laboratories in the US and Europe and one internal site. The total inter-site imprecision was determined as the square root of the sum of squares of the average total intra-site imprecision and between site (i.e. inter-site) imprecision. Table 4 shows a summary of the total inter-site imprecision (%CV), range (95% upper and lower confidence limits) of analyte recovery, and mean concentration (μM) for one DBS level across four independent sites.

Table 4: Inter-site Performance

Concentration (μM)	ALA	ARG	CIT	GLY	LEU	MET	ORN	PHE	PRO	SA	TYR	VAL
753	201	143	734	354	85	353	225	380	61	302	343	
Total Inter-site Imprecision (%CV)	9	9	7	13	7	7	8	7	8	13	7	7
Recovery (%)	76-104	89-101	98-114	77-115	81-93	86-110	90-112	89-105	78-108	58-101	73-107	

Concentration (μM)	C0	C2	C3	C4	C5	C5DC	C6	C8	C10	C12	C14	C16	C18
211	52	5.9	2.7	2.1	4.7	2.4	2.5	2.5	2.5	2.8	2.4	6.9	5.6
Total Inter-site Imprecision (%CV)	7	8	7	9	8	10	12	8	7	8	9	7	8
Recovery (%)	107-127	92-109	93-109	95-110	96-110	101-119	77-99	101-117	97-111	106-122	93-103	75-97	95-109

7 Clinical Performance at NBS Laboratories

The clinical correlation between the non-derivatized assay and the NeoGram assay was determined at two US newborn screening laboratories. Identical specimens were analyzed as paired samples by both methods. Samples included 9416 newborn neonatal samples, 104 samples with true positive diagnoses, and 320 artificially-enriched DBS. Table 5 shows the percent agreement in clinical determinations by both methods.

Table 5: Clinical Correlation

Analyte	Total # of Observations	% Agreement	Analyte	Total # of Observations	% Agreement
ALA*	2559	99.7%	C14*	9813	99.9%
ARG*	2564	100.0%	C16*	9803	99.9%
CIT*	9805	99.8%	C18*	9781	100.0%
GLY*	2474	99.8%	C4-OH/C4DC*	2564	99.5%
LEU*	9771	99.6%	C5-1	9840	100.0%
MET*	9808	99.7%	C5-OH/C4DC*	7276	98.4%
ORN*	2554	99.7%	C6DC	9840	98.1%
PHE*	9749	99.8%	C10-1	9840	100.0%
TYR*	9803	99.9%	C12-1*	2564	100.0%
VAL*	9745	99.5%	C14-1*	9840	99.9%
C0*	9461	99.9%	C14-2*	2564	99.9%
C2*	9808	100.0%	C14-OH*	2564	99.9%
C3*	9781	99.9%	C16-1*	2564	100.0%
C4*	2559	99.9%	C16-1-OH	9840	100.0%
C5*	9809	99.6%	C16-OH	9840	100.0%
C5DC	9840	97.2%	C18-1	9840	99.0%
C6	9840	100.0%	C18-1-OH	9840	100.0%
C8	9840	100.0%	C18-2*	2564	99.9%
C10	9840	99.9%	C18-OH	9840	100.0%
C12*	2559	99.9%			

*Only one of the two sites measured the indicated analytes.
#Several observations were associated with plate controls out of range. These observations were removed from the analysis and thus the lesser number of observations vs. the 9840 total.

8 Clinical Performance at NBS Laboratories

Table 6 shows a summary of the analysis of true positive samples at the two US newborn screening laboratories.

Table 6: Clinical Results

Disorder Abbreviation	Disorder Full name	Number Analyzed	Number Detected by Study Cutoffs	
			Non-derivatized	NeoGram
3MCC	3-Methylcrotonyl-CoA Carboxylase Deficiency	9	9	9
GUD	Carnitine Uptake Defect	10	10	10
GTD	Carnitine Transporter Defect	1	1	1
CPT-1	Carnitine Palmitoyltransferase I Deficiency	1	1	1
GA-1	Glutaric acidemia, type 1	9	9	9
HCV	Homocystinuria	7	7	7
IVA	Isovaleric acidemia	9	9	9
2MBDD	2-Methylbutyryl-CoA Dehydrogenase Deficiency	1	1	1
MCAD	Medium-Chain Acyl-CoA Dehydrogenase Deficiency	16	16	16
MCD	Multiple CoA Carboxylase Deficiency	3	3	3
MMA	Methylmalonic Aciduria	2	2	2
PPA	Propionic Acidemia	3	3	3
MSUD	Maple Syrup Urine Disease	2	2	2
SCAD	Short-Chain Acyl-CoA Dehydrogenase Deficiency	1	1	1
PKU	Phenylketonuria	12	12	12
LCHAD	Long-Chain 3-hydroxyacyl-CoA Dehydrogenase Deficiency	5	5	5
VLCAD	Very Long-Chain Acyl-CoA Dehydrogenase Deficiency	10	10	10
VLCAD	Very Long-Chain 3-hydroxyacyl-CoA Dehydrogenase Deficiency	1	1	1
TYR 1	Tyrosinemia Type 1	4	4	0

9 Clinical Performance – Tyrosinemia Type I

Figures 1 and 2 show the frequency distributions of succinylacetone and tyrosine concentrations from 9416 neonatal samples acquired using the non-derivatized assay at two newborn screening laboratories in the US. Four true positive samples for Tyrosinemia Type I were also acquired (whose SA and TYR concentrations are designated with arrows in Figures 1 and 2, respectively). All four contain highly elevated succinylacetone but do not contain the corresponding elevated tyrosine concentrations, highlighting the fact that SA is the primary marker for the disorder.

Figure 1

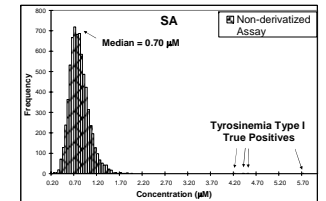
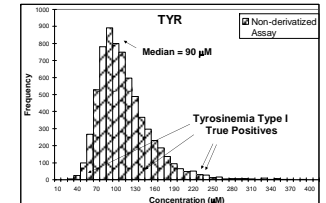


Figure 2



10 Conclusions

- Analytical performance of the non-derivatized assay is equivalent to the derivatized NeoGram assay
- The non-derivatized assay provides consistent results evidenced by the excellent correlation between sites
- The non-derivatized assay is effective in detecting IEMs in neonatal DBS
- Clinical correlation between the non-derivatized and NeoGram assays is excellent
- Measurement of succinylacetone with the non-derivatized assay allows for improved Tyrosinemia Type I screening

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