DRG® Phenylalanine (PKU) neonate (with Membrane Plate) (EIA-1477)

DRG® Phenylalanine (PKU) neonate (excluding Membrane Plate) (EIA-1478)

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Please use only the valid version of the package insert provided with the kit.

INTRODUCTION

INTENDED USE

The Phenylalanine Micrwell Enzyme Assay (PHE-MW EA) is designed for the quantitative determination of phenylalanine in neonatal blood spots.
It is for use as an aid in screening newborns for elevated phenylalanine levels.

CLINICAL PHYSIOLOGY

Phenylketonuria (PKU) is a congenital disease resulting in elevated blood levels of phenylalanine and excessive excretion of phenylpyruvic acid in urine. The disorder is characterized by irreversible mental retardation, skin abnormalities and behavioral disturbances (1). It is one of the most common hereditary amino acid disorders and affects approximately one in 15,000 newborns (2).
PKU is caused by the absence or deficiency of the hepatic enzyme, L-phenylalanine hydroxylase. This enzyme is necessary for the metabolic conversion of phenylalanine, and essential amino acid, the tyrosine. The deficiency results in an increase in both phenylalanine and its deaminated metabolite, phenylpyruvic acid, in plasma, urine cerebrospinal fluid (3-7).
Excessive accumulation of phenylalanine and related metabolites in the body, especially crebrospinal fluid, interferes with proper development of the brain. The injury to the cerebral tissue begins within the second and third week of life and progresses with time, becoming maximal at eight to nine months. Brain damage and the resulting progressive mental retardation may be minimized if the newborn is placed on a low-phenylalanine diet soon after birth (1, 8-10).
Screening for elevated levels of phenylalanine in neonates is important for early detection and treatment of PKU. Phenylalanine is frequently determined in plasma or whole blood during the first few days after birth (11). During the mid 1960's, the United States began a program requiring mass screening of newborn infants using a dried blood sport specimen (12, 13). This has since become widespread practice throughout the word and has resulted in early detection and dietary management of PKU in infants (14-18). It is now generally accepted that the mental retardation associated with PKU can be prevented by a low-phenylalanine diet, provided that the disease is detected in early infancy.
Syndromes other than PKU have been recognized to demonstrate high blood phenylalanine levels. These syndromes are referred to as atypical PKU or benign persistent hyperphenylalanine (PHP). While classical PKU is caused by a deficiency in phenylalanine hydroxylase, the atypical or variant forms of PKU are related to those enzymes affecting the production of tetrahydrobiopterin. Tetrahydrobiopterin is the cofactor required for the activity of phenylalanine hydroxylase. All patients with persistent hyperphenylalaninemia should be investigated to rule out the tetrahydrobiopterin-deficient forms of phenylketonuria (19).
The PHE-MW EA is designed to aid in newborn screening for elevated phenylalanine levels. Following enzymatic oxidation of the phenylalanine in the blood-spot sample, a simple color reaction demonstrating rapid and quantitative determination of phenylalanine levels in neonates.

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CLINICAL APPLICATION

NEWBORN SCREENING:
The PHE-MW EA is designed as an aid in screening newborns for PKU. Presumptive positive results should be confirmed by analysis with another methods.

FACTORS AFFECTING NORMAL VALUES

1. **NEWBORN AGE**:
   In patients affected with PKU, phenylalanine concentrations begin to rise progressively in the first 6-12 hours after birth (20). The clearly identify elevated phenylalanine levels, it is recommended that sampling should occur after 24 hours of age. The American Academy of Pediatrics recommends that infants initially screened before 24 hours should be re-screened for PKU (18). Other determinates of when to take patient samples depend on national requirements. Please observe local established guidelines.
2. **SAMPLE AGE**:
   Although blood spot samples are stable for up to six (6) months under proper storage conditions, prompt analysis of samples for the presence of phenylalanine should take place according to screening facility protocol. Once the sample is collected, it should be thoroughly dried (21).
3. **NEWBORN DIET**:
   A diet containing protein, such as breast milk or formula, is required for phenylalanine accumulation and subsequent identification of PKU in newborns (22,23).

PRINCIPLE OF THE TEST

The PHE-MW EA is an enzyme assay which follows an acid extraction of the phenylalanine contained in a 3/16" (5 mm) blood spot. In this assay, a single blood spot is extracted in a special semi-porous membrane bottom plate. Following extraction, all 96 extracts are simultaneously transferred to a conventional microwell plate, by means of a vacuum manifold. A neutralizing buffer is added to each acid extract, followed by addition of a combination enzyme-substrate reagent. This rapidly oxidizes the phenylalanine to phenylpyruvate, reducing NAD to NADH+ in the process. The NADH+ reduces a colorless dye to form a colored end-product with an absorbance maximum at 570 nm. The absorbance read is directly proportional to the amount of phenylalanine in the sample.

Note: All references to phenylalanine concentrations are in whole blood units.

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REAGENTS PROVIDED AND LABEL COLOR CODE

COMPONENT		LABEL COLOR	AMOUNT PER KIT	
			Single Kit	Bulk Kit
Membrane Transfer Plate		Tan	2 Plates	10 Plates
Collection-Reaction Plate	**PLATE COL**	Tan	2 Plates	10 Plates
PHE/GAl Blood Spot Standards	**STD 1-5**	Green	1 Card	3 cards
Tri-Level Blood Spot Controls	**CONTROL 1-3**	Green	1 Card	3 Cards
TCA Extraction Solution	**SOLN TCA**	Yellow	22 mL	110 mL
Phenylalanine Neutralizing Solution	**SOLN NEUT**	Yellow	11 mL	55 mL
Reagent A	**REAG A**	Red	11 mL	55 mL
Phenylalanine Reagent B	**REAG B**	Green	11 mL	55 mL

REAGENT DESCRIPTION And Preparation

Note: Some reagents contain sodium azide which has a tendency to build up in lead or copper plumbing forming potentially explosive metal azides. Always flush large quantities of water through the plumbing after the disposal of these reagents.

A. Membrane Transfer Plate (only for EIA-1477)
96-well porous microwell plates:
Storage: May be stored at room temperature or at 2-8°C with kit.
Stability: Refer to kit expiration

B. Collection-Reaction Plate
96-well uncoated microwell plates.
Storage: May be stored at room temperature or at 2-8°C with kit.
Stability: Refer to kit expiration.

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C. PHE/GAL Blood Spot Calibrators

Five standards are proved in mg/dL of whole blood. These standards are prepared in a bovine whole blood matrix containing sodium azide as a preservative and are spotted on Schleich and Schuell's filter Paper #903. Refer to foil package for concentrations.

Storage: 2-8°C. For long term storage greater than 7 days, store at –20°C. Reseal bag after use.
Stability: Refer to expiration date on foil package.

D. Tri-Level Blood Spot Controls

Three controls are provided. These controls are prepared in a bovine whole blood matrix containing sodium azide as a preservative and are spotted on Schleicher and Schuell's filter paper #903. Refer to foil package for concentration ranges.

Storage: 2-8°C. For long term storage greater than 7 days, store at –20°C. Reseal bag after use.
Stability: Refer to expiration date on foil package.

E. TCA Extraction solution

Trichloroacetic acid (TCA) in deionized water.
Storage: 2-8°C.
Stability: Refer to expiration date on kit vial.

F. Phenylalanine Neutralizing Solution

Sodium carbonate (Na_2CO_3) in deionized water.
Storage: 2-8°C.
Stability: Refer to expiration date on kit vial.

G. Reagent A

Substrate buffer with sodium azide (NaN_3) as preservative.
Storage: 2-8°C. For longer term storage greater than 7 days, store at –20°C.
Stability: Refer to expiration date on kit vial.

H. Phenylalanine Reagent B

Nicotinimide adenine dinucleotide (NAD) and phenylalanine dehydrogenase (PDH) in buffer with bovine calf serum and sodium azide (NaN_3) as a preservative. PDH is a peptide produced by microbial fermentation with *Sporosarcina sp*. The enzyme contains no material of human, animal or plant origin.

Storage: 2-8°C. For long term storage greater than 7 days, store at –20°C.
Stability: Refer to expiration date on kit vial.

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LIMITATIONS, PRECAUTIONS AND GENERAL COMMENTS

A. The reagents supplied in this kit are for IN-VITRO DIAGNOSTIC USE ONLY.

B. Strict adherence to the protocol is advised for to obtain reliable results. Any modifications made to the reagents or assay procedure are the responsibility of the user.

C. A standard curve must be established for each run. A run should consist of a maximum of two consecutive plates. For larger assays, the timing of pipetting should be staggered to ensure uniform plate processing.

D. Extrapolated value outside the range of standards (see foil package for concentration) are approximate and should be reported as „less than the value of the lowest standard“ or „greater than the value of the highest standard“.

E. This assay is designed to be used with samples which are exclusively collected on SCHLEICHER & SCHUELL (S&S) FILTER PAPER #903. Changes in filter paper lots may affect patient results. All laboratories should record lot numbers of S&S #903 paper to monitor possible changes.

F. The PHE-MW EA is to be used as an initial screening test for the quantitation of phenylalanine levels in whole blood spots. Individual screening laboratories are responsible for further test those patients with increased phenylalanine levels using documented confirmatory test PKU.

G. Several variables not directly related to serum levels of phenylalanine may affect the determined value. These include hematocrit, moisture content of the paper, rate of blood deposition and lot of filter paper utilized. For these reasons, uniform collection techniques coupled with careful examination of the blood spots before analysis is recommended (24).

H. A blood test to screen for elevated concentrations of phenylalanine should be performed on all newborn infants after the onset of feeding. For further information, please see recommendations from the American Academy of Pediatrics (18).

I. Infants receiving special treatment or prolonged hospital stay should be tested in a timely manner after the onset of feeding. Feeding status must be monitored to ensure adequate protein intake in these infants (25).

SPECIMEN COLLECTION AND HANDLING

Infant Screening programs may differ from one another in the amount of sample required. A heel sample collected from 5/8” (16 mm) diameter blood spot pre-printed on Schleicher & Schuell Filter Paper #903 is suggested. The following summary is described in detail in NCCLS publication LA4-A4.

A. Be sure the required information on the specimen card has been completed, including the infant’s name, time and date of birth, and time and date of collection. Also indicate pre- or post-term, the infant’s weight and feeding status and whether or not the infant was a twin.

B. Collect the blood from the heel of an infant usually 24 to 72 hours postpartum. Sampling times may vary from center to center. See Limitations Section (items H and I).

C. Wash the heel with soap and water and wipe dry. Swab area with alcohol and allow to air dry. Disinfectants containing iodine should be avoided.

D. Puncture infant's heel with sterile lancet and wipe away first drop of blood. Make sure the tip of lancet is no longer than 2.5 mm. Allow another drop of blood of adequate volume to form, and gently touch the specimen card to the droplet in the center of the pre-printed circle on the filter paper card. The blood volume must be enough to completely fill at least two circles on the card. View the card from the opposite side as the blood penetrates the filter paper. Avoid excessive squeezing of the heel as it may cause hemolysis and also dilute the sample with tissue fluid. Avoid tearing or disrupting of the filter paper surface.

E. Place the filter paper card horizontally on a clean surface and allow to air dry for at least 6 hours at room temperature. Avoid direct sunlight.

F. Place each specimen in its own paper envelope and transport to the laboratory within 24 hours of drying.

G. The receiving laboratory should store the sample at 2-8°C in a moisture proof environment shielded from direct light.

H. Although phenylalanine has been reported to be stable in blood spots in excess of six (6) months, not all components are as stable and neonatal blood spot storage must be considered on an analyte by analyte basis.

EQUIPMENT AND REAGENTS REQUIRED

A. A 3/16" (5 mm) or 1/8" (3 mm) diameter paper punch

B. Plate reader able to read absorbance at 550-570 nm.

C. Multi-channel and single channel micropipets to 50 µL and 100 µL.

D. Rotary horizontal shaker.

E. Vacuum manifold. (Requires a standard hose bench-top vacuum outlet or equivalent external vacuum source.

PROCEDURE

Assay Preparation

1. Prior to beginning assay, label each collection-reaction plate to match its complementary membrane transfer plate. Additionally, each laboratory should determine the time necessary for complete transfer of extract to the collection plate using the vacuum manifold. The time required should be between 30 and 60 seconds.
2. For a two plate assay (192 Wells), transfer the entire contents of REAGENT A vial to the Phenylalanine REAGENT B vial. Transfer mixture between vial A and B a few times to ensure adequate mixing. Combine REAGENT A and PHENYLALANINE REAGENT B immediately prior to use. **Do not store or re-use.**
3. If less than 22.0 mL of REAGENT A and Phenylalanine REAGENT B is required, mix equal volumes of vial A and B for desired volume. Promptly store the remaining un-mixed reagent at 2-8°C, or at –15°C for long term storage.

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Assay Steps

1. For the five (5) *Standards*, three (3) *Controls* and variable unknowns: Punch one 3/16" (5 mm) or two 1/8" (3 mm) blood spot(s) into the appropriate wells of the membrane transfer plate. Be sure to leave two empty wells for the reagent blank.
2. Add 100 µL of *TCA Extraction Solution* to each well.
3. Rotate mix for 60 minutes at room temperature (R.T.).
4. Simultaneously transfer acid extracts to empty collection-reaction plate using vacuum manifold.
5. Add 50 µL of *Phenylalanine Neutralizing Solution* to each well and shake gently by hand for 10 seconds.
6. Add 100 µL of pre-combined *Reagent A-B* to each well and shake plate gently by hand for 10 seconds.
7. Incubate 45 minutes at R.T.
8. Read at 550-570 nm and calculate the results by plotting the standard curve on linear graph paper (See "Calculations" Section).

Quality Control

BLOOD SPOT CONTROLS containing low, intermediate and high levels of phenylalanine are included in this kit. They should be included in each assay run as unknowns in order to monitor the performance and reliability of the assay. Likewise, external blood spot controls (i.e., CDC, etc.) containing phenylalanine at several different levels should be routinely included in each run. Analysis of the results obtained should be done according to acceptance criteria established by the individual laboratory.

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PROTOCOL

WELLS	SAMPLE (3/16" Blood Spot)	TCA Extract. Solution	Rotate & Incubate	Vacuum Manifold	NEUTRALIZING SOLUTON	REAGENT A – B		Read Absorbance
A1, B1	Reagent Blank	100 µL	60 minutes at R.T.	Transfer Extracts to Collection Plate	50 µL	100 µL	**Incubate** 45min.at R.T.	550-570 nm
C1, D1	Standard 1	↓			↓	↓		
E1, F1	Standard 2							
G1, H1	Standard 3							
A2, B2	Standard 4							
C2, D2	Standard 5							
E2, F2	Control I							
G2, H2	Control II							
A3, B3	Control III							
C3, D3	Unknown 1							
E3, F3	Unknown 2							
Etc.	Etc.							

CALCULATIONS

A. Average the absorbance duplicates for all standards, controls and patients. Subtract the average reagent blank absorbance from each of the averages obtained above. This yields the net absorbance (Abs.)

B. Construct the standard curve by plotting the net absorbance (y-axis) versus the concentration of the phenylalanine standard (x-axis) using linear graph paper and a weighted linear curve fit. This yields the standard curve.

C. Using the standard curve, determine the phenylalanine concentrations of each patient sample. Read patient samples (Abs. – Blank) directly off curve as mg/dL phenylalanine in whole blood. A sample assay and calibration curve are provided below.

D. Computer assisted data reduction may be used to calculate results. A weighted linear curve fit using a Blank subtract is recommended. To program your automated data reduction system, please contact your software manufacturer.

E. Unit conversion (mg/dL ←→ mmol/L):

To convert mg/dL to mmol/L, multiply by 0.06

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To convert mmol/L to mg/dL, multiply by 16.5 or divide by 0.06.

SAMPLE ASSAY

The following calculations are for example only and patient values must not be derived from this chart. The user must construct a standard curve each time the assay is run.

SAMPLE	ABS	ABS-BL	CONC (mg/dL)	PHENYLALANINE (mg/dL)
Blank	0.121			
Std. 1	0.166	0.045	1.0	
Std. 2	0.242	0.121	4.0	
Std. 3	0.286	0.165	7.0	
Std. 4	0.431	0.310	15.0	
Std. 5	0.671	0.550	27.0	
Control I	0.206	0.085		2.9
Control II	0.318	0.197		8.7
Control III	0.515	0.394		18.9

The following is a sample standard curve and related information as illustrated from a computer assisted data reduction program.

Y Axis:	ABS-BL	
X Axis:	Conc. (PHENYLALANINE9	
Curve Fit:	Weighted Linear	
Graph:	Linear vs. Linear	
Slope:	0.019392	
Y Int:	0.027391	
R Factor:	0.999417	
% CV:	2.14	
STD	Response	Conc
1	0.045	1.00
2	0.121	4.00
3	0.165	7.00
4	0.310	15.00

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5	0.550	27.00

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EXPECTED VALUES

NEONATES (24-48 hours): 0.6 to 3.6 mg/dL whole blood

Literature indicates that serum phenylalanine levels in normal, full term infants are 2.1 ± 0.5 mg/dL (4). The actual values range from 0.6 – 3.6 mg/dL. A study performed using the PHE-MW produced similar results of 2.0 ± 0.7 mg/dL. As with any diagnostic test, differences in physiological ranges may be encountered from laboratory to laboratory due to patient demographics, laboratory techniques, and population sampling. These ranges should only be used as a guideline. We recommend each laboratory establish its own ranges using a statistically significant number of characterized patient specimens.

A. Normal Range Analysis

This Phenylalanine Neonate EIA was compared with a commercially available quantitative method for phenylalanine determination. Data from this study (n = 1046) are highly compatible with a Gaussian (normal) distribution, with an estimated mean of 2.04 mg/dL and an estimated standard deviation of 0.685 mg/dL.
The 95% confidence interval for the mean is 2.00 to 2.09 mg/dL.
The actual observed range of the assay in the database is 0.13 to 4.9 mg/dL.
The 95th percentile corresponds to the value 3.23 mg/dL and the 99th percentile to the value 3.7 mg/dL.
The median and the mode are both 2.03 mg/dL.

B. Cut-Off Derivation

The table below illustrates the statistically derived cut-off for phenylalanine levels in this Phenylalanine Neonate Kit and a comparison assay at the 95th, 99th and 99.9th percentile. These data are based on results generated at an established testing facility.

	Phenylalanine Neonate (mg/dL)	Comparison Assay (mg/dL)
95^{th} percentile	3.2	2.3
99^{th} percentile	3.7	2.7
99.9^{th} percentile	4.2	3.1

C. Referral Rate Analysis

The table below demonstrates the variable presumptive positive rate observed in this Phenylalanine Neonate Kit utilizing a broad range of cut-off values for phenylalanine. The data below was generated at a state testing facility utilizing a population of normally distributed neonatal blood spot samples (n=1044). Definition of a cut-off lower than 4.0 mg/dL may enhance screening security through re-testing or presumptive positive samples. National reference intervals, which also determine the need for re-testing, might exist.

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Phenylalanine EIA Cut-Off (mg/dL)	Expected Presumptive Positive Rate (%)
3.0	8.33
3.1	6.42
3.2 (95% Normal Range)	5.36
3.3	3.93
3.4	2.87
3.5	2.01
3.6	1.44
3.7 (99% Normal Range)	0.96
3.8	0.67
3.9	0.57
4.0	0.48

PERFORMANCE CHARACTERISTICS

INTER-ASSAY VARIATION

Inter-assay variation, or precision was calculated by evaluating the same tri-level samples in multiple assay runs.

	Level I n = 42	Level II n = 42	Level III n = 42
Mean	2.85	8.63	19.0
S.D.	0.39	0.77	1.46
% C.V.	13.7	8.9	7.7

INTRA-ASSAY VARIATION

Intra-assay variation, or precision was calculated by assaying 10 replicates each of three tri-level samples.

	Level I n - = 10	Level II n = 10	Level III n = 10
Mean	3.09	9.33	20.4
S.D.	0.38	0.45	1.18
% C.V.	12.3	4.8	5.8

FUNCTIONAL SENSITIVITY

Although lower doses are distinguishable from zero, it is recommended that, based on the functional sensitivity of the assay, all values which fall below the lowest standard (Standard 1), should be reported out as “less than the value of the lowest standard”.

PARALLELISM (linearity of dilutions)

Results shown represent actual values multiplied back by the indicated dilution factor.

SAMPLE	PHE (mg/dL)	1 : 2 PHE (mg/dL)	1 : 4 PHE (mg/dL)
1	27.8	25.8	25.8
2	35.4	29.9	31.1
3	32.6	29.6	30.8

Comparison Study

Patient Sample Correlation

Results from samples with values distributed throughout the quantitative range of this assay were compared with those obtained with a commercially available method. The correlation coefficient was 0.969 (slope = 1.125, y-intercept = -0.494 mg/dL). A paired t-test did not show a difference in the values obtained by the two methods.
415 patients were selected from a statewide screening program for PKU. They were stratified over a broad range of measurements and were chosen from low, intermediate and presumptive positive sample pools, as well as from elevated and known positive PKU patients.
Two samples in this study gave poor correlation between our own and the comparison methods. However, both methods indicated that the samples were well above the usual clinical cut-off.

Relative Presumptive Positive Analysis

The table below classifies the 1044 cases of the normal range study according to normal and presumptive positive results for the 95th percentile cut-off. Utilizing the statistically derived cut-off values, the presumptive positive rates are similar for both methods (4.2% and 5.36%, respectively). Based on statistical analysis, the discordance observed between the two is due to random method variation.
No confirmed positives were encountered in this naturalistic population. We have shown, however, that inclusion of all elevated and known PKU positive samples available for reanalysis in both assays (n = 54), produced all positive results at the highest examined cut-off of 4.0 mg/dL.

	Own Group	Comparison Group	
Frequency	≤ 2.3 mg/dL	> 2.3 mg/dL	TOTAL
≤ 3.2 mg/dL	959	29	988 (94.64%)

> 3.2 mg/dL	42	14	56 (5.36%)
TOTAL	n = 1001 (95.88%)	n = 43 (4.12%)	1044 (100%)

Recovery

Results shown illustrate the percent recoveries of samples to which known amounts of phenylalanine were added.

Sample Number	Expected PHE (mg/dL)	Observed PHE (mg/dL)	% PHE Recovered
1	4.25	4.33	102
2	8.29	7.72	93
3	14.0	13.1	94
4	6.17	5.54	90
5	10.2	9.96	98
6	15.9	15.8	99
7	12.2	12.1	100
8	17.8	17.7	99
9	21.9	25.2	115
			AVERAGE RECOVERY 98.9%

Interfering Substances

The following substances were added to a blood spot standard in order to determine their effect on the result. Ascorbic acid was found to interfere with the phenylalanine assay at superphysiologic levels. No interference was observed when ascorbic acid was tested at normal physiologic levels (0.2 – 2.0 mg/dL). Additionally, tetracycline was found to present a possible interference. Infant receiving tetracycline antibiotic therapy should be evaluated accordingly, especially if elevated phenylalanine results are observed. None of the other compounds interfered at the concentrations tested.

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	Amount Added (mg/dL)	Baseline PHE (mg/dL)	Observed PHE (mg/dL)
Amoxycillin	100	4.20	4.30
Ampicillin	100	4.20	4.20
Erythromycin	100	4.20	4.20
Gentamycin	100	4.20	4.50
Penicillin	100	4.20	4.50
Tetracycline	100	4.20	6.30
Bilirubin	20	4.10	4.20
Hemoblobin	100	4.20	4.20
Triglycerides	125	3.71	3.90
Cysteine	1.44	7.13	6.66
Cystine	1.44	7.13	6.73
Tryptophan	19.4	7.13	7.37
Tyrosine	17.9	7.13	7.44
Leucine	15.8	7.13	7.87
Methionine	3.7	7.13	6.65
Ascorbic Acid	11.0	7.13	10.8
Ascorbic Acid	1.1	0.0	0.19
Ascorbic Acid	1.44	0.0	0.52

ALTERNATE PROCEDURE (For manual transfer of extracts)

1. For the five Standards, three Controls and variable unknowns: Punch one 3/16" (5 mm) blood spot or two 1/8" (3 mm) blood spot(s), into appropriate well of plate. Be sure to leave empty well for reagent blank.
2. Add 200 µL of TCA Extraction Solution to each well.
3. Rotate mix for 60 minutes at room temperature.

4. Using a single channel or multichannel pipet, transfer 100 µl of each extract to the corresponding well of a new plate.
5. Add Neutralizing Solution and Reagent A-B as indicated in package insert.

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