پادتن علم
PADTAN ELM

THE PADTAN ELM KIT FOR 96 TESTS

Neonatal PKU

Intended use:
Quantitative determination of Phenylalanine levels in dried blood samples

For *in vitro* diagnostic use.

I.Introduction

Phenylketonuria (PKU) is a hereditary metabolic disorder which is inherited in an autosomal recessive pattern. It is caused by the absence or deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) which is responsible for hydroxylation of phenylalanine to tyrosine. Due to the block of tyrosine formation, phenylalanine is metabolized via the alternative pathway through formation of phenyl pyruvate, phenyl lactic acid, phenyl acetic acid etc[1].
PKU affects approximately one in 10000 to 15000 newborns[2]. Untreated PKU prevents normal brain development and result in severe mental retardation. Newborns with phenylketonuria initially don't have any symptoms. Without treatment, though, babies usually develop signs of PKU within a few months. Phenylketonuria symptoms can be mild or severe and may include: vomitting, hyperactivity and restlessness, skin rashes, blond hair and fair skin, a musty odor in the child's sweat, skin or urine, and stunted growth[3]. Gradually, the condition worsens progressively resulting in mental retardation. Brain damage and the resulting progressive mental retardation may be minimized if the newborn is placed on a low phenylalanine diet soon after birth[4-7].Thus, considering the fact that PKU is asymptomatic in the immediate newborn period and early pre-symptomatic treatment is vital in prevention of mental problems, screening for elevated levels of phenylalanine in neonates is important for early detection and treatment of this congenital disease[8].
The Padtan Elm PKU microwell enzyme assay is useful for quantitative determination of phenylalanine in neonates and allows early detection of PKU and thus preventing infant mental retardation.

II.Principle of the test

The Padtan Elm Neonatal PKU kit is a quantitative test which is based on microwell enzymatic colorimetric assay. During the course of the assay, a 5mm disk of standards, controls and infant samples is punched off the blood spots collected on Whatman's filter paper 903. The disk is then placed into the microplate wells and elution buffer is added. During the incubation, phenylalanine present in the samples is extracted from dried blood spots. After extraction, the neutralizing buffer is transferred into new wells and eluted sample is added to each well. After neutralization, a combination of phenylalnine dehydrogenase (PDH) and a substrate reagent are added to each well. PDH oxidizes phenylalnine to phenylpyruvate, reducing NAD^+ to $NADH^+$. $NADH^+$ produced reacts with tetrazolium salt to form a colored end-product with an absorbance maximum at 492nm.
The intensity of the color is proportional to the amount of phenylalnine present in the sample. Standard curves are constructed for each assay by plotting absorbance value against the concentration of each standard. The phenylalnine concentrations of patient samples are then read from the standard curve.

III. Kit contents

The reagents provided with the Neonatal PKU Kits (Cat.No.P-PKI) are sufficient for 96 wells. The expiry date of each reagent is shown on the vial label.

Store the Kit at 2-8° C.

1.Microplate wells: 2×96 uncoated wells.
2.Standards: 6 Blood Spots dried on Whatman's filter paper 903 containing a known amount of phenylalnine which is printed beside the spots.
3.Controls: Two Blood Spots dried on Whatman's filter paper 903 containing a known amount of phenylalnine which is printed beside the spots.
4. Elution Buffer: One vial (12ml) of acidic elution buffer
5.Neutralizing solution: One vial (6 ml) of basic neutralizing solution
6.Reagent (A): One vial (0.3 ml) of phenylalnine dehydrogenase solution (PDH) with PBS
7.Reagent (B): One vial (0.3 ml) of NAD^+ solution with PBS.
8.Reagent Buffer: One vial (12 ml) of reagent buffer containing Tetrazalium salt, PBS and preservative.

IV. Materials required (but not supplied with the kit)

1.Microtiter plate spectrophotometer reader with a wavelength of 492 nm(with reference wavelength at 630 nm) and absorbance range of 0 to 3.0.
2. Precision 25,50 and 100 µl micropipettes.
3. 5mm Hole puncher
4. ELISA shaker capable of 1500rpm

V. Specimen collection and preparation

For Infant Phenylalanine screening, a heel stick sample collected on Whatman's filter paper 903 , 24 to 72 hours postpartum is suggested. The approved method of Blood collection for Newborn Screening programs according to NCCLS, LA4-LA2 publication is as following:
Clean the heel of the infant with soap and wipe dry. Use alcohol on the area and allow to air dry. Puncture infant's heel with sterile lancet and wipe away the first drop of blood. Allow a second, large drop of blood to form and lightly touch filter paper to this large drop of blood. Allow blood to soak through and completely fill the preprinted circle with a single application to this large blood drop.The blood volume must be enough to completely fill circles on the card.
Place the filter paper card horizontally on a clean surface and allow to air dry thoroughly for at least 3 hours at ambient temperature. Avoid direct sunlight. After 3 hours of drying, place each specimen in its own paper envelope with silica

VI. Assay procedures

All reagents should be brought to room temperature prior to use.

1.Punch 5 mm diameter disks off the infant's blood spots, standards and controls dried on filter paper using a 5 mm puncher. Place them into the wells.
2.Add 100µl Elution buffer to each well. Mix them by gentle tapping the plate 30 seconds.
3.Incubate the microplate for 1 hour at room temperature with constant shaking (Do not cover the wells).
4.Secure the required number of new wells into the holder , then dispense 50 µl of Neutralizing buffer into the wells.
5.Transfer 50µl of the eluated present in the primary wells (wells containing blood spots) to the newly prepared wells.
6. Add 100 µl of Reaction buffer to each well. In order to prepare Reaction buffer, mix 1 ml of Reagent buffer with 25 µl Reagent A and 25 µl Reagent B. This amount is sufficient for 8 wells. The Reaction buffer is stable up to 10 minutes after preparation. Recommend to prepare Reaction buffer after transfer of Neutralizing buffer into the wells.
7.Incubate the plate for 1 hour at room temperature in the dark.
8.Read absorbance at 492 nm (with reference wavelength at 630 nm) in a microtiter plate reader.

SUMMARY OF ASSAY PROCEDURE:

Punch 5 mm diameter disks off the standards , controls or infant's boold spots

Pipette 100µl Elution Buffer

Incubate 1 hour at RT on shaker

Pipette 50µl Neutralizing buffer into the new wells

Pipette 50µl Eluted samples

Pipette 100µl Reaction buffer

Incubate 1 hour at RT

Read at 492/630 nm

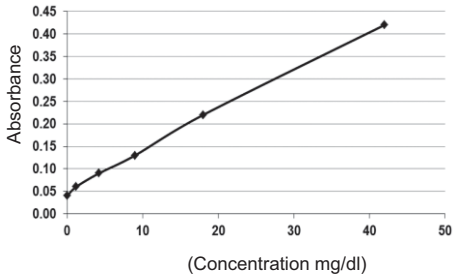
VII. Calculation of results

The results can be calculated by microtiter plate spectrophotometer reader or manual evaluation. If a microtiter plate spectrophotometer reader with data calculation program is used, refer to the plate reader and create a program using the concentration of each of the Phenylalanine standards in (mg/dl). For manual evaluation, a standard curve is constructed by plotting the absorbance (A) values obtained for each Phenylalanine standard against the corresponding Phenylalanine concentrations in mg/dl . The unknown PAH concentration in mg/dl, can then be read from the standard curve using the absorbance value of each patient specimen.

Example of calculation:

The values shown below are examples and must not be used in place of experimental data.

Specimen	Absorbance	Phenylalanine concentration (mg/dl)
Standard A	0.04	0
Standard B	0.06	1.2
Standard C	0.09	4.2
Standard D	0.13	9
Standard E	0.22	18
Standard F	0.42	42
Blood Spot A	0.107	5.9



VIII. Normal values

650 infant whole blood samples were analyzed by Padtan Elm PKU kit and normal values of neonatal Phenylalanine has been determined. According to the results obtained, in 99% of the infants, Phenylalanine concentration of whole blood was less than 2 mg/dl with the mean value of 1.4 mg/dl.

It is highly recommended that screening laboratories use the phenylalanine concentration of 4 mg/dl as the cut-off point that determined by health centers, albeit the infant's condition(age, weight, preterm birth, twins or multipless are considered.

IX. Specific assay characteristics

1. Sensitivity: The sensitivity of this kit was calculated based upon the standard curve and expressed as the minimal concentration of phenylalanine showing a significant difference from the "zero" standard. This minimal detectable concentration of phenylalanine for the present kit is 0.1 mg/dl.

2. Specificity: The specificity of Phenylalanine dehydrogenase enzyme activity was determined before and after adding 10 mg/dl of Tyrosine, Metionine, Leucin and Cystein into the reaction and measuring the corresponding dehydrogenase activity. According to the results, none of these amino acids showed any significant interference in the enzyme activity of PDH.

3. Precision: Precision was evaluated for inter and intra-assay variability. Intra-assay reproducibility was determined by measurement of 10 replicates of 3 samples in a single run. Inter-assay reproducibility was determined by replicate measurement of 3 samples in 10 separate runs. The results of standard deviation (SD) and coefficient variation (CV) are shown in the table.

Reproducibility

	Inter-assay			Intra-assay		
Sample	Mean(mg/dl)	SD	%CV	Mean(mg/dl)	SD	%CV
1	4.3	0.4	8.3	5.8	0.4	6.7
2	8.2	0.6	7.3	8.9	0.6	6.4
3	14.3	0.8	5.7	14.9	0.8	5.1

4. Accuracy: To assess the accuracy of Padtan Elm neonatal PKU kit, phenylalanine concentration of several normal and patient blood samples were measured by Fluorometric method and compared with Padtan Elm results. The difference between the results from two methods was less than 10% in all samples.

6. Recovery and dilution: Recovery is defined as the increase in concentration seen when a known concentration of an analyte is added to a sample. Several citrated blood samples with high phenylalanine concentrations were mixed with samples containing low phenylalanine concentration. Phenylalanine concentration of the samples on Whatman's filter paper 903 were then analyzed by Padtan Elm PKU kit. The percentage recovery is calculated as:

[(Measured conc. (mg/dl)/ Expected conc. (mg/dl)] X100

The percentage recovery ranged from 95% to 118.4%.

Recovery test

Added Conc.(mg/dl)	Expected Conc.(mg/dl)	Measured Conc.(mg/dl)	%Recovery
-	-	2.7	-
0.5	3.2	3.5	109.4
1.1	3.8	4.5	118.4
3.3	6.0	5.7	95

For dilution test, several blood samples containing elevated phenylalanine concentrations were tested after serially diluting with the zero standard, then were spotted on Whatman's filter paper 903 and analysed with the kit for phenylalanine concentrations. The results of one of these samples are shown in the following table. The percentage recovery ranged from 91.4 to 108.6.

Dilution test

Dilution	Expected Conc. (mg/dl)	Measured Conc. (mg/dl)	%Recovery
-	-	27.9	-
1:2	14	13.1	93.6
1:4	7.0	6.4	91.4
1:8	3.5	3.8	108.6

X. References

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WARNING!

This kit contains toxic materials, animal and human sourced components. Since no method can completely rule out the presence of blood-borne disease(e.g. HIV,HCV and HBV) therefore,all human sourced material must be considered potentially infectious.
In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary. Never pipette by month. Do not eat, smoke or apply cosmetics in the laboratory.

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