INTENDED USE

The kit has been designed for the quantitative in vitro Determination of phenylalanine in neonatal blood spots intended for screening phenylketonuria (PKU).

INTRODUCTION

Phenylketonuria (PKU) is a congenital disease caused by a deficiency of the enzyme phenylalanine hydroxylase, resulting in elevated levels of phenylalanine. Untreated patients with PKU may develop irreversible mental retardation, skin and behavioral abnormalities. Neonatal screening for elevated phenylalanine levels is important for early detection and treatment of PKU.

PRINCIPLE OF THE ASSAY

Neonatal phenylalanine assay is based on chemical reaction of phenylalanine eluted from dry blood spot. Phenylalanine reacts with ninhydrin in the presence of L-leucyl-L-alanine and generates a fluorescent compound. The reaction runs under the pH 5.88 and controlled by phthalate buffer. To stabilize the fluorescent complex and improve the signal, copper reagent is added. Fluorescence is measured with appropriate fluorometer on the microtitration plates. Excitation wavelength is 360 nm and the emission wavelength is 480 nm.

KIT CONTENTS

All reagents of the kit are stable until the expiry date indicated on the kit label, if stored at 2-8°C.

1- Phtalate buffer : 15 ml
   Ready to use. Contains Bronidox 0.2% as preservative.

2- Ninhydrin : 30 ml
   Ready to use.

3- L-Leucyl-L-Alanine : 30 ml
   Ready to use. Contains Bronidox 0.2% as preservative.

4- Copper reagent : 250 ml
   Ready to use.

5- Calibrators :
   1 paper with 4 sets of 6 calibrators, in a foil package with a desiccant. Phenylalanine in dried blood spots on filter paper S&S 903. The calibrator’s values may vary by lot. The actual values of the calibrator are indicated on the calibrator sheet in the kit.
   The calibrators are prepared from human blood with a hematocrit value of 50 %–54 % and calibrated against the 1st ISNS Reference Preparation for Neonatal Screening for thyrotropin, phenylalanine and 17-alpha-hydroxyprogesterone in blood spots.

6- Controls :
   1 paper with 5 sets of 2 controls in a foil package with a desiccant. Phenylalanine in dried blood spots on filter paper S&S 903. The controls values may vary by lot. The actual values of the calibrator are indicated on the calibrator sheet in the kit. The calibrators are prepared from human blood with a hematocrit value of 50 %–54 %.

7- Reaction plates: 10 pcs.
   Non coated white microplates.

8. Adhesive slips: 20 pcs

OTHER MATERIALS REQUIRED, BUT NOT PROVIDED

- 80 % ethyl alcohol.
- Elution plate .U bottom microplate for disk elution.
- Disk puncher with a diameter of 3 mm.
- Precision micropipettes 50 -200 μl, 200-1000 μl. 8-channel micropipette 50-300 μl.
- Microplate shaker.
- Incubator
- Microplate fluorometer
PRECAUTIONS AND WARNINGS
- This reagent kit is for in vitro diagnosis only.
- This reagent kit is for professional use only.
- Do not interchange reagents between different lots.
- All human sourced material, calibrators and controls are tested free from HBsAg, Anti-HCV and Anti-HIV. However, all materials of human origin should be considered as potentially infectious and it is recommended that all kit materials and test specimens to be handled using established good laboratory practice.
- Ninhydrin is an irritant to skin, eyes and respiratory tract. Avoid contact of the ninhydrin with skin, eyes and mucous membranes. In case of contact, flush immediately with abundant amounts of water.
- Ethanol is highly flammable. Keep container tightly closed. Keep away from sources of ignition.
- All kit components and specimens should be regarded as potential hazards to health. It should be used and discarded according to your own laboratory’s safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols.

SPECIMEN COLLECTION AND HANDLING
The specimen collection technique is described in detail in NCCLS document LA4-A3. The samples of blood are collected and dried on the filter paper reserved for neonatal screening test (Schleicher & Schuell 903). The blood sample is collected between 3-5 days after birth from newborn's heel. The heel is cleaned with 70% alcohol and punctured with a sterile blood lancet. The blood drop obtained is soaked onto the filter paper in the centre of the circle printed. Both sides of the paper have to be penetrated and saturated by this one drop. After collection of the samples, the filter papers are dried horizontally for 3-6 hours at room temperature. The dry samples should be stored at 2-8 °C.

STORAGE CONDITIONS
- All components must be stored at 2 to 8°C
- All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use.
- Do not use reagents over the expiration date.

ASSAY PROCEDURE
- Bring all reagents, controls, calibrators and samples to room temperature (20 - 25°C) before assay

Preparation of reagent:
- After elution step, prepare the needed volume of test mixture as describe on Table 1

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<thead>
<tr>
<th>Number of plates</th>
<th>Phtalate buffer (ml)</th>
<th>Ninhydrin (ml)</th>
<th>L-Alanine (ml)</th>
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Test procedure:
1- Place 3 mm punched spots (calibrator, control or sample) into the appropriate well on the Elution plate.
2- Add 100 μl of 80 % ethyl alcohol to each well, cover the plate and elute phenylalanine for 30 min at room temperature on a shaker (shaking speed ; 900 rpm).
3- Prepare the needed volume of test mixture.
4- Transfer 50 μl of eluate from Elution plate into the correct wells of the White plate.
5- Add 50 μl of test mixture, seal tightly with a new incubation cover. Shake for 1 min at room temperature.
6- Incubate the white plate 60 minutes at 60°C.
7- Add 200 μl of cold copper reagent to each well.
8- Incubate the plate for 10 min at room temperature.
9- Read the fluorescence within 10-30 min after addition of the copper reagent using microplate fluorometer. Filters; excitation 360 nm/emission 480 nm.

RESULTS
Calculation of the Results
Results are obtained from the standard curve by interpolation. The curve serve for the determination in samples measured at the same time as the calibrators. Calculation of results can be carried out manually if there is no automatic data reduction.
Figure 1: Example of calibration curve

If automatic data processing can be used, linear or cubic spline curve fitting with lin-lin axis scaling is recommended. Determine the F.I. for each well. Plot the calibrator curve using linear graph paper with concentration of calibrators on the x-axis and F.I. on the y-axis.

EXPECTED VALUES

The phenylalanine concentration measured by the ODAK phenylalanine Assay Kit was assessed by testing 400 dried blood spot specimens. Cut-off value was determined to be 2 mg/dL. Samples above this cut-off value would be regarded as presumptive positive and repeat test (confirmatory) should be performed without delay. As a screening method for phenylketonuria, elevated results are not absolutely diagnostic but indicate an urgent need for further investigation of the neonate under question. It is strongly recommended that each laboratory determines its own reference ranges and cut-off based on specimens from the laboratory routine population and also establishes a procedure for the close follow up of newborns above cut-off levels.

To convert to umol/L, use the conversion factor.
1 mg/dL = 60.6 mmol/L

PERFORMANCE CHARACTERISTICS

Analytical sensitivity:
The detection limit of phenylalanine calculated was 0.4 mg/100 ml (=25 mol/L).

Specificity:
The principle of the determination guarantees no crossreaction with other amino acids.

Measurement range:
From 0.4 to 40. The calibration curve is linear between lowest and highest calibrators.

Precision:

Intra-assay
Blood spot samples were assayed in 8 replicates in the same series. The coefficients of variation were found below or equal to 10.4 %.

Inter-assay
Blood spot samples were assayed in 8 different series. The coefficients of variation were found below or equal to 12.3 %.

LIMITATIONS

The test should not be used to detect phenylalanine in infants less than 48 hours old after birth, or those that have been transfused or been given pharmaceutical treatments, or in prematurely born infants. If the infant with PKU has not had a sufficient intake of protein prior to the test a false negative result may occur. A definite clinical diagnosis should not be based on the results of any single test.

REFERENCES:

ODAK Neonatal Phenylalanine Assay Rev 01
25.07.2011