Enzolve

Instructions for Use

Phenylketonuria (PKU) Screening Kit (400/1000 tests)

For the quantitative determination of L-phenylalanine in human newborn dried blood spot specimens

For In Vitro Diagnostic Use
For Professional Use Only

Σ **400** REF **5610-01**

Σ **1000** REF **5610-02**

IVD

2 °C 8 °C

CE 0050

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Intended use

The Enzolve Phenylketonuria (PKU) Screening Kit is an enzyme-based colorimetric test designed for the quantitative determination of the phenylalanine concentration in neonatal blood samples collected on Whatman 903® specimen collection paper.

The test is suitable for screening newborns for PKU/hyperphenylalaninaemia. Elevated results are not a diagnostic confirmation of PKU/hyperphenylalaninaemia, but indicate the urgency of further study of the newborns for these disorders.

Background

Phenylketonuria (PKU) is one of a range of hyperphenylalaninaemia hereditary disorders identified by neonatal dried blood spot mass screening and is the most common disease caused by a deficiency of an enzyme of amino acid metabolism[1]. The worldwide average incidence of PKU is 1:15,000 and varies from country to country depending on the ethnic background of the population[1]. It is a metabolic defect in which patients lack a sufficient amount of the enzyme phenylalanine hydroxylase (E.C. 1.14.16.1), which catalyses the conversion of phenylalanine to tyrosine. Increased concentrations of phenylalanine and its related metabolites in the blood and other body fluids of newborns interfere with proper development of the brain, resulting in progressive mental retardation[1-3]. This process can be prevented by early diagnosis and prompt dietary phenylalanine restriction, since delays have been correlated with increasing severity of retardation[1-3].

Apart from the enzymatic method, the three other screening methods currently used worldwide for the determination of blood phenylalanine level in newborns are: (1) the Guthrie bacterial inhibition assay (BIA)[4]; (2) the fluorescence method with the use of ninhydrin[5] and (3) tandem mass spectrometry (MS/MS)[6]. The first two methods suffer from interference from antibiotics, resulting in false negative and false positive data respectively for the BIA and fluorescence methods. Although the MS/MS method is fully automated, has high sensitivity and is free from any interference, it requires high-cost equipment and internal controls as well as highly trained personnel for its operation.

Enzolve's Phenylketonuria (PKU) Screening Kit is a cost-effective, enzyme-based colorimetric, endpoint test for quantitative determination of phenylalanine in neonatal dried blood spot (DBS) samples to be implemented in PKU mass screening programmes worldwide. It is not affected by other amino acids or commonly-occurring metabolites, or by most antibiotics, thus reducing the possibility of obtaining false positive or false negative results. It is rapid (approx. 10 min), very specific, has outstanding stability and is ready for automation.

Principle of the Assay

Enzolve's PKU Screening Kit uses an engineered recombinant microbial Phenylalanine Dehydrogenase (PheDH) (E.C. 1.4.1.20) to catalyse the oxidative deamination of blood phenylalanine with the use of NAD^+ as a cofactor[7-9].

$$\text{L-Phenylalanine} + NAD^+ \xrightarrow{\text{PheDH}} \text{Phenylpyruvate} + NADH + NH_4^+ \quad (1)$$

$$NADH + \text{Tetrazolium Dye} \xrightarrow{\text{Diaphorase}} NAD^+ + \text{Formazan} \quad (2)$$

The NADH formed is converted back to NAD^+ by the coupled Diaphorase/tetrazolium reaction system (2), which prevents product inhibition and pushes the equilibrium of the dehydrogenase reaction (1) to almost 100% completion[10,11]. The use of Enzolve's uniquely engineered Diaphorase has allowed creation of a simple, single-step, fast, accurate and sensitive colorimetric method for the determination of blood phenylalanine.

Kit Components

Component	Description	Quantity	REF	Quantity	REF
		400 tests	**5610-01**	**1,000 tests**	**5610-02**
Enzyme Reagent	Bacterial Recombinant Phenylalanine Dehydrogenase (1.9-2.1 U/ml*) and Diaphorase (3.8-4.2 U/ml*) in buffer with sodium azide as preservative	2 vials 2 ml/vial	5511-01	5 vials 2 ml/vial	5511-01
Enzyme Buffer	Buffer solution with detergent and sodium azide as preservative	1 vial 20 ml/vial	5512-01	1 vial 50 ml/vial	5512-02
Coenzyme Reagent	Dry powder of NAD^+, Tetrazolium salt, buffer and sodium azide as preservative	2 Vials 135 mg/vial	5513-01	5 vials 135 mg/vial	5513-01
Phenylalanine Standards and Controls	Human dry blood spots on Whatman 903® paper with four different phenylalanine concentrations (Range 1 – 20 mg/dL) as calibrators (S1, S2, S3, S4) and two controls with low (C1) and high (C2) phenylalanine levels within the range. Exact concentrations indicated on each card. Provided in ziplock plastic bag with desiccant. 8 spots per card.	1 set (6 cards per pack)	5516-01	1 set (6 cards per pack)	5516-01

** 1U = 1µmol substrate conversion per minute at 25°C, pH 10.5*
Kit contents to be stored at 2-8°C.

Additional Required Items

REF	**Description**
5600-07	Phenylalanine extraction solution (10x) 30% Trichloroacetic acid (TCA). Dilute 10-fold with deionised/distilled water before use
5600-02	Assay Plates (96 well, flat-bottom)
5600-01	Hole puncher (4.75 mm)
5600-06	Plate sealers
5600-03	Multi-channel pipettes
	Plate reader with 570/690 nm filters (dual wavelength) or 570 nm filter (single wavelength)

Vacuum Manifold Transfer Method also requires the following:

5600-05	Filter Plates (96 well, acrylic with 0.2 µm PVDF membrane)
5600-04	Vacuum manifold for 96-well plates
	Vacuum pump

Blood Sample Collection

Neonatal blood samples should be collected using Whatman 903® specimen collection paper following the NCCLS approved standard procedure LA4-A3[12]. The samples should be collected at least 24 hours after birth (ideally between 48 and 72 hours). For correct results the newborn should be on a proper diet that contains protein, such as breast-milk or formula[13,14].

Preparation of Reagents and Stability (Sufficient for 200 tests)

1. **Enzyme Reagent:** Into 1 vial of **Enzyme Reagent** (2 mL) add 9 mL of **Enzyme Buffer** and mix gently (total volume 11 mL). Diluted **Enzyme Reagent** is stable for 30 days at 2-8°C.
2. **Coenzyme Reagent:** Reconstitute 1 vial of **Coenzyme Reagent** with 33 mL of distilled/ deionised water and swirl gently. Do not shake vigorously. Reconstituted **Coenzyme Reagent** is stable for 30 days at 2-8°C.
3. **Working Solution:** Equilibrate diluted **Enzyme Reagent** and reconstituted **Coenzyme Reagent** to room temperature before use. Mix diluted **Enzyme Reagent** with reconstituted **Coenzyme Reagent** in the ratio of 1:3 (1 part of diluted **Enzyme Reagent** to 3 parts of **Coenzyme Reagent**). **Prepare the Working Solution freshly before use**. **Working Solution** is stable for a maximum of 8 hours at 2-8°C.

Assay Procedure

Elution of Phenylalanine from Dried Blood Spots and Sample Transfer

For manual sample transfer

1. Punch 4.75 mm (3/16") diameter discs from Blood Phenylalanine Standards S1-S4 and Controls C1-C2 provided with the kit into their respective wells in a 96-well microtitre plate

(duplicates are recommended). Punch patient specimens into the remaining individual wells and note their positions on the plate. Cover any unused wells with a plate sealer.

2. Pipette **70** µL of Phenylalanine extraction solution (3% TCA) into the wells and gently agitate the plate for 30-45 min at room temperature (18-25°C). Periodically examine the wells to ensure that the punched disks are completely immersed in the extraction solution.
3. After incubation, transfer **50** µL of the extracted material from each well into the corresponding wells of a new assay plate.

For sample transfer using vacuum manifold

1. Punch 4.75 mm (3/16") diameter discs from each of the Blood Spot Standards S1-S4 and Controls C1-C2 provided with the kit into their respective wells in a 96-well **filter** plate (duplicates are recommended). Punch patient specimens into the remaining individual wells and note their positions on the plate. Cover any unused wells with a plate sealer.
2. Pipette **60** µL of Phenylalanine extraction solution (3% TCA) into each well and gently agitate the plate for 30-45 min at room temperature. Periodically examine the wells to ensure that each disk is completely immersed in the extraction solution.
3. After incubation, transfer the extracted material from filter plate into a new 96-well assay plate applying vacuum for 2x10 seconds using a vacuum manifold. Please follow the manufacturer's instructions carefully for correct transfer of the samples.

Sample Assay

4. Pipette 200 µL of freshly prepared Working Solution into each well of the assay plate, making sure that **no bubbles are created,** and incubate the plate for 10-12 min at room temperature (18-25°C).
5. Read the absorbance of the plate at 570 and 690 nm (dual wavelength) or 570 nm only (single wavelength) immediately after incubation.

Calculations

Most modern plate readers come with software which calculates the results automatically using linear regression once given the concentrations of the Standards. However, for manual calculations, a Standard Curve should be constructed by plotting the absorption at 570 nm minus the absorption at 690 nm (or 570 nm only) of the Standards S1-S4 on the Y-axis against the phenylalanine concentration of the Standards on the X-axis. The best-fit straight line is drawn through these points to give the **Standard Curve**. The phenylalanine concentrations of the Controls (C1 and C2) and patient samples can now be read directly off this Standard Curve.

Note that all concentrations are given in mg/dL units. To convert it into µmol/dL a conversion factor 60.5 should be used (1 mg/dL = 60.5 µmol/dL).

A typical Standard Curve for the Enzolve Phenylketonuria (PKU) Screening kit is shown below (Figure 1). Please note that this graph is for illustration purposes only. Users must construct their own Standard Curve each time the assay is run.

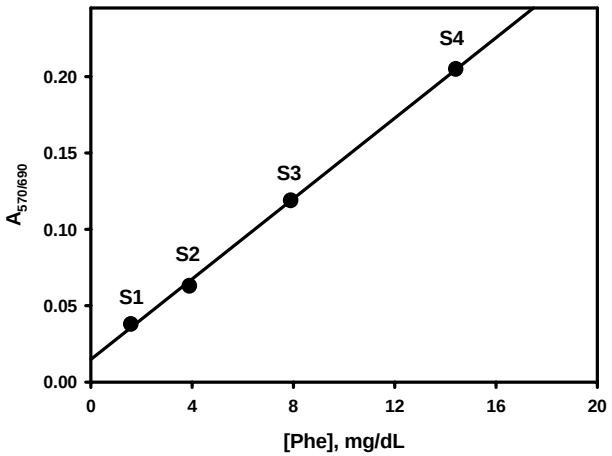

Figure 1. A typical Standard Curve for Enzolve Phenylketonuria (PKU) Screening Kit

Quality Control/Traceability

Dried Blood Spot standards and controls should be run on every plate. Internal controls in the low (C1) and high (C2) range of phenylalanine concentrations are included in the kit. These controls should be included in each assay in order to monitor the performance and reliability of the assay. Similarly, external reference controls containing phenylalanine at different levels should be routinely included.

The results of the assay are valid if the concentrations for each control are within the range quoted on the control cards. The assay results are unacceptable if **either** of these values fall out of specification. In the latter case the patient sample results should **not** be reported.

In those patient samples where the phenylalanine values are below the acceptable detection limit of the assay, a repeat test should be conducted to exclude mis-sampling or procedural errors.

Blood phenylalanine standards and controls included in Enzolve Phenylketonuria (PKU) Screening Kits are traceable to the Second ISNS Reference Preparation for Neonatal Screening (2nd ISNS-RPNS).

Expected Value Range

A total of 450 Dried Blood Spot samples were assayed using Enzolve's Phenylketonuria (PKU) Screening Kit. The actual values for phenylalanine concentrations were all less than 2.1 mg/dL and followed a Gaussian distribution with a mean of 0.69 mg/dL and a standard deviation of 0.33 mg/dL, and are within the range of values reported in the literature[16,17].

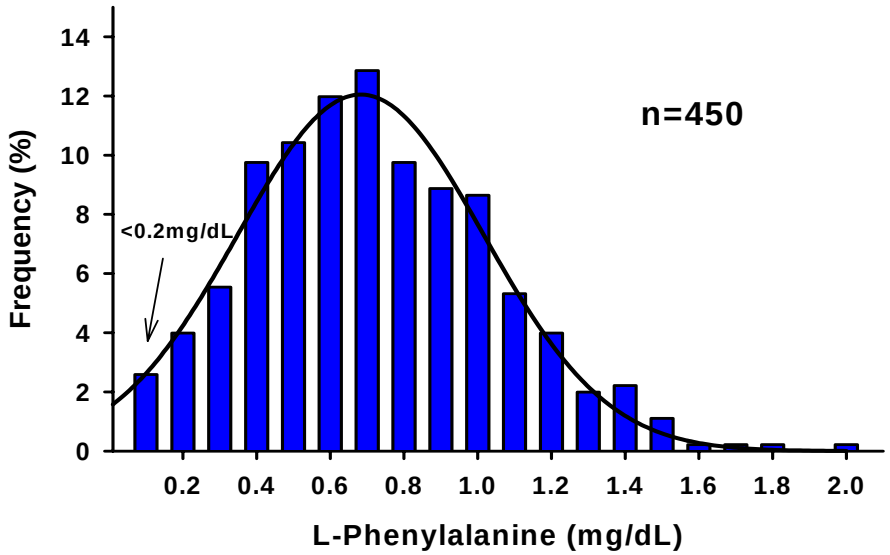

Figure 2. Distribution of phenylalanine level in blood of healthy population with its Gaussian fit

Data in the Table below represent the statistically derived cut-off values for phenylalanine levels obtained using Enzolve Phenylketonuria (PKU) Screening Kit at the 95th , 99th and 99.9th percentile.

Percentile	Cut-off values (mg/dL)
95	1.34
99	1.54
99.9	1.78

It is recommended that each laboratory establish its own normal range and statistical cut-off value.

Linearity and Sensitivity

Enzolve PKU Screening Assay is linear up to 20 mg/dL blood phenylalanine concentration assessed according to NCCLS Proposed Guideline EP6-P[15]. Following the same guideline the minimum detectable phenylalanine level was estimated, and found to be 0.2 mg/dL.

Data Comparison

Dried blood samples from patients were analysed in parallel using Enzolve Phenylketonuria (PKU) Screening Kit, another commercial enzymatic assay and the gold standard Tandem Mass spectrometry method (MS/MS). Samples for this study were selected from a normal healthy population as well as from patients already diagnosed with PKU or other mental disorders (non-PKU).
The values obtained with the Enzolve kit are slightly lower than the values obtained by the MS/MS method, while the values obtained by the competitor's product are 20% higher than the values obtained by MS/MS and with a poorer correlation coefficient (Figure 3).

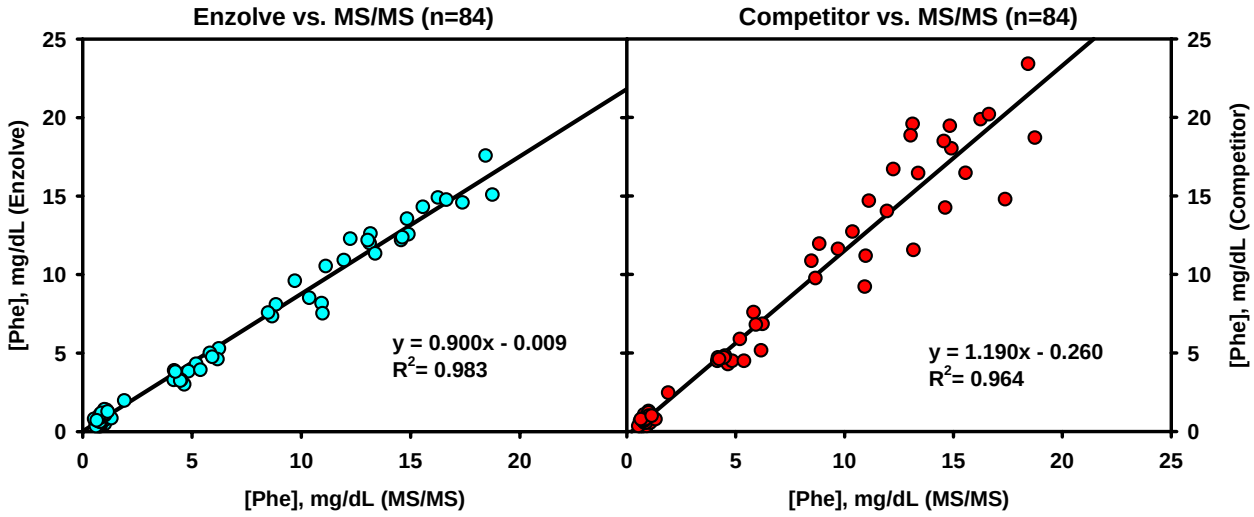

Figure 3. Comparison of the blood phenylalanine concentrations determined by two enzymatic methods (Enzolve and competitor product) with the results obtained by tandem mass spectrometry

Specificity and Interfering Substances

Commonly occurring amino acids, antibiotics, and metabolites were used to assess the specificity of Enzolve Phenylketonuria (PKU) Screening Assay. Tetracycline was found to be the only potential interfering substance. No other interference was observed in the presence of different common antibiotics and metabolites which could lead to a false positive or negative outcome.

Assay Precision

The Inter- and Intra-assay precision of the Enzolve method was calculated by evaluating the same samples in multiple assay runs (Inter-assay) or by assaying N replicates of the same samples (Intra-assay).

Type of Study	Results		
	Concentration Range (mg/dL)	CV (%)	N
Inter-Assay Variation	3.55 – 16.54	5.2 – 10.2	40
Intra-Assay Variation	3.85 – 15.43	5.3 – 11.5	20

Precautions and Comments

All solutions contain 0.095% sodium azide as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metallic compounds. When disposing of reagents containing azide, flush with a large quantity of water to prevent azide build-up.

The sample extraction solution contains 3% trichloroacetic acid (TCA) which is corrosive. Appropriate safety precautions should be taken when working with this solution.

Dried Blood Spot Standards and Controls are prepared using human blood samples which are tested and found to be negative for the presence of antibodies to HIV 1 and 2, Hepatitis B surface antigen

and Hepatitis C. However, all blood samples of human origin should be considered as a potential hazard and appropriate precautions should be taken with their handling and disposal.

Blood collected prior to 24 hr may lead to a false-negative or false-positive result.

Elevated results should be further investigated by other methods.

It is recommended that each laboratory establish its own normal range and statistical cut-off value.

Enzolve Phenylketonuria (PKU) Screening Kit is designed for use by appropriately trained and qualified laboratory personnel. It is not for self-testing or for over-the-counter sale.

It is recommended that all users become familiar with the use of the kit prior the reporting of results.

It is recommended that results are interpreted by a trained professional and other clinical information should be considered prior to any clinical intervention.

It is recommended that all standards, controls and samples should be run in duplicate until the laboratory personnel become proficient with the test procedure. It is compulsory:

a. To run Standards and Controls on every plate.
b. To keep all reagents (stock or diluted/resuspended, DBS Standards and Controls) at 2-8°C in closed containers and to equilibrate them to room temperature before use.
c. To strictly follow the protocol to obtain reliable results. Any modifications made to the reagents or assay procedure are the responsibility of the user.
d. To properly maintain and calibrate equipment used for the assay. All equipment used should be CE marked.
e. Not to use the kit or its components after the expiry date indicated on the labels.
f. Not to use diluted/reconstituted solutions after recommended stability periods or if they become turbid or discoloured.
g. Not to use any components that have been obviously broken or that are visually contaminated.
h. Not to reuse assay plates in order to reduce the potential for false-positive results.

References

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In Vitro Diagnostic Directive (IVDD 98/79/EC) Symbols

CE 0050 - European Conformity

REF - Catalogue Number

LOT - Lot Number

 - Consult Instructions for Use

IVD - For In Vitro Diagnostic Use

 - Manufacturer

 - Use by

 - Sufficient for <n> tests

 - Temperature Limit

ENZYM | REAG - Enzyme Reagent

ENZYM | BUFF - Enzyme Buffer

COENZ | REAG - Coenzyme Reagent

STD | DBS - Dried Blood Spot Standard

CONTROL | DBS - Dried Blood Spot Control