

Neonatal Phenylketonuria (PKU) Screening Assay



Enzymatic assay for the quantitative determination of Phenylalanine levels in new born dried blood spots (FOR IN VITRO DIAGNOSTIC USE ONLY)

1. INTENDED USE

Born Safe™ Neonatal PKU Screening Assay is an enzymatic assay for the quantitative determination of phenylalanine concentrations in neonates using blood spot samples dried on Whatman S&S 903 filter paper. This kit is particularly suitable for use in a neonatal screening program to measure Phenylalanine concentrations as an aid in identifying Phenylketonuria in new-borns. Elevated results are not diagnostic per se of phenylketonuria, but indicate the urgent need for further study of the new born from which a presumptive positive sample was received.

The kit is not intended for use in monitoring the circulating phenylalanine concentrations of phenylketonuric patients for the purpose of assessing dietary control, nor for confirmatory testing.

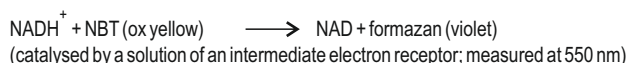
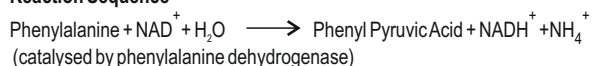
2. SUMMARY AND EXPLANATION OF THE ASSAY

Phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH) activity.¹ PKU is characterized by an inability of the body to utilize the essential amino acid, phenylalanine. Patients with this disorder have insufficient hepatic phenylalanine hydroxylase, an enzyme that acts as a catalyst in the conversion of phenylalanine to tyrosine.² As a result, phenylalanine and its toxic metabolites accumulate in the blood, brain and in urine; eventually causing a variety of symptoms - the major one being mental retardation if left untreated.³ Once diagnosed, treatment with a special diet that restricts dietary Phenylalanine should be initiated promptly. Delays in treatment have been correlated with increase in the severity of the retardation.^{7,8} The overall average incidence of PKU is 1:10,000 (range 1:2600 to 1:25000) depending on the ethnic background of the population.⁴ Limited data suggests that the incidence of PKU and non-PKU hyperphenylalaninaemia in non-Caucasians is 5-fold less than in Caucasians. In general screening practice a dried blood spot specimen with a phenylalanine concentration in excess of 4.0 mg/dL is classified as 'presumptive positive'. This definition of presumptive positive varies. Classical PKU is characterised as a circulating phenylalanine concentration of greater than 20 mg/dL with a tyrosine concentration of less than 2 mg/dL,⁵ assuming an adequate dietary load i.e. breast or formula feeding for 48 hours prior to sampling. Non-PKU hyperphenylalaninaemia, classified as circulating phenylalanine concentrations greater than 2 mg/dL but less than 8 mg/dL⁶ or greater than 10mg/dL but less than 20 mg/dL,⁵ requires repeat testing and follow-up to ascertain whether the raised phenylalanine concentration is 'benign' due to immaturity of the clearance system, or 'malignant' which is unresponsive to dietary restriction. Its rate of incidence is approximately one in 10 000 newborns in the United States.

3. PRINCIPLE OF THE ASSAY

The Phenylalanine from cellulose paper (dried blood spot samples) is extracted with trichloroacetic acid (Elution buffer). After extraction, the eluted sample is combined with the enzyme reagent Phenylalanine dehydrogenase. This enzyme reagent catalyses the NAD-dependent oxidative deamination of Phenylalanine to phenylpyruvate and ammonia. The NADH produced, reacts with a colour reagent in which a tetrazolium salt gets reduced producing a distinct colour. This colour can be measured colorimetrically with a photometer at 550 nm and is directly proportional to the concentration of Phenylalanine present in the sample.

Reaction Sequence



4. PRESENTATION

REF	Pack Size
1122010096	96 Assays

5. KIT COMPONENTS:

Reagents: (96 T Pack size)

- Calibrators and Controls blood spots:** 1 + 1 sets of blood spots cards of human whole blood spotted onto Whatman S&S 903 paper containing 5 calibrators and 2 controls. Refer to the quality control sheet for the exact concentrations of the Calibrators and acceptable range values of the Controls.
- Elution Buffer:** 1 x 10.0 ml of TCA 3% w/v. Ready to use.
- Enzyme Reagent:** 4 x 1.0 ml of Phenylalanine dehydrogenase lyophilized with buffer and a stabilizer. Reconstitute each vial with 1.0 ml of distilled water. After reconstitution, the reagent can be stored at 2-8°C for one month.
- Coenzyme Reagent:** 4 x 1.0 ml of Lyophilized NAD. Reconstitute each vial with 1.0 ml of distilled water. After reconstitution, the reagent can be stored at 2-8°C for one month.
- Colour Reagent:** 1 x 8.0 ml of tetrazolium salt. Ready to use.

Preservative: NaN_3 (< 0.1%).

- Colour Booster:** 1 x 1.0 ml of a solution of an intermediate electron receptor in buffer. Ready to use. Preservative NaN_3 (< 0.1%).
- Dilution Buffer:** 1 x 2.0 ml of buffer. Ready to use. Preservative NaN_3 (< 0.1%).

Reagents	Quantity	Physical State
Calibrator and Controls	1 set each	Dried blood spots
Elution Buffer	1 x 10.0 ml	Ready to use
Enzyme Reagent	4 x 1.0 ml	Lyophilized
Coenzyme Reagent	4 x 1.0 ml	Lyophilized
Colour Reagent	1 x 8.0 ml	Ready to use
Colour Booster	1 x 1.0 ml	Ready to use
Dilution Buffer	1 x 2.0 ml	Ready to use

Accessories

- Round bottom microtiter plates** (Elution Plates).
- Flat-bottom microtiter plates** with superior optical quality (Assay Plates).

6. STORAGE AND STABILITY OF THE KIT

- Store all reagents at 2-8°C when not in use. Calibrators and Controls should be stored protected from moisture and light in the original bag with desiccant. Stable at 2-8°C until expiry date stated on the label. Make sure that the plastic bag remains sealed during storage.
- We recommend that the Blood Spots (Calibrators and Controls) should be preferably stored at -20°C with desiccants when not in use for prolonged periods.**
- Unopened reagents will retain reactivity until expiration date shown on the label. Do not use reagents beyond this date.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or de-ionized water.
- Adjustable, automatic micropipettes with disposable tips.
- Microtiter plate reader equipped with 550 nm filter in endpoint reading mode.
To be Procured Separately
- Blood spot puncher 3.2 mm.
- Orbital plate shaker (900 rpm).
- Blood spots collection cards [Whatman Schleicher & Schuell 903 recommended; CLSI NBS01-A6 compliant].

8. WARNINGS AND PRECAUTIONS

A thorough understanding of the pack insert is mandatory before performing the test for the first time. Adherence to the protocol specified herein is necessary to ensure optimal performance of the product. Any deviation from the assay procedure may affect the results.

Operating: In order to obtain reproducible results, the following rules must be observed:

- Do not mix reagents of different lots.
- Do not use reagents beyond their expiry date.
- Use thoroughly clean labware.
- Use distilled water, stored in clean containers.
- Avoid any contamination among samples; for this purpose, disposable tips should be used for each sample and reagent.
- Keep all reagents at normal refrigerator temperature (2-8°C) in closed containers when not in use, but ensure that all reagents are equilibrated to 18-25°C before use. Keep Blood Spot Standards and Controls at normal refrigerator temperature (2-8°C) in the original foil pouch containing desiccant when not in use, but ensure that spots are equilibrated to 18-25°C before use.

Safety: In order to avoid personal and environmental contamination, the following precautions must be observed:

- Use disposable gloves while handling potentially infectious material and performing the assay.
- Do not pipette reagents by mouth.
- Do not smoke, eat, drink or apply cosmetics during the assay.
- All material of human origin used for the preparation of this kit tested negative for HBsAg, anti-HIV and anti-HCV. Since no test at present can guarantee complete absence of these viruses, all samples and reagents used for the assay must be considered potentially infectious. Therefore, the assay waste must be decontaminated and disposed of, in accordance with established safety procedures.
- Disposable ignitable material must be incinerated; disposable non-ignitable material must be sterilized in autoclave for at least 1 hour at 121°C. Liquid wastes must be added with sodium hypochlorite at a final concentration of 3%. Let the hypochlorite act for at least 30 minutes. Liquid wastes containing acid must be neutralized with appropriate amounts of base, before treating with sodium hypochlorite.
- Avoid splashing and aerosol formation; in case of spilling, wash carefully with a 3% sodium hypochlorite solution and dispose of this cleaning liquid as potentially

infectious waste.

- Some reagents contain sodium azide as preservative; to prevent build-up of explosive metal azides in lead and copper plumbing, reagents should be discarded by flushing the drain with large amounts of water.
- Caution:** Elution Buffer containing trichloroacetic acid (TCA), is highly acidic and corrosive. Protective gloves and safety glasses should be worn while using this reagent.

9. SPECIMEN COLLECTION AND HANDLING

Blood samples should ideally be collected between the third and fifth days of life (48 to 120 hours after birth) and should be taken directly from a heel prick onto filter paper. Bilirubin (≤ 40 mg/dL; ≤ 0.684 mmol/L), ascorbic acid (≤ 3 mg/dL; ≤ 0.17 mmol/L), and D-glucose (≤ 800 mg/dL; ≤ 44 mmol/L) do not interfere. Haemoglobin concentrations ≥ 150 g/L to ≤ 200 g/L do not interfere with the assay.

Neonatal screening programs differ from one another in the type of specimen required, the recommendation is a blood spot, approximately 12.7 mm ($\frac{1}{2}$ inch) in diameter, collected by heel prick and spotted onto filter paper (Whatman Schleicher & Schuell 903). The specimen collection device must comply with national regulations. A method based on dried blood samples requires skillful collecting, handling and transport of samples. The collection technique is described in detail in CLSI document LA4-A5,¹⁰ and the main points are listed below.

- Blood from the new-born's heel should be collected **ONLY** from the medial (closest to the body center-line) or lateral portion (furthest from the body center-line) of the planter surface (walking surface).
- Blood collection from other areas of the infant's foot, e.g. arch, may result in nerve, tendon or cartilage injury.
- Clean the skin with an alcohol swab and allow to air-dry.
- Puncture the infant's heel with a sterile lancet or with a heel incision device to the depth of approximately 2.0 mm. Puncturing deeper than 2.0 mm on small infants may cause bone damage.
- Wipe away the first drop of blood. Gently touch the filter paper against a large drop of blood and, in one step, allow a sufficient quantity of blood to soak through to completely fill a pre-printed circle on the filter paper. Examine both sides of the filter paper to make sure that the blood penetrated and saturated the paper. Excessive milking or squeezing the puncture may cause haemolysis of the specimen or an admixture of tissue fluids with the specimen. Do not layer successive drops of blood in the collection circle (this causes caking).
- Allow the blood specimen to air-dry in a horizontal position for at least 4 hours at ambient temperature (18-25°C). Do not heat or stack the specimens during the drying process.
- Arrange transport of the collection card to the screening laboratory within 24 hours of collection.
- Store in sealed paper envelopes or containers that will provide protection from moisture, light, heat and contact with other materials.
- Phenylalanine in dried blood spots is stable at 2-8°C without loss of phenylalanine. Storage at -20°C under desiccated conditions further improves the stability for prolonged period.^{11,12,13,14,15,16}
- The sample discs should be punched from similar areas on each individual blood spot. Do not punch sample discs from areas that include printed marks or that are near the edges of the blood spot.
- Be sure that the required information on the specimen collection card has been completed. The minimum pre-printed information required on the collection device includes:
 - last name (and first, if available), sex, birth date (optional: time of birth), birth weight and age of the infant; (indicate if < 24 h), and patient identification number
 - the first and last name of the mother
 - date of specimen collection (optional: time of collection)
 - the name and address of the submitter (optional: birth facility)
 - the name and phone number of the physician (health care provider)
 - the name of the new born screening program and address
 - each card should have a unique serial number and an expiration date.
- Specimens should not be placed in hermetically sealed containers (e.g. plastic or foil bags). If required, sufficient desiccant packages must be included. Humidity and moisture are detrimental to the dried blood spot specimen.
- Before placing the specimens in a container for transport, the dried blood spots on the collection cards should be separated by a physical barrier from the blood spots on the cards in the stack immediately above and below. The blood spots can also be protected by a fold-over cover attachment or by placing glassine paper between the specimens.

Note: Transport the specimen to the laboratory within 24 hours after collection.

10. REAGENTS PREPARATION

ENZYME-COENZYME SOLUTION

- Reconstitution:** First reconstitute, one Enzyme vial and one Coenzyme vial with 1.0 ml of distilled or de-ionized water each. Swirl gently to reconstitute the Reagents. DO NOT shake the Enzyme Reagent vigorously, as this may cause loss of enzyme activity and thus poor performance. After reconstitution, the reagent can be stored at 2-8°C for one month.
- Preparation:** Mix 2 parts of Enzyme with 2 parts of Coenzyme and 1 part of

dilution buffer. The following table gives the volumes required for each of the three components to run specific number of tests (volumes in μ l).

No. of tests	Enzyme (μ l)	Co-enzyme Reagent (μ l)	Dilution Buffer (μ l)	Enzyme- Co-enzyme solution. Total vol. (μ l)
10	400	400	200	1000
20	800	800	400	2000
40	1600	1600	800	4000
80	3200	3200	1600	8000
100	4000	4000	2000	10000

Do not keep or use the reconstituted Enzyme, Coenzyme, or the combined Enzyme-Coenzyme working solution for any longer than the specified periods of time.

COLOR REAGENT MIXTURE

Prepare the mixture by adding 1 part of Colour Booster to 10 parts of Colour reagent.

No of tests	Colour booster (μ l)	Colour Reagent (μ l)	Colour reagent mixture Total volume (μ l)
10	80	800	880
20	160	1600	1760
40	320	3200	3520
80	640	6400	7040
100	800	8000	8800

After reconstitution keep the Colour Reagent mixture away from the direct light (i.e. wrapped in aluminium foil); stable for 4 hours at 2-8°C. Not to be left out of the refrigerator longer than needed. Take the colour reagent out of the refrigerator just prior to use. Take out just the quantity you are going to use for the day. Return the rest of the colour reagent in the refrigerator.

11. ASSAY PROCEDURE

A. ELUTION STEP:

- Bring all reagents (except the colour reagent) to room temperature before pipetting.
- Punch 2 spots of C0 for blank and 2 blood spots of **Calibrators (C1-C5), Controls (L1, L2) and Samples** (each 3.2 mm diameter) Put 2 discs into the respective wells of the round bottom microtiter plate.
- Pipette **100 μ l** of **Elution Buffer** into each well. Ensure that each disk is fully immersed in the liquid.
- Incubate the microtiter plate on an orbital plate shaker for **30 minutes** at room temperature (**20-26°C**).
- During elution, reconstitute and prepare the reagents (section 10), and a flat bottom microtiter plate.

B. SAMPLE TRANSFER AND ASSAY:

- After the incubation, remove the plate from the plate shaker and transfer **40 μ l** of the eluate from each well to the corresponding wells of the flat bottom microtiter plate.
- Pipette **100 μ l** of the **Enzyme-Coenzyme** solution prepared in section 10 to each well. Mix well, avoiding the formation of foam.
- Incubate **30 minutes** at room temperature (**20-26°C**).
- Add **80 μ l** of **Colour Reagent** mixture prepared in section 10 to each well. Mix well to avoid the formation of foam.
- After **10 minutes** of incubation at room temperature keeping the plate away from light, measure the microplate at 550-570 nm (optimal: 550 nm), endpoint mode, single measurement. There is no need to wait longer than 10 minutes.

Please note the following:

- This assay is to be performed at room temperature (20-26°C). At higher temperatures (over 28°C) an abnormally high blank may be observed.
- A high blank may also be observed if the colour reagent stage is prolonged more than 20 minutes.

12. CALCULATION OF RESULTS

Draw a calibration curve, by plotting the calibrators concentration (x-axis) against the absorbance OD obtained for each calibrator (y-axis). The obtained OD of the standards are plotted against their concentration. The standard curve is calculated by a linear regression function. Using computer programs, the curve is best described by a 2-point linear regression fit with linear axes. Corresponding Phenylalanine concentrations in mg/dL are obtained by interpolating the absorbances of each sample on the calibration curve.

13. QUALITY CONTROL

The assay run is acceptable if the concentrations for each control are within the ranges quoted on the controls package. The assay is unacceptable if the measured values for either of the kit controls fall out of specification. If the assay is unacceptable, patient specimen results should not be reported. These controls provide valuable information regarding the validity of the test according to manufacturer. Users may wish to include further in-house controls and/or reference materials if available. The stability and

storage conditions of these additional controls and the criteria for assay acceptance/rejection should be determined by each laboratory. The participation in National External Quality Assurance Schemes is strongly recommended. As part of good QA practice, it is recommended that repeat testing be considered periodically where patient results register less than the detection limit of the assay in case these represent mis-sampling or procedural errors.¹⁷

14. EXPECTED VALUES AND INTERPRETATION CRITERIA

The determination of presumptive positives for phenylketonuria is based on the use of a cut-off value, which distinguishes between presumptive negative and presumptive positive results. An equivocal zone is used to reflect the imprecision of the assay. Test results may vary based on infant age at the time the blood is drawn¹³ as well as other conditions. Caution must be exercised in correlating the laboratory result to clinical status with specimens from new born less than 48 hours after birth, premature and low birth weight newborns and hospitalised sick newborns. The L-phenylalanine concentration measured in blood spots from these classes of new born may be unrelated to the true clinical status of the new born. Guidelines as to the procedure for collection of specimens from newborns of various classes are available. However, it is recommended that each laboratory determine the procedure most appropriate for its working practice. Please note that the values given in this section should be used only as a guideline and it is recommended that each laboratory should establish its own reference range and statistical cut off value based on their local demographic population (e.g. specimens from laboratory routine population). Confirmation by a diagnostic test procedure should be performed to confirm the diagnosis of PKU. A review of various published NBS programs study outcomes and referring to the CDC-Newborn Screening Quality Assurance Program (NSQAP) summarized PT reported cut off values, following guidelines are suggested.

Suggested Cut off Values

Interpretation	Concentration (mg/dl)
Presumptive Negative	2.1 mg/dl
Equivocal	2.1- 3.0 mg/dl
Presumptive Positive	>3.0 mg/dl

15. LIMITATIONS

Born Safe™ Neonatal PKU Screening Assay is a tool to screen neonates for elevated levels of phenylalanine. It is important to confirm the diagnosis of phenylketonuria by a follow-up plasma sample analysed using a different method. Therapeutic decisions cannot be decided based on the results themselves. Interpretation of results from the specimen must be cautiously interpreted due to L-phenylalanine concentrations not always correlated to true clinical status. In certain circumstances the concentration of L-phenylalanine measured in a dried blood spot specimen may be unrelated to the clinical status of the newborn and may result in 'false negative' or 'false positive' results. An increased incidence of false negative results may be expected when the sample is collected less than 72 hours after birth or the newborn was not receiving an adequate protein-containing diet for at least 24 hours prior to sampling. An increased incidence of false positives may occur in heterozygotes due to reduced clearance capacity, in newborns of phenylketonuric mothers due to the increased maternal L-phenylalanine load or in premature newborns as a result of the immaturity of the L-phenylalanine clearance systems. In all scenarios outlined above, if a false negative or false positive result is suspected, a repeat analysis of another disc from the same card must be performed immediately. If the result is again equivocal in any respect, a repeat specimen must be obtained or, if the newborn has been discharged from the place of birth, arrangements should be made to obtain a fresh specimen as soon as possible so that the true clinical status of the newborn can be ascertained.

Conditions which are known to cause anomalous analytical assay results are:

- sample spot not uniformly saturated with blood.
- sample spots punched too close to the edge of the blood spot.
- poorly collected and improperly dried specimens.
- non-eluting blood spot due to deterioration of sample caused by exposure to heat and humidity.
- contamination of blood spot filter paper with faecal material.

Nutritional status that may affect the results:

- blood samples obtained from infants who have not ingested sufficient protein may produce false negative results.
- samples obtained from infants who are on total parenteral nutrition may produce false positive results.

Note: Samples collected from patients who have transient or hereditary tyrosinemia may have elevated values for phenylalanine. Variables such as haematocrit, prematurity and age of the infant may affect the interpretation of phenylalanine values produced.

16. COMPLAINTS

Complaints can be accepted in written format (preferably on the manufacturer's complaint form). All details of the test kit, as well as the test results, can be included. A copy of the complaint form is available from Tulip Diagnostics Pvt Ltd. upon request.

17. REFERENCES

1. Chang, P.L., Lewis, S., Heathcote, J. and Whelan, D.T. (1991): Molecular diagnosis of phenylketonuria. *JIFCC* **3** (2), 58–65.

2. Koch, R. and de la Cruz, F. (1991): The danger of birth defects in the children of women with phenylketonuria. *J NIH Research* **3**, 61–63.
3. Okano, Y., Eisensmith, R.C., Guttler, F. et al. (1991): Molecular basis of phenotypic heterogeneity in phenylketonuria. *New Engl J Med* **7**, 1232–1237.
4. Scriver C R, Kaufman S, Eisensmith R C, Woo S L C. (1995) *in: The Metabolic Basis of Inherited Diseases I* (Scriver C R, Beaudet A L, Sly W S, Valle D. eds.) Seventh edition. McGraw Hill 1015.
5. Kirkman H N, Carroll C L, Moore E G, Matheson M S. (1982) *Am. J. Hum. Genet.* **34** 743. Cauca
6. Mabry C C. (1990) *Ann. Clin. Lab. Sci.* **20** 392.
7. Waisbren S E, Mahon B E, Schnell R R, Levy H L. (1987) *Pediatrics* **79** 351.
8. Rylance G. (1989) *Postgrad. Med. J.* **65** (Suppl 2) S7.
9. Smith I, Beasley M G, Ades A E. (1990) *Arch. Dis. Childhood* **65** 472.
10. Clinical and Laboratory Standards Institute (2007): Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard – Fifth Edition; CLSI Document LA4-A5. CLSI, Wayne, Pennsylvania 19087-1898, USA.
11. Madira, W.M., Xavier, F., Stern, J., Wilcox, A.H., and Barron, J.L. (1992): Determination and assessment of the stability of phenylalanine and tyrosine in blood spots by HPLC. *Clin. Chem.* **38**, 2162–2163.
12. Lundsjö, A., Hagelberg, S., Palmér, K., and Lindblad, B.S. (1990): Amino acid profiles by HPLC after filter paper sampling: 'appropriate technology' for monitoring of nutritional status. *Clin. Chim. Acta* **191**, 201–210.
13. McCabe, E.B.B., McCabe, L., Mosher, G. and Allen, R.J. (1983): Newborn screening for phenylketonuria: Predictive validity as a function of age. *Pediatr.* **72**, 390–398.
14. Searle, B., Mijuskovic, M.B., Widelock, D., Davidow, B. (1967): A manual Colorimetric paper disc method for detecting phenylketonuria. *Clin. Chem.* **13**, 621–625.
15. Spierto, F.W., Hearn, T.L., Gardner, F.H., and Hannon, W.H. (1985): Phenylalanine analyses of blood-spot control materials: Preparation of samples and evaluation of interlaboratory performance. *Clin. Chem.* **31** (2), 235–238. 24 13905231-11 (en)
16. Chase, D.H., Adam, B.W. Smith, S.J., Alexander, J.R., Hillman, S.L., and Hannon, W.H. (1999): Validation of accuracy-based amino acid reference materials in dried blood spots by tandem mass spectrometry for newborn screening assays. *Clin. Chem.* **45**, 1269–1277.
17. Westgard, J.O. et al. (1981): A multi-rule chart for quality control. *Clin. Chem.* **27**, 493–501.

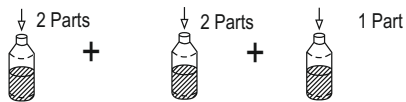
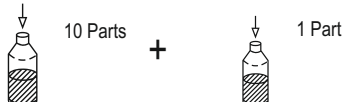
Summary Protocol

BornSafeTM

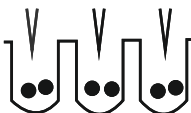
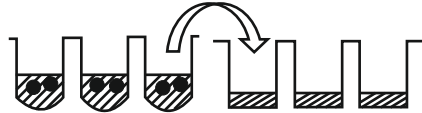
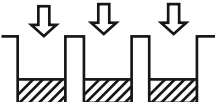
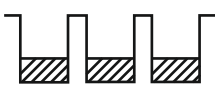
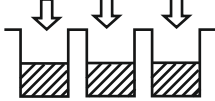
PKU



REAGENT PREPARATION

Reconstitution of Enzyme vial		Each vial with 1 ml distilled /deionized water
Reconstitution of Co-Enzyme vial		Each vial with 1 ml distilled /deionized water
Enzyme- Co-Enzyme solution		2 Parts Enzymes + 2 Parts Co-Enzyme + 1-Part Dilution Buffer
Colour Reagent Mixture		10 Parts Colour Reagent + 1-Part Colour Booster

ASSAY PROCEDURE

1. Punch out Calibrators, Controls and unknown in 'U' bottom microtiter plate		2 blood spots into each wells of round bottom microtiter plate
2. Add Elution Buffer		Add 100µl. Ensure that each disk is fully immersed in Elution Buffer
3. Incubate		30 min at RT (20°-26°C) on an orbital plate shaker (900rpm)
4. Transfer to corresponding well of flat bottom microtiter plate		Add 40µl of the eluate from each well
5. Add Enzyme-Co-Enzyme solution		Add 100 µl. Gently mix, avoiding the formation of foam
6. Incubate		30 min at RT (20°-26°C)
7. Add Colour Reagent Mixture		Add 80 µl. Gently mix, avoiding the formation of foam
8. Incubate		10 min at RT, away from light
9. Read/Measure		Place the plate in a microplate reader and read at 550nm

SYMBOL KEYS

	Temperature Limitation		Consult Instructions for use		Date of Manufacture		Batch Number / Lot Number
	Manufacturer		In vitro Diagnostic Medical Device		This side up		Caution
	Use by		Catalogue Number		Contains sufficient for <n> tests		

Manufactured by:

Coral Clinical Systems
A Division of Tulip Diagnostics (P) Ltd.

Bldg. 'D', Plot No. M-46, Phase III B, Verna Industrial Estate, Verna, Goa - 403 722, INDIA.
Regd. Office: Gitanjali, Tulip Block, Dr. Antonio Do Rego Bagh, Alto Santacruz, Bambolim Complex P.O., Goa - 403 202, INDIA.