DRG® Phenylalanine (PKU) Screening Assay

- **(ENZ-4502 = 480 tests)**
- **(ENZ-4743 = 2,400 tests)**

Revised 26 July 2011 rm (Vers. 10.1) **USA: RUO**

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

1 INTRODUCTION

1.1 Intended Use

The **DRG Phenylalanine (PKU) neonatal Screening Assay Kit** is an enzymatic Assay for measurement of L-Phenylalanine in human newborn blood spots. For neonatal screening on Phenylketonuria.

2 PRINCIPLE OF THE TEST

The Phenylalanine of the blood spots is eluated using Trichloracetic acid (3 %) from the cellulose paper.
After that the Phenylalanine is transformed by the enzyme Phenylalaninedehydrogenase to Phenylpyruvate. This reaction is coupled to the reduction of the coenzyme NAD+, present in the reaction mixture. The reduced NADH transforms in a redox reaction the added Tetrazolium salt to the yellow substrate Formazane.
The amount of Formazane developed is proportional to the concentration of Phenylalanine in the sample. The color of the substrate can be measured with a photometer at 450 nm.

3 WARNING AND PRECAUTIONS

- All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
- Allow the reagents to reach room temperature (21-26°C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the samples will not be affected.
- Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
- Do not use reagents beyond expiry date as shown on the kit labels.
- All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
- Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
- Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guideline or regulation.
- For information on hazardous substances included in the kit please refer to Material Safety Data Sheets. Material Safety Data Sheets for this product are available upon request directly from DRG.

4 REAGENTS

The kit components are suitable to run 480 tests (ENZ-4502) or 2400 tests (ENZ-4743).

4.1 Reagents provided

4.1.1 Reagents provided for ENZ-4502 (5 x 96)

1. ***Standard A – E***, 5 cards, 1 card per standard
   Concentrations: ~1; ~3; ~6; ~12; ~16 mg/dL
   Conversion: 1 mg/dL = 60.5 µmol/L
   Calibrated against 1st ISNS-RPNS for TSH, phenylalanine and 17α-hydroxyprogesterone).
   Contains human blood.
   Schleicher & Schuell paper No. 903. 12 Blood Spots / card.
   Exact concentrations see vial labels or QC Datasheet.
2. ***Control low and high***, 2 cards
   Control 1: ~ 3,0 mg/dL, Control 2: ~ 9,0 mg/dL
   Contains human blood.
   Schleicher & Schuell paper No. 903. 12 Blood Spots / card.
   Exact Concentrations / ***acceptable ranges see QC Datasheet or vial labels.***
3. ***Enzyme Reagent***, 5 vials, 5 x 0.5 mL, lyophilized.
   Contains phenylalanine dehydrogenase, Buffer, stabilizers.
   See „Preparation of Reagents“.
4. ***Coenzyme Reagent***, 5 vials, 5 x 0.5 mL, lyophilized.
   Contains Coenzyme NAD+, Buffer, stabilizers.
   See „Preparation of Reagents“.
5. ***Enzyme Diluent***, 2 vials, 2 x 30 mL; Ready to use.
   Contains TRIS-Buffer, 0.01 % NaN_3.
6. ***Substrate Reagent***, 2 vials, 2 x 30 mL; Ready to use.
   Contains tetrazolium salt.
7. ***Neutralization Buffer***, 1 vial, 14 mL; Ready to use.
   Contains carbonate buffer

4.1.2 Reagents provided for ENZ-4734 (25 x 96)

1. ***Standard A – E***, 25 cards, 5 cards per standard
   Concentrations: ~1; ~3; ~6; ~12; ~16 mg/dL
   Conversion: 1 mg/dL = 60.5 µmol/L
   Calibrated against 1st ISNS-RPNS for TSH, phenylalanine and 17α-hydroxyprogesterone).
   Contains human blood.
   Schleicher & Schuell paper No. 903. 12 Blood Spots / card.
   Exact concentrations see vial labels or QC Datasheet.
2. ***Control low and high***, 10 cards (5 x low, 5 x high)
   Control 1: ~ 3,0 mg/dL, Control 2: ~ 9,0 mg/dL
   Contains human blood.
   Schleicher & Schuell paper No. 903. 12 Blood Spots / card.
   Exact Concentrations / ***acceptable ranges see QC Datasheet or vial labels.***
3. ***Enzyme Reagent***, 25 vial, 25x0.5 ml, lyophilized
   Contains phenylalanine dehydrogenase, Buffer, stabilizers.
   See „Preparation of Reagents“.
4. ***Coenzyme Reagent***, 25 vial, 25x0.5 ml, lyophilized
   Contains Coenzyme NAD+, Buffer, stabilizers.
   See „Preparation of Reagents“.
5. ***Enzyme Diluent***, 10 vial, 10x30 ml; Ready to use.
   Contains TRIS-Buffer, 0.01 % NaN_3.
6. ***Substrate Reagent***, 10 vial, 10x30 ml; Ready to use.
   Contains tetrazolium salt.
7. ***Neutralization Buffer***, 3 vial, 3x24 ml; Ready to use
   contains carbonate buffer

4.2 Materials required but not provided

- Micropipettes (Multipette Eppendorf or similar devices, < 3% CV). Volumes: 25; 85; 100; 1000 µL
- Microtiterplate round bottom
- Blood spot puncher 3 mm or 5 mm
- Trichloracetic acid (3 %; w/v); from 99.5 % Trichloracetic acid, p.a. quality (e.g. Fluka, REF 91228)
- Blood collection cards (Schleicher & Schuell 903 recommended)
- Microtiter Plate (flat bottom)
- Microtiter plate shaker (300-500 rpm; Amplitude 1.5-3.0 mm)
- Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength 640 nm)
- Bidistilled or deionised water
- Paper towels, pipette tips and timer

4.3 Storage Conditions

The kit is shipped at ambient temperature and should be stored at 2-8°C.
Keep away from heat or direct sun light.
When stored at 2-8°C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
Opened reagents must be stored at 2-8°C.
The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.
Opened kits retain activity for 7 days if stored as described above.

4.4 Reagent Preparation

Allow all reagents to reach room temperature prior to use.

4.4.1 Preparation of Enzyme Reagent (Stock Solution)

The Stock Solution is stable for 7 days at 2-8°C.
Pipette 0.5 mL of bidist. water into the vial of the *Enzyme Reagent*, cap the vial and mix gently to dissolve.
Mix 2 minutes without foaming.

4.4.2 Preparation of Coenzyme Reagent (Stock Solution)

The Stock Solution is stable for 7 days at 2-8°C.
Pipette 0.5 mL of bidist. water into the vial of the *Coenzyme Reagent*, cap the vial and mix gently to dissolve.
Mix 2 minutes without foaming.

4.4.3 Preparation of Enzyme Solution

Prepare only the amount of Enzyme Solution that is needed for the actual test run.
If you are using several vials of the Enzyme Reagent Stock Solution, it is highly recommended to pool the solution and to establish the ready for use Enzyme Solution from this pool.
The Enzyme Solution is stable for 6 hours at 18–25°C. The stability can not be extended by storing at 2–8°C.

Recommended amount for the Enzyme ready for use solution:

No. of Microtiter Plates	½	1	2	5
Stock Solution (mL)	0.25	0.5	1.0	5.0
Diluent (mL)	2.5	5.0	10.0	50.0

Dilute Enzyme Reagent Stock Solution with *Enzyme Diluent* according to the table.
Mix 2 minutes carefully without foaming.

4.4.4 Preparation of Coenzyme Solution

Prepare only the amount of Coenzyme Solution that is needed for the actual test run.
If you are using several vials of the Coenzyme Reagent Stock Solution, it is highly recommended to pool the solution and to establish the ready for use Coenzyme Solution from this pool.
The Coenzyme Solution is stable for 6 hours at 18–25°C. The stability can not be extended by storing at 2–8°C.

Recommended amount for the Coenzyme ready for use solution:

No. of Microtiter Plates	1/2	1	2	5
Stock Solution (mL)	0.25	0.5	1.0	5.0
Diluent (mL)	2.5	5.0	10.0	50.0

Dilute Coenzyme Reagent Stock Solution with *Enzyme Diluent* according to the table.
Mix 2 minutes carefully without foaming.

4.5 Disposal of the Kit

The disposal of the kit must be made according to the national regulations.

4.6 Damaged Test Kits

In case of any severe damage to the test kit or components, DRG has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN COLLECTION AND PREPARATION

Blood Spots can be used in this assay.

5.1 Specimen Collection

Blood from the newborn's heel should be collected only from the medial or lateral section of the plantar surface.
The usual precautions for blood collection should be observed.
After puncture of the heel the first drop of blood should be wiped away with a sterile gauze. Touch the collection card against a large hanging drop of blood and allow a sufficient quantity of blood to soak into the filter paper in one step and so to fill the pre-printed circle completely.
Repeat the procedure to fill the required number of pre-printed circles on the collection card.
Allow the blood spots to air-dry for 3 h at room temperature away from direct sunlight.

CAUTION:
Because the standards are established with filter cards from Schleicher & Schuell No. 903 and there is a significant influence of the results by the filter card material, it is recommended to use these cards also for the samples.
Don't squeeze the puncture site during the collection because this will cause hemolysis or dilution of the blood with tissue fluid. Don't apply successive drops of blood to the same pre-printed circles. Don't touch or smear the blood spots.
Take care that the blood spot samples are visually okay (e.g. no blood smears, no coagulates, no finger-prints on the spots).

CAUTION:
The blood should be collected between the 3rd and the 5th days of life (72 to 120 hours after birth). Blood samples of newborns less than 72 hours after birth may give false negative results.

5.2 Specimen Storage

If Backup Specimen are needed, blood spots may be stored for up to 6 month at 2-8°C.

Keep away from heat or direct sun light.

5.3 Elution of Blood Spots

- Punch one 5 mm (Ø = 3/16") bloodspot or one or two 3 mm (Ø 1/8") blood spot(s) of standards, controls and samples into the appropriate round bottom wells of the microtiterplate.
- Pipette 100 µL of Trichloracetic Acid (3 %) into each well. Assure that each disc is fully immersed in the liquid.
- Incubate 60 min at RT (18–25 °C) on an orbital shaker (300–500 U/min.; Amplitude 1.5–3 mm).

6 ASSAY PROCEDURE

6.1 General Remarks

- Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
- Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
- Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
- Take care that the blood spot samples are visually okay (e.g. no blood smears, no coagulates, no finger-prints on the spots).
- Use a pipetting scheme to verify an appropriate plate layout.
- Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.

6.2 Test Procedure for one 3 mm Bloodspots

Each run must include a standard curve.

1. Pipette 25 µL of *Neutralization Buffer* into each well of the microtiter plate.
2. Pipette 85 µL of each *Standard*, *Control* and sample Blood Spot eluate into the respective wells. Shortly shake the plate.
3. Pipette 50 µL of freshly prepared Enzyme Solution into each well. (See "Preparation of Reagents".)
4. Pipette 50 µL of freshly prepared Coenzyme Solution into each well. (See "Preparation of Reagents".)
5. Incubate 60 min at room temperature (18–25 °C) on an orbital shaker (300–500 rpm; Amplitude 1.5–3 mm).
6. Pipette 100 µL of *Substrate Reagent* into each well.
7. Measure Optical Density with a photometer at 450 nm (Reference-wavelength: 640 nm)
   10 minutes after pipetting of the Substrate Reagent.

6.3 Test Procedure for one 5 mm or two 3 mm Bloodspots

Each run must include a standard curve.

1. Pipette 25 µL of *Neutralization Buffer* into each well of the microtiter plate.
2. Pipette 60 µL of each *Standard, Control* and sample Blood Spot eluate into the respective wells. Shortly shake the plate.
3. Pipette 50 µL of freshly prepared Enzyme Solution into each well. (See "Preparation of Reagents".)
4. Pipette 50 µL of freshly prepared Coenzyme Solution into each well. (See "Preparation of Reagents".)
5. Incubate 60 min at room temperature (18–25 °C) on an orbital shaker (300–500 rpm; Amplitude 1.5–3 mm).
6. Pipette 100 µL of *Substrate Reagent* into each well.
7. Measure Optical Density with a photometer at 450 nm (Reference-wavelength: 640 nm)
   10 minutes after pipetting of the Substrate Reagent.

6.4 Calculation of Results

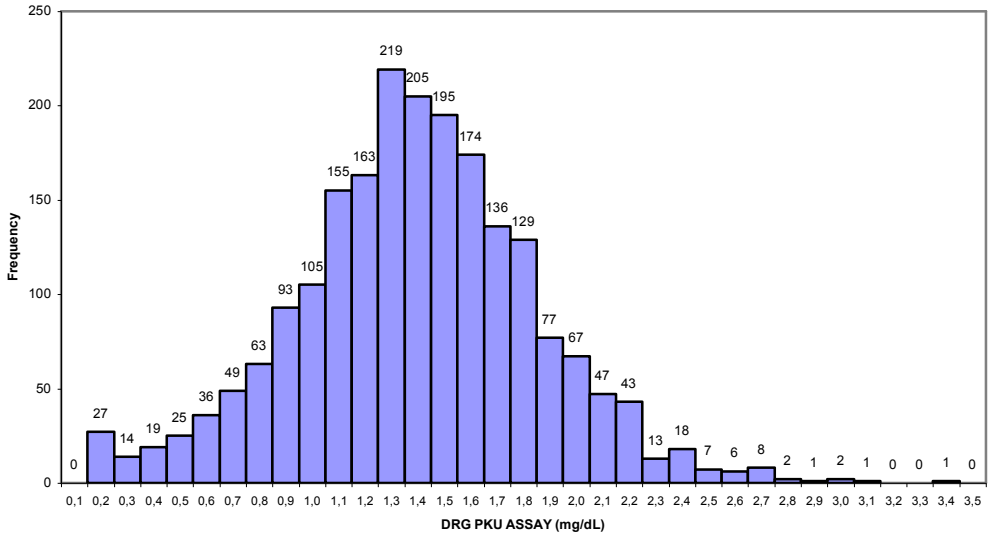
1. The obtained OD of the standards are plotted against their concentration.
   The standard curve is calculated by a linear regression or a weighted linear regression function.
   Using computer programs, the curve is best described by a 2-point linear regression fit with linear axes.
2. For the calculation of the regression curve, apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used).
3. The concentration of the samples can be read directly from the regression function.
4. Samples showing signals above the highest standard have to be confirmed by a reference method.

6.4.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

One 3 mm Bloodspot		
Standard	Phenylalanine (mg/dL)	Mean OD
1	1.35	0.04
2	2.93	0.06
3	6.08	0.10
4	11.33	0.16
5	15.73	0.22

One 5 mm Bloodspot or two 3 mm Bloodspots		
Standard	Phenylalanine (mg/dL)	Mean OD
1	1.35	0.09
2	2.93	0.13
3	6.08	0.23
4	11.33	0.39
5	15.73	0.54



7 REFERENCES / LITERATURE

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2. RIVM report 230011004/2005; *First ISNS Reference Preparation for Neonatal Screening for thyrotropin, phenylalanine and 17α-hydroxyprogestrone in blood spots;* LH Elvers, JG Loeber, JL Dhondt, M Fukushi, WH Hannon, T Torresani, D Webster

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