Instructions for Use

Phenylalanine (PKU) neonatal Screening Assay

Enzymatic Assay for the *in-vitro-diagnostic* quantitative determination of L-Phenylalanine in human newborn blood spots. For neonatal screening on Phenylketonuria.

REF RE80015 RE80019

480 2400

2-8°C

EU: IVD 0197 U.S.: For research use only. Not for use in diagnostic procedures.

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1. INTENDED USE

Enzymatic Assay for the in-vitro-diagnostic quantitative determination of L-Phenylalanine in human newborn blood spots. For neonatal screening on Phenylketonuria.

2. SUMMARY AND EXPLANATION

Phenylketonuria (PKU) is one of the most often hereditary diseases of the metabolism of the amino acids. It is transmitted autosomal recessive to the descendant with an incidence of circa 1:2600 to 1:25000 dependent on the observed population group.

The main cause of the disease – 90 to 99% of all cases – is a decrease or the absence of the activity of the enzyme complex Phenylalaninehydroxylase which is responsible for the transformation of the essential amino acid Phenylalanine into Tyrosine. The latter is a precursor of the Catecholamines, Melanine and the thyroid hormones. Because of the blocking of this metabolism Phenylalanine is transformed by an alternative pathway to Phenylpyruvate and –acetate which are excreted by the kidneys.

The disease becomes apparent by a mental retardation of the patients beginning in the first weeks of their life. Responsible for this development is the improper myelinization of the neurons in the brain because of the change in the protein metabolism. Other signs of the Phenylketonuria are the lacking of pigmentation of the dermis and its adnexis because of the disturbance of the synthesis of Melanine and therefore its predisposition of skin diseases.

It is important to make the diagnosis of Phenylketonuria in the newborns because the cerebral damage can be prevented by a low Phenylalanine diet. Therefore a screening test for the detection of elevated Phenylalanine concentrations in the blood has to be made between the 2rd and the 5th day of the infants life. If this one is positive it is followed by a confirmatory assay to detect the special mutations on the chromosome 12.

Many screening tests were developed for monitoring patients for Phenylketonuria. The first one called to its inventor GUTHRIE is based on the neutralization of an growth inhibiting factor of Bacillus subtilis by high Phenylalanine levels.

Because of some disadvantages of this method it is replaced by assays in which the Phenylalanine in the blood of the patients takes part in a chemical reaction developing a fluorescinating or a coloured substance which can be measured quantitavely. The procedures can be used more conveniently in comparison to the more sophisticated and more expensive method of the HPLC.

3. TEST PRINCIPLE

The Phenylalanine of the blood spots is eluated quantitavely 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 yellow Tetrazolium salt to the violet 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 570 nm.

4. WARNINGS AND PRECAUTIONS

1. For in-vitro diagnostic use only. For professional use only.
2. 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.
3. In case of severe damage of the kit package please contact IBL or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details. Material Safety Data Sheets for this product are available on the IBL-Homepage or upon request directly from IBL.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
8. All reagents of this kit containing human serum or plasma have been tested and were found negative for anti-HIV I/II, HBsAg and anti-HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

5. **STORAGE AND STABILITY**

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sunlight. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

6. **SPECIMEN COLLECTION AND STORAGE**

**Blood Spots**

| 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. |

⚠️ 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 (see LIMITATIONS OF THE PROCEDURE), it is recommended to use these cards also for the patient 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). |

⚠️ The optimal collection point of time is 48 to 72 hours after birth. The blood sample should not be collected before the 36. and not after 72. hour after birth. In this time frame failed sample collection must be catch up without any further delay. In case of discharge before the 36. hour after birth or relocation the first sample should be taken. An earlier determination point of time as the 36. hour after birth raises the risk of false negative diagnostic findings. In case of discharge before the 36. hour after birth the parents (Care beneficiary) must be informed about exigency of second laboratory determination in due time. Extract from German Child Direction (BAnz. Nr. 60). National and country specific guidelines to sample collection point of time must be considered. |

| Storage: 2-8°C | Keep away from heat or direct sun light. | Stability: 6 mon |
7. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>RE80019</th>
<th>RE80015</th>
<th>Symbol</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x</td>
<td>1 x</td>
<td>ENZ LYO</td>
<td>Enzyme lyophilized</td>
</tr>
<tr>
<td>1 x 220 mL</td>
<td>1 x 45 mL</td>
<td>ENZDIL</td>
<td>Enzyme Diluent</td>
</tr>
<tr>
<td>1 x 260 mL</td>
<td>1 x 55 mL</td>
<td>SUBS</td>
<td>Substrate</td>
</tr>
<tr>
<td>5 x 5</td>
<td>1 x 5</td>
<td>CAL A-E</td>
<td>Standard A-E</td>
</tr>
<tr>
<td>5 x 2</td>
<td>1 x 2</td>
<td>CONTROL1+2</td>
<td>Control 1+2</td>
</tr>
<tr>
<td>1 x 8 mL</td>
<td>1 x 2 mL</td>
<td>DYE CONC</td>
<td>Dye Stock Solution</td>
</tr>
</tbody>
</table>

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV). Volume: 25; 75; 100; 1000 µL
2. Round-bottom polystyrene test tubes (12 x 75 mm)
3. Rack for test tubes
4. Shaker for test tubes
5. Trichloracetic acid (3%; w/v); from 99.5% Trichloracetic acid, p.a. quality (e.g. Fluka, REF 91228)
6. 0.5 M NaOH solution; (e.g. 1 N NaOH Titrisol Sol., Merck, REF 9956)
7. Blood collection cards (Schleicher & Schuell 903 recommended)
8. Blood spot puncher, 5 mm (1/5”) (e.g. Sauer, Hannover, Germany)
9. Microtiter Plate (flat bottom)
10. Microtiter plate shaker (300-500 rpm; Amplitude 1.5-3.0 mm)
11. Microtiter plate reader capable of reading absorbance at 570 nm (reference wavelength 690 nm)
12. Bidistilled or deionised water
13. Paper towels, pipette tips and timer

9. PROCEDURE NOTES

1. 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.
2. 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.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each component and specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Take care that the blood spot samples are visually okay (e.g. no blood smears, no coagulates, no fingerprints on the spots).
5. It is advised to determine samples in duplicate to be able to identify potential pipetting errors.
6. Use a pipetting scheme to verify an appropriate plate layout.
7. 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.
10. **PRE-TEST SETUP INSTRUCTIONS**

10.1. **Preparation of Enzyme Stock Solution**

⚠️ The Stock Solution is stable for 5 d at 2-8°C. Prepare only the amount of Enzyme Solution that is needed for the actual test run.

**Pipette 12 mL of bidist. water into the vial of the Enzyme, cap the vial and mix gently to dissolve. Mix 2 min. without foaming.**

10.2. **Preparation of Enzyme Solution**

⚠️ If you are using several vials of the Enzyme 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 8 h 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:**

<table>
<thead>
<tr>
<th>No. of Microtiter Plates</th>
<th>1/3</th>
<th>2/3</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Solution (mL)</td>
<td>0.8</td>
<td>1.6</td>
<td>2.4</td>
<td>4.8</td>
<td>7.2</td>
<td>9.6</td>
<td>12</td>
</tr>
<tr>
<td>Diluent (mL)</td>
<td>2.7</td>
<td>5.3</td>
<td>8.0</td>
<td>16.0</td>
<td>24.0</td>
<td>32.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

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

10.3. **Preparation of Colored Trichloracetic Acid (3%)**

Dilute Dye Stock Solution (51x) with Trichloracetic acid (3%) 1:51 (e.g. add 2 mL Dye Concentrate to 100 mL of Trichloracetic acid (3%)).

The solution is stable until the expiry date of the kit at 18-25°C.

10.4. **Elution of Blood Spots**

Punch Blood Spot Standards, Controls and samples (each 5 mm Ø = 1/5”) and put each disc into one of the polystyrene tubes. Label all tubes.

**Pipette 100 µL of Colored Trichloracetic Acid (3%)** into each tube. Assure that each disc is fully immersed in the liquid.

**Incubate 30–60 min at RT (18–25 °C) on an orbital shaker (300–500 U/min.; Amplitude 1.5–3 mm).**

11. **TEST PROCEDURE**

1. **Pipette 25 µL of 0.5 M NaOH into each well of the microtiter plate.**

2. **Pipette 75 µL of each Standard, Control and sample Blood Spot Eluate into the respective wells.** Shortly shake the plate.

3. **Pipette 100 µL of freshly prepared Enzyme Solution into each well.**

4. **Incubate 30 min at RT (18–25°C).**

5. **Pipette 100 µL of Substrate into each well. Shake plate for 3 min on an orbital shaker (300-500 rpm; Amplitude 1.5–3 mm).**

6. **Measure Optical Density with a photometer at 570 nm (Reference-wavelength: 690 nm) within 3-5 min after pipetting of the Substrate Reagent.**

12. **QUALITY CONTROL**

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All kit controls must be found within the acceptable ranges as stated on the labels and the QC certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls. It is recommended to participate at appropriate quality assessment trials.

In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.
13. **CALCULATION OF RESULTS**

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.

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).

The concentration of the samples can be read directly from the regression function.

Samples showing signals above the highest standard have to be confirmed by a reference method.

**Conversion:**

1 mg/dL = 60.5 µmol/L

**Typical Calibration Curve**

(Example. Do not use for calculation!)

<table>
<thead>
<tr>
<th>Standard</th>
<th>Phenylalanine (mg/dL)</th>
<th>OD_{Mean}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.28</td>
<td>0.036</td>
</tr>
<tr>
<td>B</td>
<td>2.73</td>
<td>0.065</td>
</tr>
<tr>
<td>C</td>
<td>4.91</td>
<td>0.102</td>
</tr>
<tr>
<td>D</td>
<td>9.31</td>
<td>0.179</td>
</tr>
<tr>
<td>E</td>
<td>13.67</td>
<td>0.247</td>
</tr>
</tbody>
</table>

**14. INTERPRETATION OF RESULTS**

Based on the assumption that the expected values of phenylalanine follow a normal distribution blood spots with phenylalanine concentrations greater than 2.5 mg/dL (98% percentile) would be subsequently considered as "presumptive positive". Confirmation of the results would require repetition of the assay of 2% of the studied population.

If the result of the repeated measurement (in duplicate) is above the threshold of 3.0 mg/dL, a new sample should be collected and analyzed applying a confirmatory assay.

Based on classification scheme test results in the concentration range of 2.5 mg/dL to 3.0 mg/dL could potentially be diagnosed false negative and should be affirmed by additional measurement.

Various societies for neonatal screening recommend different cut-off values for repetition of the measurement and the application of confirmatory assays. Depending on the application of samples of different populations of newborns it is highly recommended that each laboratory establishes its own range of normal values and that this distribution of values is co-ordinated with the recommendations of the responsible society of this geographic region.

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations and diagnostic tests.
15. **EXPECTED VALUES**

Distribution of Phenylalanine Concentrations in Arbitrary Blood Spot Samples of Newborns

\[ x = 1.6 \text{ mg/dL} \]
\[ SD = 0.48 \text{ mg/dL} \]
\[ n = 6693 \]

Population = Poland

<table>
<thead>
<tr>
<th>percentile</th>
<th>mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>90%</td>
<td>2.16</td>
</tr>
<tr>
<td>95%</td>
<td>2.35</td>
</tr>
<tr>
<td>98%</td>
<td>2.51</td>
</tr>
<tr>
<td>99%</td>
<td>1.45</td>
</tr>
</tbody>
</table>

It is recommended that each laboratory establishes its own range of normal values.

16. **LIMITATIONS OF THE PROCEDURE**

Specimen collection has a significant effect on the test results. See SPECIMEN COLLECTION AND STORAGE for details.

Screening Test. Any result with an elevated concentration has to be indicated as ‘presumptive positive’ and has to be confirmed with further sampling and testing.

A false negative result of this assay cannot be excluded with absolute certainty. Any anamnestic or clinical hint of Phenylketonuria has to lead to a repeated measurement.

A direct influence of applied drugs to the patients on the phenylalanine results cannot be excluded. Therefore, it is highly recommended in these cases to review even a normal phenylalanine value with a confirmation test if there are anamnestic or clinical signs of a phenylketonurie in a patient.

It is recommended to use S&S 903 filter cards for the blood spot samples. When using other filter cards, the correction factor must be taken into account. For example, results with filter cards 2992 are ca. 20% different:

Standards (2992) = 1.20 x (S&S 903) + 0.181; \( r = 0.996; n = 349 \)

For cross-reactivities, see PERFORMANCE.

The following blood components do not have a significant effect on the test results up to the below stated concentrations:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>5</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>500</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>3000</td>
</tr>
</tbody>
</table>
17. PERFORMANCE

### Analytical Specificity (Cross Reactivity)
No cross-reactivities were found with the typical substances tested.

### Functional Sensitivity (Limit of Detection)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Functional Sensitivity, 20%; $y = 26.33 \times x^{-0.6304}$ $R^2 = 0.82$</th>
</tr>
</thead>
</table>

### Precision

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-Assay</td>
<td>2.68 – 9.60</td>
</tr>
<tr>
<td>Inter-Assay</td>
<td>2.79 – 9.36</td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.8 – 11.0</td>
</tr>
<tr>
<td>V (%)</td>
<td>6.4 – 15.1</td>
</tr>
</tbody>
</table>

### Linearity

Samples showing concentrations above the highest standard have to be confirmed by a reference method. A dilution of the sample is not possible, because there does not exist a blood without phenylalanine as diluent. Other diluents than extracts of phenylalanine free blood will probably have matrix effects.

### Recovery

<table>
<thead>
<tr>
<th>Mean (%)</th>
<th>Range (%)</th>
<th>% Recovery after spiking</th>
</tr>
</thead>
<tbody>
<tr>
<td>107</td>
<td>92 - 127</td>
<td></td>
</tr>
</tbody>
</table>

### Method Comparison versus Other Assay

\[
iBL-Assay = 0.88 \times \text{Other Assay} - 0.21 \quad r = 0.95; \quad n = 98
\]

18. PRODUCT LITERATURE REFERENCES

LIABILITY: Complaints will be accepted in each mode—written or vocal. Preferred is that the complaint is accompanied with the test performance and results. Any modification of the test procedure or exchange or mixing of components of different lots could negatively affect the results. These cases invalidate any claim for replacement. Regardless, in the event of any claim, the manufacturer’s liability is not to exceed the value of the test kit. Any damage caused to the kit during transportation is not subject to the liability of the manufacturer.