Introduction

GSP® Neonatal Phenylalanine kit (3308-0010/3308-001B) is a fully automated enzymatic assay for the GSP® system. Optimized for the quantitative determination of phenylalanine concentration in blood specimens dried on filter paper, the kit is an effective aid in screening newborns for phenylketonuria (Figure 1.). Analytical performance of the GSP Neonatal Phenylalanine kit was determined in verification studies conducted at PerkinElmer, Turku, Finland. In order to establish population distribution data and screening performance for the GSP Neonatal Phenylalanine kit two studies were conducted in newborn screening laboratories. In these studies GSP Phenylalanine kit was compared to manual Phenylalanine kit (NP-1000/-4000).

Key benefits over existing manual assays:

- Automated assay
- Only two hands-on steps for reconstitution
- 24 hrs valid calibration curve
- Control step to detect missing sample disks
- Resorufin step added in the reaction to decrease the unspecific background fluorescence
- All reagents and QC material are bar-coded
- Simultaneous screening of any other GSP assays in any order
- Reagent on-board stability 4 days / 4 plates

Figure 1. Workflow comparison of manual phenylalanine assay and automated GSP Neonatal Phenylalanine assay
Full selection of reagents in one kit

Two different sizes of kits are available as 12 plates kit (3308-0010) and a bulk kit for 60 plates (3308-0018)

- Neonatal Phenylalanine Calibrators - 7 [15] dried blood spot cassettes each containing 1 set of dried blood spots
- Neonatal Phenylalanine Controls - 4 [20] dried blood spot cassettes each containing 3 sets of dried blood spots
- Neonatal Phenylalanine Substrate Reagent - 3 [15] vials, lyophilized
- Neonatal Phenylalanine Substrate Reagent - 3 [15] vials, lyophilized
- Neonatal Phenylalanine Assay Buffer - 3 [15] bottles, ready-for-use
- Barcode labels for the plates

Validated assay method

The GSP Neonatal Phenylalanine assay is based on the fluorescent phenylalanine dehydrogenase method, which differs from the fluorescent ninhydrin method used in manual Neonatal Phenylalanine kit (NP-1000/NP-4000). In the first reaction phenylalanine dehydrogenase converts phenylalanine in sample to phenylpyruvate generating NADH. In the presence of NADH, resazurin dye is reduced to fluorescent resorufin, which is measured using an excitation wavelength of 505 nm and an emission wavelength of 580 nm (Figure 3.). Due to longer excitation wavelength than with manual phenylalanine assay, unspecific background fluorescence is reduced for improved performance.

GSP Neonatal Phenylalanine kit assay protocol

After punching of the samples and reconstitution of the Phenylalanine Substrate and Enzyme Reagents, the assay is fully automated from plate loading to completion.

The assay time for one plate is 2 h 28 min and around 2-3 plates can be processed per hour after an initial 3 hour dwell time (Figure 4.):

- Dispensing Extraction Solution (20 µL/well)
- Shaking (30 s)
- Dispensing Assay Buffer (100 µL/well)
- Dispensing Substrate Reagent (5 µL/well)
- Dispensing Enzyme Reagent (5 µL/well)
- Incubation (1 h)
- Shaking (30 s)
- Measurement (Phenylalanine concentration)
- Measurement (Elution Control to check the sample disk is present in a well)
Lower limits of detection, measuring range and linearity

The analytical limits were determined in accordance with CLSI document EP17-A2 [2] and linearity was determined in accordance with CLSI document EP06-A [3]. Analytical limits, measuring range and linearity of the GSP Neonatal Phenylalanine kit are summarized in Table 2. The Limit of Blank (LoB) is defined as the 95th percentile of a distribution of blank samples (n=150), the Limit of Detection (LoD) is based on 216 determinations of low level samples and the Limit of Quantitation (LoQ) is defined as the lowest concentration with a total CV equal to or less than 24%.

Table 2. Analytical limits, measuring range and linearity

<table>
<thead>
<tr>
<th>Specimen stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>The influence of storage time, temperature, and humidity on phenylalanine concentration was studied using several artificial samples spiked with phenylalanine. Phenylalanine concentrations were measured at different time points after different storage conditions. Storage under desiccated conditions is recommended (Figure 6.), [4].</td>
</tr>
</tbody>
</table>

Specimen stability

The change of phenylalanine concentration during storage at different temperatures and humidity conditions over time (% of reference without storage at the zero time point).
Internal and external studies were conducted to produce normal newborn population distribution data for the GSP Neonatal Phenylalanine kit, to assess the screening performance and to compare GSP Neonatal Phenylalanine assay to manual Phenylalanine assay (NP-1000/-4000).

### Internal study

An internal study comprising of 2212 leftover routine newborn screening DBS samples and 13 confirmed PKU positive newborn specimens was conducted at PerkinElmer, Turku, Finland. Phenylalanine concentration was measured with both the GSP Neonatal Phenylalanine kit (3038-0010/-B) and manual Neonatal Phenylalanine kit (NP-1000/-4000). All the positive samples were classified as screening positive using higher 99.9% percentile. The frequency distribution for routine specimens (excluding confirmed PKU specimens) is shown in Figure 9. and Table 4.

### External study 1

Phenylalanine concentration of 2064 newborn screening specimens and 22 archived confirmed high phenylalanine specimens were measured using GSP Neonatal Phenylalanine kit in a European newborn screening laboratory. Population range, mean, median and cut-off values corresponding to 99th, 99.5th and 99.9th percentile were calculated. The frequency distributions of the newborn dried blood spot specimens of measured phenylalanine concentrations are visualised in Figure 7. and descriptive statistics in Table 3.

The 22 positive samples consist of 7 PKU cases with classical phenylketonuria or deficient cofactor (PKU) and 15 hyperphenylalaninemia (HPA) cases. All of the 22 retrospective high phenylalanine specimens were classified as screening positive by the GSP Neonatal Phenylalanine kit by using the higher 99th percentile and 99.5th percentile cut-off values.

### External study 2

518 routine newborn screening dried blood spot specimens and 37 archived confirmed high phenylalanine specimens were included in an external study 2 conducted in China and the phenylalanine concentration was measured with GSP Neonatal Phenylalanine assay. The frequency distributions of the newborn specimens for PKU concentrations are visualised in Figure 8. and descriptive statistics in Table 4.

---

**Table 4.** Range, mean and median values with upper percentiles for the routine and retrospective positive samples using both GSP Neonatal Phenylalanine kit and manual PKU kit in internal study (excluding confirmed positive PKU specimens).

<table>
<thead>
<tr>
<th>Platform</th>
<th>Median (µmol/L)</th>
<th>Mean (µmol/L)</th>
<th>Routine samples (n=2212)</th>
<th>Confirmed positive (n=13)</th>
<th>Upper percentiles (µmol/L blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>95%</td>
<td>99%</td>
<td>99.5%</td>
</tr>
<tr>
<td>GSP PKU</td>
<td>67.9</td>
<td>68.5</td>
<td>0.8</td>
<td>845.1*</td>
<td>99.3</td>
</tr>
<tr>
<td>Manual PKU (HPLC)</td>
<td>64.7</td>
<td>64.1</td>
<td>18.1</td>
<td>526.3*</td>
<td>91.7</td>
</tr>
<tr>
<td>Manual PKU (ISNS)</td>
<td>84.6</td>
<td>83.8</td>
<td>23.6</td>
<td>689.6*</td>
<td>120.0</td>
</tr>
</tbody>
</table>

*This sample was drawn from an HPA patient. The lowest sample result with classical PKU or deficient cofactor was 322.9 µmol/L.
Method comparison

GSP Neonatal Phenylalanine kit is calibrated against ISNS Reference preparation for Neonatal Screening [5], whereas manual PKU kit is calibrated against HPLC calibration. The method comparison data from internal verification study shows that there is a level difference between GSP and manual Phenylalanine assays (Figure 10) and no conversion factor between the methods is available.

At GSP Neonatal Phenylalanine concentration levels below 120 µmol/L (or <2 mg/dL) the manual PKU results are ~15 % higher results than the GSP method, whereas at GSP Neonatal Phenylalanine concentration are ~15 % lower results than the GSP method.

![Figure 10. Deming regression analysis results between the GSP and manual Phenylalanine methods using GSP Neonatal Phenylalanine as a reference.](image)

<table>
<thead>
<tr>
<th></th>
<th>Result</th>
<th>95% Confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>16.57</td>
<td>9.05 to 24.09</td>
</tr>
<tr>
<td>Slope</td>
<td>0.77</td>
<td>0.74 to 0.81</td>
</tr>
</tbody>
</table>

References


The product is not available in the USA, Canada, Russia and some Asian and Latin American countries.