



*Last revision: 2018/06*

## **MucoPAP**

PAP assay kit for Cystic Fibrosis newborn screening

**INSERM Patent**

**Enzyme-linked immunosorbent assay (ELISA)**

Instruction manual and reagents for 96 assays

Manufactured by:

**DYNABIO SA,**

**Luminy Biotech Entreprises**

**Case 922 – 163, avenue de Luminy**

**13288 Marseille cedex 09**

**FRANCE**

**REF** MPK01

**IVD**

**CE**

## SYMBOLS



For *in vitro* diagnostic use



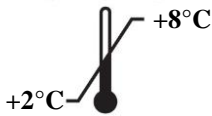
Lot number



Catalog number



Expiry date (yyyy/mm/dd)



Store between +2°C and +8°C



Contains reagents for 96 assays



Note: see instruction manual



Manufacturer

## TABLE OF CONTENTS

<b>INTRODUCTION.....</b>	<b>4</b>
<b>PRINCIPLE OF THE ASSAY.....</b>	<b>4</b>
<b>EQUIPMENT AND PRODUCTS NOT PROVIDED REQUIRED FOR THE ASSAY .....</b>	<b>4</b>
<b>KIT COMPOSITION.....</b>	<b>5</b>
<b>DESCRIPTION OF REAGENTS.....</b>	<b>6</b>
<b>SAMPLE COLLECTION AND TREATMENT.....</b>	<b>6</b>
<b>CAUTION FOR USE .....</b>	<b>6</b>
<b>RECOMMENDATIONS FOR USE.....</b>	<b>7</b>
<b>PREPARATION OF SAMPLES.....</b>	<b>7</b>
<b>PREPARATION OF REAGENTS .....</b>	<b>7</b>
<b>ASSAY PROCEDURE .....</b>	<b>8</b>
<b>CALCULATION OF RESULTS .....</b>	<b>9</b>
<b>Calibration.....</b>	<b>9</b>
<b>Quality control .....</b>	<b>9</b>
<b>Analysis of results of newborn samples.....</b>	<b>10</b>
<b>LIMITS OF THE ASSAY .....</b>	<b>10</b>
<b>INTERPRETATION OF RESULTS.....</b>	<b>10</b>
<b>PERFORMANCES.....</b>	<b>10</b>
<b>PERFORMANCES AND ANALYTICAL CHARACTERISTICS .....</b>	<b>11</b>
<b>WARRANTY .....</b>	<b>12</b>
<b>BIBLIOGRAPHY .....</b>	<b>12</b>
<b>SUMMARY OF ASSAY .....</b>	<b>13</b>

## INTRODUCTION

The Pancreatitis-associated protein (PAP) is synthesized in pancreas during pancreatic stress. In cystic fibrosis (CF), the pancreas is already diseased *in utero* and PAP is synthesized before birth. Several studies have shown that PAP concentration is indeed elevated in the blood of CF newborns (1, 2, 3, 4, 5).

PAP assay on calibrated screening cards allows detection of newborns at risk of developing cystic fibrosis.

## PRINCIPLE OF THE ASSAY

The MucoPAP kit is designed to assay PAP in dried blood spots of newborns from screening cards calibrated and approved by competent authorities. It is a sandwich enzyme-linked immunosorbent assay (ELISA), in which the standard range and the internal control are supplied lyophilised, to be reconstituted with sterile water and diluted to obtain precise PAP concentrations described in this manual.

The wells of the microtitration plate are coated with anti-PAP antibodies. In a first step, the range, the internal control and the eluates of newborn blood spots are deposited in the wells and the PAP they contain is allowed to bind to specific antibodies. All proteins not specifically bound are eliminated by washing. Then anti-PAP antibodies coupled to biotin are allowed to attach to the bound PAP. After washing, antigen-antibodies complexes are detected by an avidin-peroxidase conjugate. After a last washing step, the addition of a chromogenic substrate of peroxidase enzyme leads to the release of a blue degradation product. Enzymatic reaction is stopped by the addition of an acid solution, which transforms coloration from blue to yellow. The intensity of yellow coloration, measured by spectrophotometry at 450 nm, is proportional to the quantity of PAP present in the initial sample and bound during the first step.

## EQUIPMENT AND PRODUCTS NOT PROVIDED REQUIRED FOR THE ASSAY

### Equipment:

- Vortex mixer
- Semi-automatic or automatic plate washer
- Plate reader for absorbance measurement, equipped with a 450 nm filter (and eventually with a 630 nm reference filter to subtract blue coloration residual intensity to optical density measured at 450 nm)
- Computer with paired printer
- Single and multi-channel micropipettes
- 1L plastic bottle (for washing buffer)
- Manual or automatic puncher (3 mm diameter) to sample screening cards
- Two litres of sterile water

### Disposable material:

- U-bottom 96 well plates (for elution of blood spots)
- Micropipette tips
- Disposable 10 mL pipettes
- Five disposable reagent reservoirs (one per reagent): PBS, biotinylated antibodies, avidin-peroxidase, chromogenic substrate and acid
- Newborn blood spots on screening cards calibrated and approved by competent authorities

## KIT COMPOSITION

Each kit contains reagents for 96 assays. Expiry date is mentioned on all labels of the kit.

The microtitration plate is strippable, allowing adaptation of the assay to the number of samples to be assayed. However, each run must include a standard range and internal control.

REAGENTS	STORAGE BEFORE OPENING	CHARACTERISTICS OF USE	STORAGE AFTER OPENING
96 wells microtitration plate (8 x 12 wells horizontal strips)	Keep protected from light in the sealed package between +2°C and +8°C until expiry date.	Coated with anti-PAP antibodies. Ready to use.	Store between +2°C and +8°C, in plastic bag with dessicant provided in the kit, for a maximum of 30 days.
Standard range of PAP	Stable if stored between +2°C et +8°C until expiry date.	Lyophilisate to be gently dissolved in 1 mL of sterile water, directly in the vial.	Keep at -20°C for 30 days maximum.
Internal control		Lyophilisate to be gently dissolved in 11 mL of sterile water, directly in the vial.	
Dilution buffer for standard PAP range preparation			
Biotinylated anti-PAP antibodies		Vial containing 15 mL. Ready to use.	Keep between +2°C et +8°C for 30 days maximum.
Avidin-peroxidase conjugate			
Chromogenic substrate (TMB)		Vial containing 11 mL. Ready to use.	
Acid (H <sub>2</sub> SO <sub>4</sub> )		Dissolve in 1 L of sterile water. Keep 15 mL for elution of blood spots.	Keep the prepared washing buffer at -20°C for 30 days maximum.
PBS tablet			
Tween 20 (10 % solution)		Add to the remaining 985 mL of PBS solution to generate the washing buffer.	

The kit can be used within 30 days following opening if the recommendations listed above are followed.

## DESCRIPTION OF REAGENTS

REAGENTS	DESCRIPTION
Microtitration plate (96 wells in horizontal strips, 8 x 12 wells)	Wells coated with PAP-specific mouse monoclonal antibodies
Range and internal control	Recombinant human protein PAP (rhPAP) in phosphate buffer containing bovine proteins and protective agents
Dilution buffer for standard PAP range preparation	Tris-HCl buffered saline containing bovine proteins and protective agents
Biotinylated antibodies to PAP	PAP-specific mouse monoclonal antibodies conjugated to biotin, in a phosphate buffered solution containing protective agents
Avidin-peroxidase conjugate	Avidin conjugated to peroxidase enzyme, in a phosphate/citrate buffer containing protective agents
Chromogenic substrate (TMB)	Solution of chromogenic substrate of peroxidase enzyme 3,3',5,5'-tétraméthylbenzidine (TMB)
Acid (H <sub>2</sub> SO <sub>4</sub> )	Diluted sulphuric acid solution
PBS tablet	Phosphate buffered saline
Tween 20 solution (10 %)	Concentrated detergent solution

## SAMPLE COLLECTION AND TREATMENT

Blood samples must be obtained by heel pricking and directly collected on approved filter paper (method of reference).

The method and devices used for sample collection must comply with local regulation.

It is recommended to consult local regulation about sampling and time after birth at which sample must be taken, in accordance with the local newborn screening program. This program also determines the period after sample collection during which PAP assay can be done.

The quality of results obtained on dried blood samples depends greatly on the care taken at collecting, manipulating, transferring and storing samples. A document (6) describes the appropriate collection methods to correctly deposit blood on standardized filter paper. It also provides instructions to correctly manipulate, transport and store samples to ensure a good quality of newborn screening results.

## CAUTION FOR USE

This kit must be used for *in vitro* diagnostic purposes only, by properly trained staff provided with suitable protective equipment.

Dried blood spots from newborns as well as biotinylated antibodies contain products of human or animal origin, respectively. They must be considered as potentially infectious and used with adequate care.

Waste should be disposed according to local law.

Do not pipette by mouth.

Do not eat, drink or smoke during the test.

The following reagents may be toxic or irritant and must be handled to avoid any contact with the skin, eyes and mucosae: PBS tablet, chromogenic substrate (TMB) and acid solution. In case of accidental contact, rinse the affected parts immediately with plenty of water.

## RECOMMENDATIONS FOR USE

Avoid any biological or chemical contamination of samples.

Never use outdated reagents.

Do not mix reagents from different lots.

Equilibrate all reagents at room temperature (+19°C to +22°C) and stir them before use to homogenize the content.

Avoid cross contamination between reagents: use a different reservoir for each reagent (reservoirs not provided).

Respect strictly the indicated incubation time at all steps.

Washing steps must be thorough to avoid background increase.

Never let the plate dry down as this would alter the quality of results.

The lyophilized reagents (standard range, internal control, dilution buffer, biotinylated antibodies and avidin-peroxidase) must be put in solution at least 10 minutes before use, to ensure complete dissolution and adequate homogeneity of the reagent.

Do not expose chromogenic substrate TMB to air and light before use.

If the kit is damaged during shipment (broken/spilled vials, reinflated aluminium bags), please contact Dynabio S.A. by email at [info@dynabio.com](mailto:info@dynabio.com) or by phone at +33 (0)4 86 94 85 04.

## PREPARATION OF SAMPLES

The day before the assay:

Dissolve the PBS tablet in 1L of sterile water. After complete homogenization, save 15 mL for blood spots elution: the remaining 985 mL are stored between +2°C and +8°C until the assay on the next day, to prepare the washing buffer.

*Samples to assay:* punch in the screening card a disc of 3 mm in diameter, imperatively on the periphery of the blood spot, in a region with thorough blood impregnation, without overload or double-deposit. Put the disc in a well of a 96 U-bottom well plate (not provided). To obtain a duplicate assay, punch another disc on the same blood spot. Add 150 µL of PBS per well. Allow 16 hours for elution (overnight) between +2°C and +8°C.

## PREPARATION OF REAGENTS

On the day of the assay: After overnight incubation, all the eluates need to be carefully homogenized by up and down pipetting before taking the 100 µL sample to assay.

**Microtitration plate:** Packaged under vacuum, it must be equilibrated at room temperature before removal from its wrapping. Once opened, the plate must be identified by the user not to mistake it with another plate processed on the same day. All strips of the plate must also be identified (from A to H) to avoid switching them in case they fall from their frame while flicking the plate during washing steps.

**Washing buffer** (PBS/0.1% Tween): Add the whole content of the supplied Tween 20 (10%) vial to the rest of PBS dissolved the day before (985 mL remaining) and homogenize.

**PAP standard range:** The range is prepared from recombinant human freeze-dried PAP. The lyophilisate is reconstituted with 1 mL of sterile water to obtain 0.5 µg/L of PAP. This standard solution at 0.5 µg/L is then used to prepare a reference range presenting concentrations from 0.125 to 0.0078 µg/L, by successive dilutions in the dilution buffer provided for that purpose.

**Internal control:** The internal control is also prepared from recombinant human freeze-dried PAP. The lyophilisate is reconstituted with 1 mL of sterile water to obtain 0.02 µg/L of PAP. When calculating results, this control is expected to be at a concentration of  $0.02 \times 50 = 1$  µg/L. An elution factor (x50) is indeed applied to newborn samples due to the

elution of PAP in PBS (3  $\mu\text{L}$  of blood in a 3 mm disc diluted in 150  $\mu\text{L}$  of PBS). To ease the treatment of results by analysis software, this same factor is also applied to the internal control.

**Dilution buffer:** The lyophilisate is dissolved in 11 mL of sterile water directly in the vial. It is ready to use after complete dissolving and homogenisation to prepare standard range dilutions.

**Biotinylated antibodies:** The lyophilisate is dissolved in 11 mL of sterile water directly in the vial. It is ready to use after complete dissolving and homogenisation.

**Avidin-peroxidase:** The lyophilisate is dissolved in 11 mL of sterile water directly in the vial. It is ready to use after complete dissolving and homogenisation.

**Chromogenic substrate (TMB):** Ready to use solution after homogenization.

**Acid ( $\text{H}_2\text{SO}_4$ ):** Ready to use solution after homogenization.

## ASSAY PROCEDURE

A reference PAP range is prepared from the standard lyophilized solution of PAP previously reconstituted at 0.5  $\mu\text{g/L}$  with 1 mL of sterile water. The dilution buffer provided with the kit is used to prepare serial dilutions of this standard solution to obtain the following PAP concentrations: 0.125 / 0.062 / 0.031 / 0.015 / 0.0078  $\mu\text{g/L}$ . Each dilution from 0.125 to 0.0078  $\mu\text{g/L}$  will be assayed in duplicate (100  $\mu\text{L/well}$ ).

**Preparation of the PAP reference range** (example for one PAP range assayed in duplicate):

150  $\mu\text{L}$  of standard solution at 0.5  $\mu\text{g/L}$  + 450  $\mu\text{L}$  of dilution buffer = 0.125  $\mu\text{g/L}$

then:

300  $\mu\text{L}$  of dilution at 0.125  $\mu\text{g/L}$  + 300  $\mu\text{L}$  of dilution buffer = 0.062  $\mu\text{g/L}$

then:

300  $\mu\text{L}$  of dilution at 0.062  $\mu\text{g/L}$  + 300  $\mu\text{L}$  of dilution buffer = 0.031  $\mu\text{g/L}$

then:

300  $\mu\text{L}$  of dilution at 0.031  $\mu\text{g/L}$  + 300  $\mu\text{L}$  of dilution buffer = 0.015  $\mu\text{g/L}$

then:

300  $\mu\text{L}$  of dilution at 0.015  $\mu\text{g/L}$  + 300  $\mu\text{L}$  of dilution buffer = 0.0078  $\mu\text{g/L}$

Background value of the assay (blank corresponding to 0  $\mu\text{g/L}$  of PAP) will be determined by depositing 100  $\mu\text{L/well}$  of dilution buffer, in duplicate.

Range, background, internal control and newborn samples to assay are deposited in the wells (100  $\mu\text{L/well}$ , in duplicate). The plate is covered with the adhesive supplied with the kit and incubated 3 hours at room temperature ( $\sim 21^\circ\text{C}$ ).

Then, wells are washed 5 times with the washing buffer (PBS/0.1% Tween as described previously), as follows:

- Thoroughly draw up the wells
- Fill with  $\sim 300$   $\mu\text{L}$  of washing buffer
- Repeat the first two steps 4 times
- After the last wash, eliminate residual liquid by inverting the plate (in a sink or in a container for liquid waste) and tapping it on absorbent paper.

*Note:* it is recommended to use an automatic or semi-automatic plate washer.

The reconstituted solution of biotinylated anti-PAP antibodies is then immediately deposited on the plate (100  $\mu\text{L/well}$ ) and incubated for 30 minutes at room temperature, after covering the plate with the adhesive.

The plate is then washed 5 times with washing buffer as described above.

Then the solution of avidin-peroxidase conjugate is added to each well (100  $\mu\text{L/well}$ ) and incubated 15 minutes at room temperature, after covering the plate with the adhesive.

The plate is then washed 5 times with washing buffer as described above.



The chromogenic substrate (TMB) is then added (100 µL/well): after covering it with the adhesive, the plate is immediately placed in the dark and incubated 15 minutes at room temperature.

After this 15 minutes TMB incubation protected from light, a blue coloration of variable intensity has appeared in the wells: without washing the plate, add directly 100 µL/well of acid solution to stop the enzymatic reaction. This final addition brings the total volume to 200 µL/well and transforms the coloration from blue to yellow.

The absorbance of each well of the plate must be read within 30 minutes after the reaction is stopped, using a spectrophotometer equipped with a 450 nm filter.

*Note: Some spectrophotometers are programmed to read a first time at 450nm and a second time with a reference filter at 630nm. The absorbance at 630nm is then subtracted from the one at 450nm to eliminate residual blue coloration intensity. However, the 630nm reference filter is not mandatory.*

## CALCULATION OF RESULTS

### Calibration

A standard range must be added to every run of assays. If the run of the day involves several plates, the range must be included in each plate.

To generate the range curve, the background value of the assay must be first calculated, as the mean value of all blank determinations (0 µg/L). The mean background value is then subtracted from the value of each replicate of all points of the range.

Background must also be subtracted from each replicate of control and samples before calculating the mean of duplicates.

The table hereunder gives an example of a PAP range generated with a background giving an optical density of 0.075 (results provided for information only):

PAP (µg/L)	Absorbance (Optical Density at 450 nm)				
	Replicate 1	Replicate 2	Replicate 1 - mean background	Replicate 2 - mean background	Mean
0	0.076	0.074			
0.0078	0.318	0.300	0.243	0.225	0.234
0.015	0.506	0.503	0.431	0.428	0.429
0.031	0.908	0.859	0.833	0.784	0.809
0.062	1.630	1.562	1.555	1.487	1.521
0.125	2.981	2.920	2.906	2.845	2.875

The standard curve is constructed using the function [PAP] = f(mean absorbance), by plotting the mean optical density measured for each point of the range versus the theoretical PAP concentrations and applying a 4-parameter logistic adjustment. The use of a computer program to define the parameters of this function from range values is recommended. The concentration of PAP in control and eluates of blood spots is determined by extrapolation from this function.

**Warning:** The values obtained using this equation have to be multiplied by 50 to get the PAP blood concentrations of newborns. Indeed, a 3 mm diameter disc of standardized filter paper contains 3 µL of blood. Each disk is then eluted in 150 µL of PBS, corresponding to a 1/50 dilution. To come back to blood PAP concentration, the value measured in the eluate of blood spot must be multiplied by this factor.

### Quality control

Assaying internal control is recommended to ensure the quality of results. Control must be processed exactly as newborn samples, which means that its theoretical concentration in the assay is 0.02 µg/L but that it must be multiplied by 50: it is then expected at 1 µg/L. This control must be included in each assay, like the standard range. If the run of the day involves several plates, control must be included in each plate. It is recommended that result obtained for the control is within +/-20% from its theoretical value:

Control – Theoretical concentration	Lower limit	Higher limit
Internal control - 1 µg/L	0,8 µg/L	1,2 µg/L

Results for newborn samples can be validated only if control value fit this acceptance criterion.

In case of recurrent problems with assay performance, please contact Dynabio S.A. by email at [info@dynabio.com](mailto:info@dynabio.com) or by phone at +33 (0)4 86 94 85 04.

### **Analysis of results of newborn samples**

Calculation of PAP concentration in newborn blood: if the protocol described above is carefully followed, and if samples come from calibrated screening cards punched with a 3 mm device, PAP blood concentration is directly deduced from the equation of the range [PAP] = f(mean absorbance) and then by multiplying this result by 50 as explained above.

### **LIMITS OF THE ASSAY**

Information given by blood PAP concentration obtained with the MucoPAP kit must be used in conjunction with information given by other assays (e.g. IRT) as part of a strategy for newborn screening of cystic fibrosis. It must be interpreted in the light of other available clinical information.

Situations leading to potentially abnormal assay results:

- the screening card is not thoroughly filled with blood,
- the sample was punched too close from the edge of the blood spot,
- the sample was punched in the center of the blood spot instead of its periphery,
- the sample is not correctly collected or dried,
- the sample was exposed to excessive heat or humidity,
- the screening card is contaminated with stools.

It is suggested to refer to sections « Caution for use » and « Recommendations for use ».

### **INTERPRETATION OF RESULTS**

Evaluation of PAP concentration in blood spots is used to identify a population of newborns at risk for cystic fibrosis. Strategies presently implemented in several countries involve in general three tiers. The first one is an assay of Immunoreactive Trypsinogen (IRT) in all newborns. The second one is PAP assay in newborns with elevated IRT. In newborns with high IRT and PAP, the third tier is either a diagnostic test (sweat test) or a search of CF-associated mutations in the CFTR gene, followed by a sweat test in newborns bearing those mutations.

An exhaustive review of the performances of available strategies was conducted by the French Haute Autorité de Santé and published in 2015 as « *Place de la stratégie couplant les dosages de la TIR et de la PAP dans le dépistage systématique de la mucoviscidose en France* » available online at : [http://www.has-sante.fr/portail/jcms/c\\_1739994/fr/](http://www.has-sante.fr/portail/jcms/c_1739994/fr/).

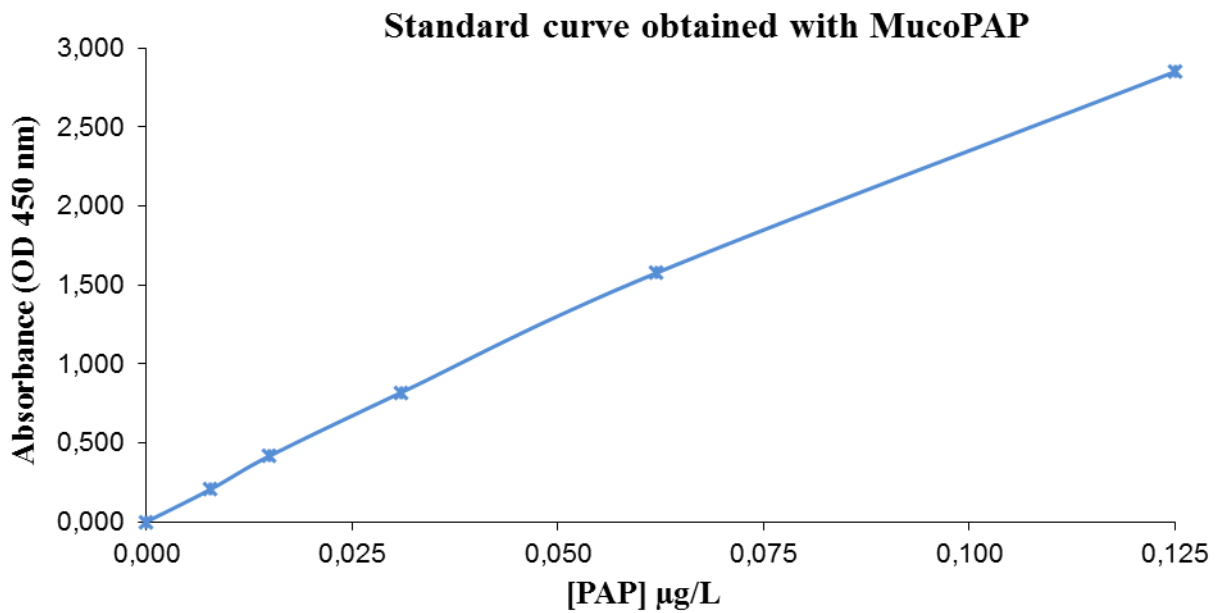
It is recommended to review this document before implementing a CF newborn screening strategy involving PAP assay.

### **PERFORMANCES**

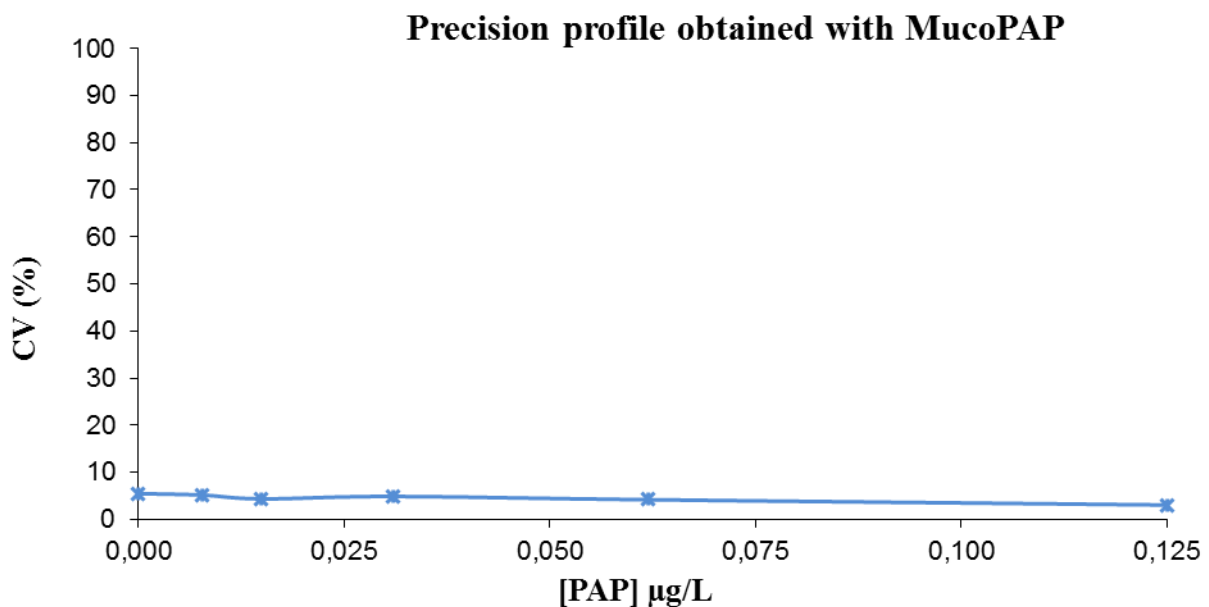
In newborns with elevated IRT (>50 µg/L) most CF had PAP > 1,1 µg/L, (except for mild forms and for meconium ileus). CF newborns represent 25% of babies with elevated IRT and PAP >1,1 µg/L. In that group, non-CF babies were often premature, babies with severe intestinal infection or with trisomy (Sarles et al J.Pediatr. 2005 147, 302-305)

## PERFORMANCES AND ANALYTICAL CHARACTERISTICS

**Standard range:** A typical standard range of the MucoPAP kit is shown below. It was determined by assaying each point of the range in duplicate, using five different lots, corresponding to ten replicates.



**Precision profile:** Precision profile of the MucoPAP kit was established by assaying each point of the range in duplicate, using five different lots of kits, corresponding to ten replicates. It is shown in the graph below.



**Repeatability and reproducibility:** Repeatability gives an estimate of the intra-lot variation and reproducibility an estimate of the inter-lot variation.

Repeatability and reproducibility of MucoPAP kit were established by assaying three different controls on five different lots. The controls used for this determination are:

- the internal control supplied with the kit, expected at  $0.02 \times 50 = 1 \mu\text{g/L}$ , assayed on 4 replicates in each lot ;
- an external control expected at  $0.04 \times 50 = 2 \mu\text{g/L}$ , assayed on 10 replicates in each lot ;
- another external control expected at  $0.10 \times 50 = 5 \mu\text{g/L}$ , assayed on 10 replicates in each lot.

Results of repeatability and reproducibility study are presented in the following table:

<b>Expected value of the control (µg/L)</b>	<b>Measured value (µg/L)</b>	<b>Repeatability CV (%)</b>	<b>Reproducibility CV (%)</b>
<b>1</b>	1,046	4,4	5,2
<b>2</b>	1,990	4,0	4,8
<b>5</b>	4,877	4,9	7,2

**Detection and quantitation limits:** Detection and quantitation limits of the MucoPAP assay (expressed as micrograms of PAP per liter of blood) are respectively 0.13 µg/L and 0.24 µg/L, given that:

- detection limit is defined as 3 standard deviation above the mean value of blank,
- quantitation limit is defined as 10 standard deviation above the mean value of blank.

**Cross-reaction:** No cross reaction was observed in the MucoPAP assay with IL2, IL6, IFN $\gamma$ , TNF $\alpha$  or *Escherichia coli* proteins.

**Hook effect:** No hook effect for PAP concentrations up to 1000 µg/L, expressed as micrograms of PAP per liter of blood.

## **WARRANTY**

Any change or modification in the procedure recommended by the manufacturer may affect the results. In that instance, Dynabio S.A. disclaims all liability expressed, implicit or established by law, including liability resulting from the sale or transport prior to use. In that instance, Dynabio S.A. cannot be held responsible for resulting direct or indirect damages.

## **BIBLIOGRAPHY**

1. Iovanna *et al.* C R Acad Sci III. 1994;7:561-4.
2. Sarles *et al.* Arch Dis Child 1999;80:F118-22.
3. Barthelley *et al.* Arch. Pédiatr 2001;8:275-281.
4. Sarles *et al.* J Pediatr 2005;147:302-305.
5. Sarles *et al.* J Cyst Fibros 2014;13:384-90.
6. Blood collection on filter paper for newborn screening programs Approved Standards (reference NBS01-A6, 6th edition, 2013). Clinical and Laboratory Standards Institute.

## SUMMARY OF ASSAY

**Remember to prepare eluates of newborn blood spots the day before assay  
in a plate with U-shaped wells (not provided with the kit)**

1. Warm up to room temperature the assay plate and the elution plate.
2. Prepare dilution buffer, range and control.
3. After room temperature is reached, remove the plate from its wrapping, and deposit after homogenization the range, the blank, the control, and eluates from newborns (100  $\mu$ L/well, in duplicate).
4. Incubate 3h at room temperature.
5. Prepare wash buffer (add Tween in PBS prepared the day before).
6. After the 3h incubation, wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
7. Distribute the biotinylated antibodies (100  $\mu$ L/well).
8. Incubate 30 min at room temperature.
9. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
10. Distribute avidin-peroxidase conjugate (100  $\mu$ L/well).
11. Incubate 15 min at room temperature.
12. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
13. Distribute chromogenic substrate TMB (100  $\mu$ L/well).
14. Incubate 15 minutes at room temperature in the dark.
15. Without washing, stop the reaction by adding  $H_2SO_4$  (100  $\mu$ L/well).
16. Read absorbance at 450 nm.

## NOTES