*IVD*
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# MucoPAP-F

PAP assay kit for Cystic Fibrosis newborn screening

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### INTRODUCTION

The Pancreatitis-associated protein (PAP) is synthesized in pancreas only 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:

Iovanna et al. C R Acad Sci III. 1994;7:561-4.

Sarles et al. Arch Dis Child 1999;80:F118-22.

Barthellemy et al. Arch. Pédiatr 2001;8:275-281.

Sarles et al. J Pediatr 2005;147:302-305.

Sarles et al. J Cyst Fibros 2014;13:384-90.

PAP assay on Guthrie cards might therefore help screening CF newborns.

### PRINCIPLE OF THE ASSAY

The MucoPAP-F kit is designed to assay PAP in dried blood spots of newborns from Guthrie cards. It is a sandwich time resolved fluoro-immunoassay, in which the standard range and the internal controls are supplied as dried blood spots absorbed on standardised screening cards (Perkin Elmer IDB 226).

The wells of the microtitration plate are coated with anti-PAP antibodies. In a first step, the eluates of blood spots are deposited in the wells and PAP 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-antibody complexes are detected by a streptavidin-europium conjugate. The addition of a fluorescence enhancement solution allows the release of europium from streptavidin and its capture into highly fluorescent chelates, which emit at 620nm when excited at 337nm. The fluorescence intensity is proportional to the quantity of PAP bound in the first step.

**DYNABIO S.A.**
Luminy Biotech Entreprises - Case 922 - Parc de Luminy 13288 Marseille cedex 09
Téléphone : 33 (0)4 86 94 85 04 / Télécopie : 33 (0)4 86 94 85 03
Internet www.dynabio.com

## KIT COMPOSITION

| REAGENTS | CHARACTERISTICS |
|---|---|
| 96 wells microtitration plate (8x12) coated with anti-PAP antibodies. | Ready to use. |
| Standard range of recombinant PAP (rhPAP). | Blood spots to be punched and eluted in 150µl of PBS overnight (16h) at +4°C. |
| Biotinylated anti-PAP antibodies freeze-dried in a protein medium. | Reconstitute carefully with 11 mL of sterile $H_2O$ directly in the vial. |
| Assay Buffer. | Use to dilute streptavidin-europium conjugate 1/1000. |
| Solution of streptavidin-europium conjugate (0.1 mg/mL). | Dilute this conjugate 1/1000 in Assay Buffer. Prepare extemporaneously the volume required. |
| Fluorescence enhancement solution. | 22 mL (2 vials of 11 mL each). Ready to use. |
| PBS (tablet). | Dissolve in 1L $H_2O$. Save 20 mL for blood spot elution. |
| Solution of Tween 20 (10 %). | Add to the remaining 980 mL of PBS to obtain the washing buffer. |
| Internal Controls (3 internal assay standards). | Blood spots to be punched and eluted in 150µl of PBS overnight (16h) at +4°C. |

## STORAGE AND STABILITY

The assay kit should be kept at +4°C until the expiration date.

Reconstituted lyophilisate and washing solution should be used immediately or stored at -20°C.

After opening, unused plate strips, range and control bloodspots must be stored at +4°C in the supplied plastic bags with dessicant.

**Do not store streptavidin-europium diluted in assay buffer.** Prepare extemporaneously the required volume of diluted conjugate depending on the number of wells used. In these conditions, the assay kit can be used for 30 days after opening.

## MATERIALS NOT SUPPLIED WITH THE KIT AND NEEDED FOR THE ASSAY

Two litres of sterile water.
Material:
Vortex mixer,
A plate shaker,
A semi-automatic or automatic plate washer,
A plate reader for fluorescence measurement, equipped with a 337nm filter for excitation and a 620nm filter for emission,
Multi-channel micropipette,
1L plastic bottle (for washing buffer storage),
Disposable material:
U-bottom 96 well plates (for elution of blood spots),
Disposable 10 mL pipettes and micropipette tips,
**3 disposable reagent reservoirs: 1 per reagent (biotinylated antibodies/streptavidin-europium/enhancement solution).**

## PRECAUTIONS FOR USE

Do not pipette by mouth.
Do not eat, drink or smoke during the test.
The following reagents may be toxic and irritant and must be handled to avoid any contact with the skin, eyes and mucosae:
PBS tablet, assay buffer, streptavidin-europium conjugate and enhancement solution.
In case of accidental contact, rinse the affected parts immediately with plenty of water.

## RECOMMENDATIONS

Never allow the plate to dry since well drying may impair the quality of the results.
Special care should be taken to the washing steps in order to avoid non-specific signal.
Do not use reagents after their expiration date.
Do not mix reagents from different batches.
Avoid biological and chemical contamination of reagents and samples.
To avoid any cross-contamination between reagents, use a different reservoir for each reagent.
As a heat-labile reagent, enhancement solution must be stored at +4°C.
Equilibrate every reagent at room temperature before use.
Freeze-dried biotinylated antibodies must be reconstituted at least 10 minutes before use in order to obtain total and homogeneous dissolution.
Shake reagents gently before use.
Respect strictly incubation times.

## PREPARATION OF SAMPLES AND STANDARDS

**On the day before the assay:**

Dissolve the PBS tablet in 1L of sterile water**.** After complete dissolution, save 20 mL for blood spots elution: the remaining 980 mL are stored at +4°C until the assay on the next day, to prepare the washing buffer.

***Samples to assay***: Punch in the Guthrie card a disc of 3 mm in diameter, in a region with thorough blood impregnation, without overload. To obtain a duplicate assay, punch two distinct discs on the same blood spot. Put each disc in a well of a 96 U-bottom well plate. Add 150 µl of PBS per well. Allow 16 h for elution (overnight) at +4°C.

***Standard PAP range:*** Like the samples to assay, the 6 standard concentrations must be punched in duplicate (3 mm diameter discs) from the card supplied in the kit. Put each disc in a well of a 96 U-bottom well plate. Add 150 µl of PBS per well. Allow 16 h for elution (overnight) at +4°C. The standard PAP concentrations in the 6 blood spots are: 6.25 / 3.13 / 1.56 / 0.78 / 0.39 and 0 µg/L.

***Internal Controls:*** Like the range, the 3 internal controls must be punched in duplicate from the card supplied in the kit. Put each disc in a well of a 96 U-bottom well plate. Add 150 µl of PBS per well. Allow 16 h for elution (overnight) at +4°C. When assayed with the supplied blood spot range, the PAP concentrations in the 3 control blood spots are respectively 1 µg/L (Low Control), 2 µg/L (Medium Control) and 3 µg/L (High Control).

## 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.

***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 (980 mL remaining).

***Biotinylated antibodies:*** The lyophilisate is dissolved in 11 mL of sterile water directly in the vial.

***Assay Buffer*:** Assay buffer is used to prepare a 1/1000 dilution of streptavidin-europium conjugate (see below).

***Streptavidin-Europium conjugate:*** Dilute this conjugate 1/1000 in Assay Buffer to get 0.1 µg/mL. The volume of diluted conjugate to be prepared depends on the number of wells used. This dilution has to be prepared extemporaneously, during the incubation of biotinylated antibodies in the plate.

***The enhancement solution*** is ready to use.

## ASSAY PROCEDURE

The PAP range is obtained after elution in PBS of 6 discs punched in blood spots supplied in the kit. This range includes the following concentrations: 6.25 / 3.13 / 1.56 / 0.78 / 0.39 and 0 µg/L.

Each eluated standard concentration is assayed in duplicate (100 µl/well). The two wells filled with the standard 0 µg/L will be used to evaluate the background value.

Eluates of newborn samples, as well as eluates of the range and internal controls, are deposited in the wells in duplicate (100 µl/well) after homogeneisation, and incubated 3 h at room temperature (~21°C) under shaking, after covering the plate with supplied adhesive. 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 ~300 µl of washing buffer
- Repeat the first two steps 4 times
- After the last wash, eliminate residual liquid by inverting the plate and tapping it on absorbent paper.

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

The reconstituted solution of biotinylated anti-PAP antibodies is then immediately deposited on the plate (100 µl/well) and incubated for 30 minutes at room temperature under shaking (the plate is covered with adhesive). The plate is then washed 5 times with washing buffer as described above. Then 100 µl of the 0.1 µg/mL solution of streptavidin-europium conjugate in assay buffer is added to each well and incubated 30 minutes at room temperature under shaking. The plate is washed 5 times with washing buffer as described above.
After the plate has been tapped dry, the enhancement solution is added (**200 µl/well**) and the plate placed under shaking. Do not cover the plate with adhesive as it may quench the signal.

After at least 30 minutes of incubation, the fluorescence signal of each well is measured on a spectrofluorometer, using a 337nm filter for excitation and a 620nm filter for emission. Plates should not be read in the first 30 minutes after addition of enhancement solution as signal is not completely stable. The signal remains stable until the next day if the plate is stored at +4°C overnight.

**Important note**: the incubation time of enhancement solution proposed in this protocol (30 minutes) has been determined using a Fluostar Omega reader (BMG Labtech). This time may differ with the type and sensitivity of the spectrofluorometer used. Users may adjust the duration of fluorescence enhancement according to their requirements.

## CALCULATION OF RESULTS

The concentration of PAP in eluates is determined by extrapolation from the standard curve, constructed by plotting the mean fluorescence counts of each point of the range versus the theoretical concentrations. The use of a computer program to define, from range values, the parameters of the [PAP] = f(fluorescence counts) function, and use of that function to calculate PAP concentrations in eluates is recommended.

### *EXAMPLE OF RESULTS*

*NB: This is only an example. A reference curve must be obtained for each plate.*

| PAP µg/L | Fluorescence counts | Fluorescence counts (- background) |
|---|---|---|
| 0 | 4125 | / |
| 0.39 | 10893 | 6768 |
| 0.78 | 18216 | 14091 |
| 1.56 | 32815 | 28690 |
| 3.13 | 61356 | 57231 |
| 6.25 | 117948 | 113823 |

## ANALYSIS OF RESULTS

Calculation of PAP concentrations in newborn blood: if the protocol given above is followed strictly and if the samples come from PerkinElmer IDB 226 screening cards and punches of ∅3mm, the PAP concentration in newborn blood is directly obtained by using the equation of the standard curve [PAP] = f(fluorescence counts).

## QUALITY CONTROL

Regarding the results obtained for controls supplied in the kit, it is recommended that their concentrations do not vary from more than +/-20% from the values indicated on the control card and in the leaflet. These limits are indicated in the table below:

| Control | Lower threshold | Upper threshold |
|---|---|---|
| Low – 1 µg/L | 0.8 µg/L | 1.2 µg/L |
| Medium – 2 µg/L | 1.6 µg/L | 2.4 µg/L |
| High – 3 µg/L | 2.4 µg/L | 3.6 µg/L |

## SUMMARY OF THE ASSAY

- Do not forget to prepare all the eluates the day before –

1 Prepare buffers and homogenize the eluates.

2 After equilibration at room temperature, open the aluminium bag and deposit in duplicate the eluates of the range, internal controls and newborn samples in the plate (100 µL/well).

3 Incubate 3h at room temperature under shaking.

4 Perform 5 washes, tap dry on absorbent paper.

5 Distribute the biotinylated antibody (100 µL/well).

6 Incubate 30 min at room temperature under shaking.

7 Perform 5 washes, tap dry on absorbent paper.

8 Distribute streptavidin-europium conjugate (100 µL/well).

9 Incubate 30 min at room temperature under shaking.

10 Perform 5 washes, tap dry on absorbent paper.

11 Distribute the enhancement solution (200 µL/well).

12 Incubate at least 30 min at room temperature under shaking.

13 Read fluorescence at 620 nm after excitation at 337nm.