

## MucoPAP

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. Four 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.

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

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

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

#### PRINCIPLE OF THE ASSAY

The MucoPAP kit is a sandwich enzyme-linked immunoassay (ELISA) optimized to assay PAP in dried blood spots on Guthrie cards.

The wells of the microtitration plate are coated with antibodies to PAP. 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 an avidin-peroxidase complex and are visualized by the addition of a chromogenic substrate. The intensity of the colour reaction is proportional to the quantity of PAP bound in the first step.

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## KIT COMPOSITION

REAGENTS	CHARACTERISTICS
One 96-well microtitration plate coated with anti-PAP antibodies.	Ready to use
Standard solution of recombinant PAP (rhPAP), freeze-dried in a protein buffer.	Reconstitute with the volume of sterile apyrogenic H <sub>2</sub> O mentioned on the vial label.
Dilution buffer, freeze-dried in a protein medium.	Reconstitute in the vial with 11 ml of sterile H <sub>2</sub> O.
Solution of biotinylated anti-PAP antibodies freeze-dried in a protein medium.	Reconstitute carefully with 11 ml sterile H <sub>2</sub> O.
Solution of avidin-POD freeze-dried in a protein medium.	Reconstitute carefully with 11 ml sterile H <sub>2</sub> O.
Solution of chromogenic substrate TMB-H <sub>2</sub> O <sub>2</sub> .	Ready to use
H <sub>2</sub> SO <sub>4</sub> solution.	Ready to use
PBS (tablet).	Dissolve in 1L H <sub>2</sub> O. Save 15 ml for blood spot elution.
Solution of Tween 20 (10 %).	Add to the remaining 985 ml de PBS to obtain the washing buffer.
Control Serum	Reconstitute with the volume of sterile H <sub>2</sub> O mentioned on the vial label.

## STORAGE AND STABILITY

The assay kit should be kept at 4°C until the printed expiration date. Once lyophilisates are reconstituted, they should be stored at -20°C, except for the avidin-POD solution witch remains stable at 4°C. The wash solution can be stored at -20°C. With these storage 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 distilled water.

Material:

Vortex mixer.

A semi-automatic or automatic plate washer

A plate reader for measuring absorbance at 450 nm, equipped with a 630 nm reference filter

Disposable tip micropipettes, single- and multi-channel.

1L bottle (washing buffer storage)

Disposable material:

U-bottom 96 well plates (elution of blood spots)

Pipettes 10 ml

1.5 ml microcentrifuge tubes (preparation of the range)

## PRECAUTIONS FOR USE

Use limited to research.

Do not pipette by mouth.

Do not eat, drink or smoke during the test.

Reagents containing H<sub>2</sub>O<sub>2</sub>, the H<sub>2</sub>SO<sub>4</sub> solution and the PBS tablet may be toxic and irritant. They must be handled to avoid any contact with the skin, eyes and mucosae.

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.

Equilibrate every reagent at room temperature.

Freeze-dried reagents 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.

## SAMPLE PREPARATION

Punch in the Guthrie card a disc 3 mm in diameter, in a region with thorough blood impregnation, without overload. Put the disc in a well of a 96 U-bottom well plate. Add 150 µl PBS buffer. Allow 16 h for elution (overnight) at 4°C.

## PREPARATION OF REAGENTS

*The plate* packaged under vacuum must be equilibrated at room temperature before removal from its wrapping.

*The wash buffer* (PBS/0.1% Tween) is obtained by dissolving the PBS tablet in 1L of distilled water. After complete dissolution, save 15 ml for blood elution then add to the rest of the buffer the whole content of the Tween 20 vial.

*The standard range* is prepared from freeze-dried rhPAP. This standard solution is reconstituted with the volume of distilled water mentioned on the vial label. This gives a solution at 4 ng/ml that is further diluted in dilution buffer to prepare the standard range.

*The dilution buffer* is to be reconstituted by adding to the vial 11 ml of distilled water.

*Biotinylated antibodies* The lyophilisate is dissolved in 11 ml of distilled water.

*Avidin-POD* The lyophilisate is dissolved in 11 ml of distilled water.

*The substrate-chromogen solution* (TMB-H<sub>2</sub>O<sub>2</sub>) is ready to use.

*The Control Serum* (internal control) is reconstituted with the volume of distilled water mentioned on the vial label.

## ASSAY PROCEDURE

A PAP range is prepared from the reconstituted reference solution (4 ng/ml). It will include solutions with concentrations ranging from 0.25 to 0.015 ng/ml. The dilution buffer provided with the kit will be used to prepare successive dilutions.

## PREPARATION OF THE PAP RANGE

Dilute 1/2 the standard PAP solution. That solution (PAP = 2 ng/ml) will be used to prepare serial dilutions with the dilution buffer.

500 µl of solution at 2 ng/ml + 500 µl buffer = 1 ng/ml

500 µl of solution at 1 ng/ml + 500 µl buffer = 0.5 ng/ml

500 µl of solution at 0.5 ng/ml + 500 µl buffer = 0.25 ng/ml

500 µl of solution at 0.25 ng/ml + 500 µl buffer = 0.125 ng/ml

500 µl of solution at 0.125 ng/ml + 500 µl buffer = 0.06 ng/ml

500 µl of solution at 0.06 ng/ml + 500 µl buffer = 0.03 ng/ml

500 µl of solution at 0.03 ng/ml + 500 µl buffer = 0.015 ng/ml

Each dilution will be assayed in duplicate (100 µl/puits). Background value will be obtained in duplicate (100 µl buffer/well)

Samples, the PAP range and the control sample are deposited in wells (100 µl/well) and incubated 3 h at room temperature (~21°C), after covering the plate with adhesive. Then, wells are washed 5 times with the wash buffer, as follows:

- Thoroughly draw up the wells
- Fill with ~300 µl wash 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 at 100 µl/well and incubated for 30 minutes at room temperature. The plate is covered with adhesive. The plate is then washed 5 times with PBS/0.1 % Tween (as described above) and then 100 µl of the reconstituted avidin-POD solution is immediately added to each well.

After 15 minutes incubation at room temperature, the plate is washed 5 times with PBS/0.1% Tween. During the last wash, washing buffer should be left 1 min in the wells. After the plate has been tapped dry, the chromogenic substrate is added at 100 µl/well. After 10 to 15 minutes of incubation in the dark, the color reaction is stopped by adding H<sub>2</sub>SO<sub>4</sub>, 100 µl/well. The absorbance of each well is read on a spectrophotometric plate reader at 450 nm, using a 630 nm filter as reference. **Plates should be read immediately after the reaction is stopped**

## CALCULATION OF RESULTS

The concentration of PAP in each sample is determined by extrapolation from the standard curve. This curve is constructed by plotting the mean absorbance values of each point of the range versus the theoretical concentrations. The use of a computer programme to define, from range values, the parameters of the [PAP] = f(D.O.450nm) function, and use 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 ng/ml	Absorbance (D.O. 450 nm)	Absorbance (- background)
0	0.039	
0.015	0.192	0.153
0.03	0.346	0.307
0.06	0.647	0.608
0.125	1.191	1.152
0.25	1.925	1.886

## ANALYSIS OF RESULTS

1/ Calculation of PAP concentrations in blood : If the protocol given above is followed strictly and if the samples come from screening cards made from Whatmann paper ref. BFC180-B997243 (approved by the French Screening Association), raw data (PAP concentrations in eluates) have to be multiplied by a factor of 30 to obtain PAP blood concentrations. This factor must also be applied to the Control Serum.

2/ Ongoing studies show that, with the present test, median blood PAP concentration in newborns is ~0.8 ng/ml. This data is only indicative.

## SUMMARY OF THE ASSAY

- 1 Prepare buffers and the PAP range
- 2 After equilibration at room temperature, open the aluminium bag and deposit the samples and the range in the plate (100 µl/well) Incubate 3h at room temperature
- 3 Draw up liquid, 5 washes
- 4 Distribute the biotinylated antibody (100 µl/well)
- 5 Incubate 30 min at room temperature
- 6 Draw up liquid, 5 washes
- 8 Distribute avidin-POD (100 µl/well)
- 9 Incubate 15 min at room temperature
- 10 Draw up liquid, 5 washes. Dry.
- 11 Distribute the substrate-chromogen mix (100 µl/well).
- 12 Incubate 10-15 min in the dark
- 13 Stop the reaction with H<sub>2</sub>SO<sub>4</sub> (100 µl/well).
- 14 Read absorbance at 450 nm.