IVD
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MucoPAP II

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 II kit is designed to assay PAP in dried blood spots of newborns from Guthrie cards. It is a sandwich enzyme-linked immunoassay (ELISA), 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 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
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.
Solution of avidin-POD freeze-dried in a protein medium.	Reconstitute carefully with 11 ml of sterile H_2O directly in the vial.
Solution of chromogenic substrate TMB-H_2O_2.	Ready to use.
H_2SO_4 solution.	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. Once lyophilisates are reconstituted, they should be used immediately or stored at -20°C, except for the avidin-POD solution which remains stable at +4°C. The washing solution can be stored at -20°C. After opening, unused plate strips, range and control bloodspots must be stored at +4°C in plastic bags with dessicant (supplied in the kit). 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 sterile 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,
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,
Disposable reagent reservoirs.

PRECAUTIONS FOR USE

Do not pipette by mouth.
Do not eat, drink or smoke during the test.
Reagents containing H_2O_2, the H_2SO_4 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 before use.
Freeze-dried reagents must be reconstituted at least 10 minutes before use in order to obtain total and homogeneous dissolution.
Do not expose chromogen TMB to air and light before use.
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.

Avidin-POD: The lyophilisate is dissolved in 11 ml of sterile water directly in the vial.

The substrate-chromogen solution (TMB-H_2O_2) is ready to use.

H_2SO_4 solution to stop the reaction 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 eluted 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 homogenisation, and incubated 3 h at room temperature (~21°C), 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 semi-automatic 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 (after covering the plate with adhesive).
The plate is then washed 5 times with washing buffer as described above.
Then 100 µl of the reconstituted avidin-POD solution is added to each well and incubated 15 minutes at room temperature.
The plate is washed 5 times with washing buffer as described above.
After the plate has been tapped dry, the chromogenic substrate is added (100 µl/well). After 15 minutes of incubation in the dark, the color reaction is stopped by adding 100 µl/well of H_2SO_4 solution (bringing the total volume to 200 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 within 30 minutes after the reaction is stopped.**

CALCULATION OF RESULTS

The concentration of PAP in eluates is determined by extrapolation from the standard curve, constructed by plotting the mean absorbance values 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(D.O.450nm) 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	Absorbance (D.O. 450 nm)	Absorbance (- background)
0	0.080	
0.39	0.152	0.072
0.78	0.284	0.204
1.56	0,566	0,486
3.13	1,114	1,034
6.25	2,006	1,926

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(D.O.450nm).

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.
4 Perform 5 washes, tap dry on absorbent paper.
5 Distribute the biotinylated antibody (100 µl/well).
6 Incubate 30 min at room temperature.
7 Perform 5 washes, tap dry on absorbent paper.
8 Distribute avidin-POD (100 µl/well).
9 Incubate 15 min at room temperature.
10 Perform 5 washes, tap dry on absorbent paper.
11 Distribute the substrate-chromogen mix (100 µl/well).
12 Incubate 15 min in the dark at room temperature.
13 Stop the reaction with H_2SO_4 (100 µl/well).
14 Read absorbance at 450 nm.