IVD
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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.

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 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
8x12 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 H_2O mentioned on the vial label.
Dilution buffer, freeze-dried in a protein buffer.	Reconstitute carefully in the vial with 11 ml of sterile H_2O.
Solution of biotinylated anti-PAP antibodies freeze-dried in a protein buffer.	Reconstitute carefully in the vial with 11 ml of sterile H_2O.
Solution of avidin-POD freeze-dried in a protein buffer.	Reconstitute carefully in the vial with 11 ml of sterile H_2O.
Solution of chromogenic substrate TMB-H_2O_2.	Ready to use.
H_2SO_4 solution.	Ready to use.
PBS (tablet).	Dissolve in 1L sterile H_2O. Save 15 ml for blood spot elution.
Solution of Tween 20 (10 %).	Add to the remaining 985 ml of PBS to obtain the washing buffer.
Control Serum.	Reconstitute with the volume of sterile H_2O 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 which remains stable at +4°C. The washing solution can be stored at -20°C. Unused strips must be stored in the plastic bag with dessicant at +4°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 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
- Micropipettes, single- and multi-channel,
- 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.

SAMPLE PREPARATION

The day before the assay: punch in the Guthrie card a disc of 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 of PBS. 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 washing buffer (PBS/0.1% Tween) is obtained by dissolving the PBS tablet in 1L of sterile 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 sterile water mentioned on the vial label. This gives a solution at 0,5 µg/L 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 sterile water.

Biotinylated antibodies are to be reconstituted by adding to the vial 11 ml of sterile water.

Avidin-POD is to be reconstituted by adding to the vial 11 ml of sterile water.

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

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

ASSAY PROCEDURE

A PAP range is prepared from the reconstituted reference solution (0.5 µg/L). It will include solutions with concentrations ranging from 0.125 to 0.0078 µg/L. The dilution buffer provided with the kit will be used to prepare successive dilutions.

Preparation of the PAP range

Example for one PAP range assayed in duplicate:

The standard PAP solution at 0.5 µg/L has to be diluted 1/4 in dilution buffer to give a concentration of 0.125 µg/L:

150 µL of solution at 0.5 µg/L + 450 µL buffer = 0.125 µg/L

This solution at 0.125 µg/L is then used to prepare serial 1/2 dilutions in dilution buffer:

300 µL of solution at 0.125 µg/L + 300 µL buffer = 0.062 µg/L

300 µL of solution at 0.062 µg/L + 300 µL buffer = 0.031 µg/L

300 µL of solution at 0.031 µg/L + 300 µL buffer = 0.015 µg/L

300 µL of solution at 0.015 µg/L + 300 µL buffer = 0.0078 µg/L

Each dilution from 0.125 to 0.0078 µg/L is assayed in duplicate (100 µl/well). Background value (0 µg/L) is obtained in duplicate (100 µl of dilution buffer/well).

The PAP range (0.125 to 0 µg/L), samples and the control serum are deposited in the wells (100 µl/well) and incubated 3 hours at room temperature (~21°C), after covering the plate with 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 in the plate at 100 µl/well. The plate is covered with adhesive and incubated for 30 minutes at room temperature. The plate is then washed 5 times with washing buffer.

After the plate has been tapped dry, 100 µl of the reconstituted avidin-POD solution is added to each well and let for incubation during 15 minutes at room temperature.The plate is then washed 5 times with washing buffer. 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 and the plate is placed in the dark. After 15 minutes of incubation, the color reaction is stopped by adding H_2SO_4, 100 µl/well (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 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

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

Range of PAP (µg/L)	Absorbance (D.O. 450 nm)	Absorbance (- background)
0	0.039	
0.0078	0.117	0.078
0.015	0.192	0.153
0.031	0.346	0.307
0.062	0.647	0.608
0.125	1.191	1.152

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, **raw data (PAP concentrations in eluates) have to be multiplied by a factor of 50 to obtain PAP blood concentrations** (a disc of PerkinElmer IDB226 paper contains 3 µl of blood. Elution in 150 µl of PBS corresponds to a 1/50 dilution of blood).
This factor must also be applied to the freeze-dried Control Serum to obtain the concentration indicated on the label.

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