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MucoPAP-F

PAP assay kit for Cystic Fibrosis newborn screening

INSERM Patent

Time-resolved fluoro-immunoassay (TRF-IA)

Instruction manual and reagents for 96 assays

Manufactured by:

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FRANCE

REF MPK03

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

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INTRODUCTION

The Pancreatitis-Associated Protein (PAP, also known as Reg3A) 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 having cystic fibrosis.

PRINCIPLE OF THE ASSAY

The MucoPAP-F assay kit is designed for quantitative determination of PAP in dried blood spots of newborns from screening cards calibrated and approved by competent authorities. It is a sandwich time resolved fluoro-immunoassay (TRF), in which the standard range and the internal controls are supplied as dried blood spots deposited on standardised screening cards, as the newborn samples to assay.

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 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 a streptavidin-europium conjugate. After a last washing step, the addition of a fluorescence enhancement solution allows the release of europium from streptavidin and its capture into highly fluorescent chelates, which emit at 620 nm when excited at 337 nm. The fluorescence intensity 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
- Plate shaker (orbital / 300 rpm = rotations per minute)
- Semi-automatic or automatic plate washer
- Plate reader for fluorescence measurement, equipped with a 337 nm filter for excitation and a 620 nm filter for emission
- Computer paired with the reader for results analysis
- Single and multi-channel micropipettes
- One litre container (for washing buffer),
- Manual or automatic hole-puncher (3mm diameter) to sample screening cards
- Two litres of distilled water

Disposable material:

- U-bottom 96 well plates (for elution of blood spots),
- Micropipette tips
- Disposable 10 mL pipettes
- Four disposable identified reagent reservoirs (one per reagent): PBS, biotinylated antibodies, streptavidin-europium and enhancement solution
- 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 controls.

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 bags with dessicant provided in the kit, for a maximum of 30 days.
Standard range of PAP: 0 µg/L 0.39 µg/L 0.78 µg/L 1.56 µg/L 3.13 µg/L 6.25 µg/L		Blood spots on calibrated filter paper to be punched and punches eluted in 150 µL of PBS overnight (16h), between +2°C and +8°C.	
Internal controls: Low 1 µg/L Medium 2 µg/L High 3 µg/L			
Biotinylated anti-PAP antibodies	Stable if stored between +2°C et +8°C until expiry date.	Lyophilisate to be gently dissolved in 11 mL of distilled water, directly in the vial.	Keep at -20°C for 30 days maximum.
Assay buffer (for conjugate dilution)		Vial containing 11 mL. Use to dilute 1/1000 the streptavidin-europium conjugate.	Keep between +2°C et +8°C for 30 days maximum.
Streptavidin-europium conjugate (0.1 mg/mL)		Dilute 1/1000 in the corresponding assay buffer. Prepare extemporaneously the required volume.	Discard the remaining 1/1000 dilution.
Fluorescence enhancement solution		2 vials, each containing 11 mL. Ready to use.	Keep between +2°C and +8°C for 30 days maximum.
PBS tablet		Dissolve in 1 L of distilled water. Keep 20 mL for elution of blood spots.	Keep the prepared washing buffer at -20°C for 30 days maximum.
Tween 20 (10 % solution)		Add to the remaining 980 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 controls	Filter paper with 2 series of 6 dried blood spots for the range and 2 series of 3 dried blood spots for the controls, containing known amounts of PAP
Biotinylated antibodies to PAP	PAP-specific mouse monoclonal antibodies conjugated to biotin, in a phosphate buffered solution containing protective agents
Assay buffer for dilution of the streptavidin-europium conjugate	Tris-HCl buffered saline containing bovine proteins, a detergent and antibacterial agents
Streptavidin-europium conjugate (0.1 mg/mL)	Europium conjugated to streptavidin, in Tris-HCl buffered saline containing protective and antibacterial agents
Fluorescence enhancement solution	Solution containing acetic acid, detergent and chelators
PBS tablet	Phosphate buffered saline
Tween 20 (10 % solution)	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). If blood cannot be directly dropped on filter paper, do not use blood collected on EDTA or citrate, as such anticoagulants chelate europium and will alter the results of the assay.

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 and 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, range and controls as well as biotinylated antibodies contain products of human or animal origin. 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, assay buffer, streptavidin-europium conjugate and enhancement solution. In case of accidental contact, rinse the affected parts immediately with plenty of water.

RECOMMENDATIONS FOR USE

Establish a plate layout defining the sequence of samples in the wells (range, blank, controls and newborn eluates) and follow it strictly to avoid switching punches.

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 biotinylated antibodies must be put in solution at least 10 minutes before use, to ensure complete dissolution and adequate homogeneity of the reagent.

The fluorescence enhancement solution is heat-labile and must imperatively be stored between +2°C and +8°C.

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

PREPARATION OF SAMPLES, RANGE AND CONTROLS

The day before the assay:

Dissolve the PBS tablet in 1L of distilled water. After complete homogenization, save 20 mL for blood spots elution: the remaining 980 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.

Standard PAP range: Like the samples to assay, punch for each standard concentration a disc of 3 mm in diameter from the card supplied in the kit, imperatively on the periphery of the blood spot. The six standard concentrations must be punched in duplicate. Put each disc in a well of the 96 U-bottom well plate (not provided). Add 150 µL of PBS per well. Allow 16 hours for elution (overnight) between +2°C and +8°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, imperatively on the periphery of the blood spot (3 mm diameter discs). Put each disc in a well of the 96 U-bottom well plate (not provided). Add 150 µL of PBS per well. Allow 16 hours for elution (overnight) between +2°C and +8°C. The PAP concentration in the three 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. Micropipettes tips have to be changed between each sample, standard PAP range or internal controls homogenized eluate.

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% solution) vial to the rest of PBS dissolved the day before (980 mL remaining) and homogenize.

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

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

Streptavidin-Europium conjugate: Dilute this conjugate 1/1000 in Assay Buffer to get a concentration of 0.1 $\mu\text{g}/\text{mL}$. 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.

Enhancement solution: Ready to use after homogenization.

ASSAY PROCEDURE

The PAP range is obtained after elution in PBS of the six standard concentrations punched from the supplied blood spots. This range includes the following concentrations: 6.25 / 3.13 / 1.56 / 0.78 / 0.39 and 0 $\mu\text{g}/\text{L}$, obtained in duplicate.

Each standard concentration, eluted in duplicate, is deposited in the assay plate after homogenization (100 $\mu\text{L}/\text{well}$). Micropipettes tips have to be changed between each standard PAP range homogenized eluate. The two wells filled with the standard 0 $\mu\text{g}/\text{L}$ will be used to evaluate the background value.

Eluates of newborn samples, as well as eluates of internal controls, are deposited in duplicate in the assay plate after homogenization (100 $\mu\text{L}/\text{well}$). Micropipettes tips have to be changed between each sample or internal controls homogenized eluate.

The assay plate is incubated 3 hours at room temperature (+19°C to +22°C) under orbital shaking (300 rpm), 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 (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 $\mu\text{L}/\text{well}$) and incubated for 30 minutes at room temperature under shaking (orbital, 300 rpm), after covering the plate with the adhesive.

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

Then the solution of streptavidin-europium conjugate diluted at 0.1 $\mu\text{g}/\text{mL}$ in assay buffer is added to each well (100 $\mu\text{L}/\text{well}$) and incubated 30 minutes at room temperature under shaking (orbital, 300 rpm), after covering the plate with the adhesive.

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

The enhancement solution is then added in the plate (**200 µL/well**). Do not cover the plate with adhesive as it may quench the signal.

After at least 30 minutes of incubation under shaking (orbital, 300 rpm), the fluorescence signal of each well is measured on a spectrofluorometer, using a 337 nm filter for excitation and a 620 nm filter for emission. Plates should not be read in the first 30 minutes after addition of enhancement solution as signal is not completely stable.

NB: The minimum incubation time of the enhancement solution suggested in this protocol was established using a Fluostar Omega reader (BMG Labtech).

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 controls and samples before calculating the mean of duplicates.

The table hereunder gives an example of a PAP range generated with a mean background of 4125 counts (results provided for information only):

PAP (µg/L)	Fluorescence				
	Replicate 1	Replicate 2	Replicate 1 - mean background	Replicate 2 - mean background	Mean
0	4200	4050			
0.39	10617	11169	6492	7044	6768
0.78	20409	16023	16284	11898	14091
1.56	33079	32551	28954	28426	28690
3.13	58947	63764	54822	59639	57231
6.25	118902	116993	114777	112868	113823

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

Quality control

Assaying internal controls is recommended to ensure the quality of results. Controls must be processed exactly as samples. Three controls with each a different PAP concentration (low, medium, high) are provided with the kit. Controls must be included in each assay, like the standard range. If the run of the day involves several plates, controls must be included in each plate. It is recommended that results obtained for controls are within +/-20% from their theoretical values:

Controls – Theoretical concentration	Lower limit	Higher limit
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

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

In case of recurrent problems with assay performance, please contact Dynabio S.A. by email at 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 fluorescence intensity).

LIMITS OF THE ASSAY

Information given by blood PAP concentration obtained with the MucoPAP-F 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.

The sample must not contain EDTA or citrate that may chelate europium.

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/.

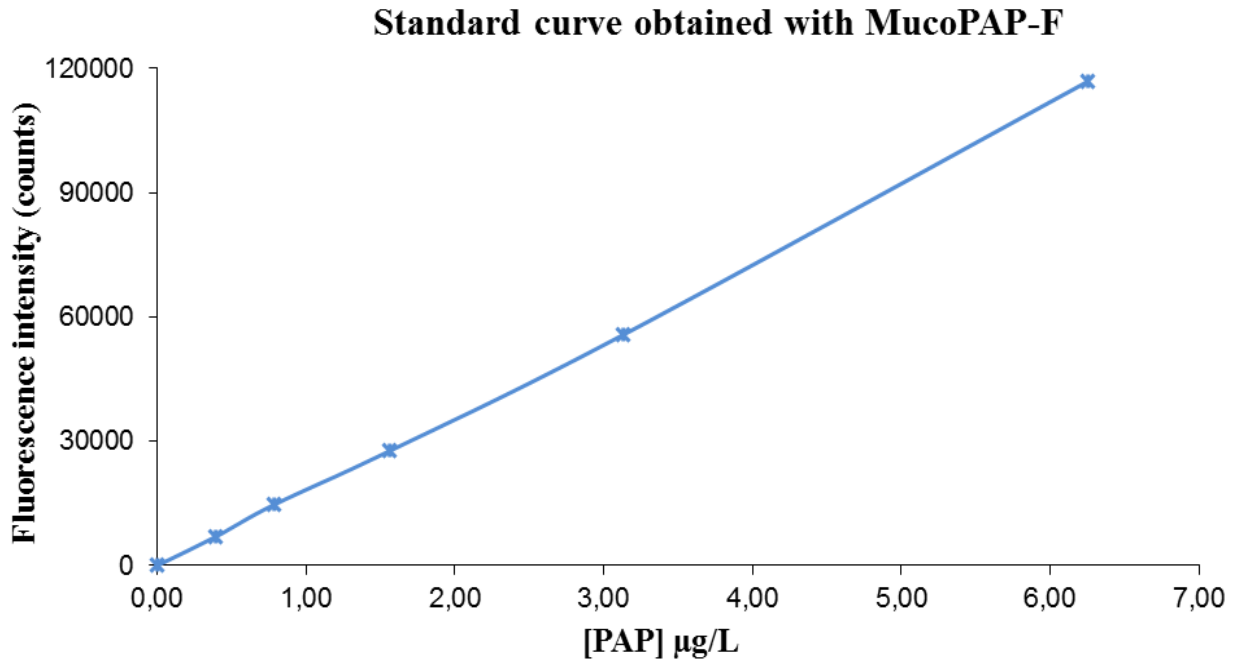
It is recommended to review this document before implementing a CF newborn screening strategy involving PAP assay.

PERFORMANCES

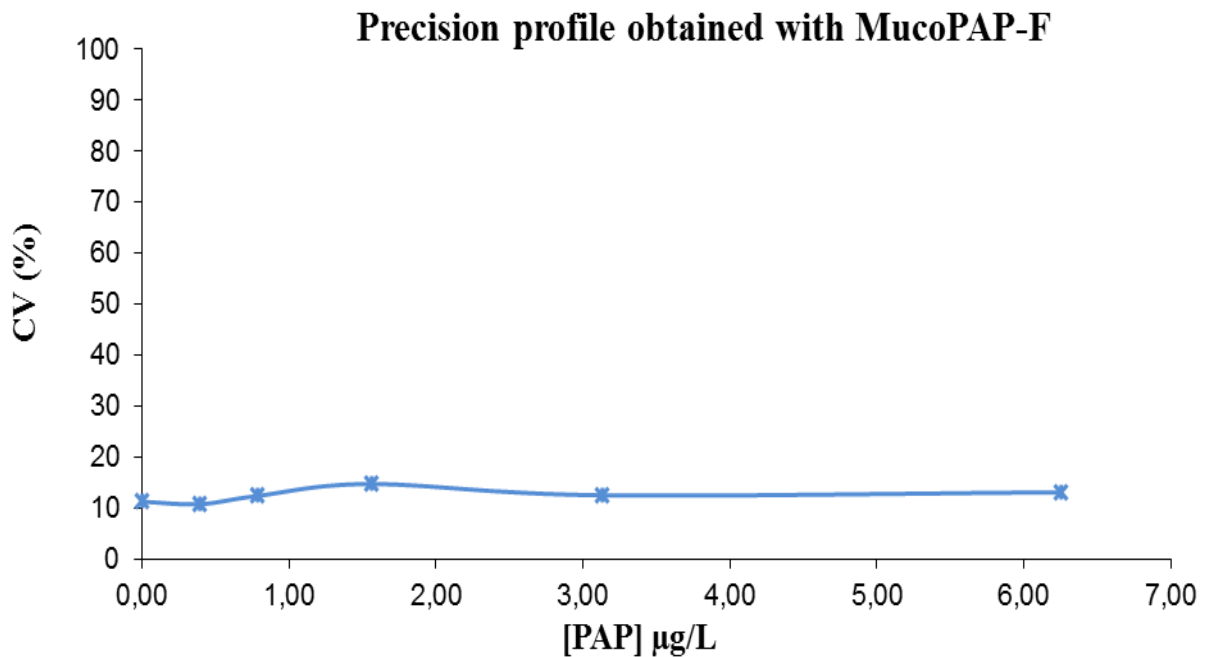
In newborns with elevated IRT (>50 µg/L), most CF had PAP > 1.75 µg/L (except for mild forms and for meconium ileus). CF newborns represent about 25% of babies with elevated IRT and PAP >1.75 µg/L. In that group, non-CF babies were often premature, babies with severe intestinal infection or with trisomy (4, 7).

ANALYTICAL CHARACTERISTICS

Standard range: A typical standard range of the MucoPAP-F kit is shown below. It was established using four different lots of kits and punching nine times the six points of the range of each lot.



Precision profile: Precision profile of the MucoPAP-F kit was established using four different lots of kits and punching nine times the six points of the range of each lot. It is shown in the graph below.



Repeatability and reproducibility: Repeatability and reproducibility of the MucoPAP-F kit were established using 5 different lots of kits and punching 11 times each of the three internal controls provided with the kits. Repeatability gives an estimate of the intra-lot variation (n = 11) and reproducibility an estimate of the inter-lot variation (n = 5).

Expected value of the control (µg/L)	Measured value (µg/L)	Repeatability (%)	Reproducibility (%)
1	0.990	11.5	13.9
2	2.100	9.8	9.7
3	3.150	8.0	10.2

Detection and quantitation limits: Detection and quantitation limits of the MucoPAP-F assay (expressed as micrograms of PAP per liter of blood) are respectively 0.24 µg/L and 0.32 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-F assay with IL2, IL6, IFN γ , TNF α 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.

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SUMMARY OF ASSAY

Remember to prepare eluates of blood spots in PBS (150 µL/well) the day before the 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. After room temperature is reached, remove the plate from its wrapping, and deposit after homogenization the eluates from range, controls and samples to be assayed (100 µL/well, in duplicate).
3. Incubate 3h at room temperature under orbital shaking (300 rpm).
4. Prepare wash buffer (add Tween in PBS prepared the day before).
5. 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.
6. Distribute the biotinylated antibodies (100 µL/well).
7. Incubate 30 min at room temperature under orbital shaking (300 rpm).
8. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
9. Distribute streptavidin-europium conjugate (100 µL/well).
10. Incubate 30 min at room temperature under orbital shaking (300 rpm).
11. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
12. Distribute the enhancement solution (200 µL/well).
13. Incubate 30 min at room temperature under orbital shaking (300 rpm).
14. Read fluorescence at 620 nm after excitation at 337 nm.

NOTES