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PancrePAP

IMMUNOENZYMATIC KIT FOR HUMAN PAP ASSAY

INSERM Patent

For Research Use Only

Enzyme-linked immunosorbent assay (ELISA)
Instruction manual and reagents for 96 assays

Manufactured by:
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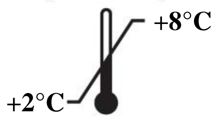
REF **PPK01**

SYMBOLS

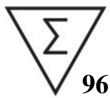
Lot number

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) was initially described as a protein synthesized by the pancreas during pancreatic stress (1). It was also studied in oncology (2).

PRINCIPLE OF THE ASSAY

The PancrePAP kit is designed for quantitative determination of PAP in serum, plasma or even whole blood samples from patients. It is a sandwich enzyme-linked immunosorbent assay (ELISA), in which the standard range and the internal control are supplied lyophilised, to be reconstituted with distilled water and diluted to obtain precise PAP concentrations described in this manual.

The wells of the microtitration plate are coated with anti-PAP antibodies. In a first step, the range, the internal control and the samples to assay 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 an avidin-peroxidase conjugate. After a last washing step, the addition of a chromogenic substrate of peroxidase enzyme leads to the release of a blue degradation product. Enzymatic reaction is stopped by the addition of an acid solution, which transforms coloration from blue to yellow. The intensity of yellow coloration, measured by spectrophotometry at 450 nm, 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
- Semi-automatic or automatic plate washer
- Plate reader for absorbance measurement, equipped with a 450 nm filter (and eventually with a 630 nm reference filter to subtract blue coloration residual intensity to optical density measured at 450 nm)
- Computer paired with the reader for results analysis
- Single and multi-channel micropipettes
- One litre container (for washing buffer)
- Two litres of distilled water

Disposable material:

- Micropipette tips
- Disposable 10 mL pipettes
- Four disposable reagent reservoirs (one per reagent): biotinylated antibodies, avidin-peroxidase, chromogenic substrate and acid

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 control.

REAGENTS	STORAGE BEFORE OPENING	CHARACTERISTICS OF USE	STORAGE AFTER OPENING
96 wells microtitration plate (6 x 16 wells vertical 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 bag with dessicant provided in the kit, for a maximum of 30 days.
Standard PAP range	Stable if stored between +2°C et +8°C until expiry date.	Lyophilisate to be gently dissolved in 1 mL of distilled water, directly in the vial.	Keep at -20°C for 30 days maximum.
Internal control		Lyophilisate to be gently dissolved in 800 µL of distilled water, directly in the vial.	
Dilution buffer for standard PAP range preparation (10X concentrate)		Dilute 1/10 in distilled water before use.	
Biotinylated anti-PAP antibodies		Lyophilisate to be gently dissolved in 11 mL of distilled water, directly in the vial.	
Avidin-peroxidase conjugate			
Chromogenic substrate (TMB)		Vial containing 15 mL. Ready to use.	Keep between +2°C and +8°C for 30 days maximum.
Acid (H_2SO_4)		Vial containing 11 mL. Ready to use.	
PBS tablet		Dissolve in 1 L of distilled water.	Keep the prepared washing buffer at -20°C for 30 days maximum.
Tween 20 (10 % solution)		Add to the litre 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, 6 x 16 wells)	Wells coated with PAP-specific mouse monoclonal antibodies
Range and internal control	Recombinant human protein PAP (rhPAP) in phosphate buffer containing bovine proteins and protective agents
Dilution buffer for standard PAP range preparation	Tris-HCl buffered saline containing bovine proteins and protective agents
Biotinylated antibodies to PAP	PAP-specific mouse monoclonal antibodies conjugated to biotin, in a phosphate buffered solution containing protective agents
Avidin-peroxidase conjugate	Avidin conjugated to peroxidase enzyme, in a phosphate/citrate buffer containing protective agents
Chromogenic substrate (TMB)	Solution of chromogenic substrate of peroxidase enzyme 3,3',5,5'-tétraméthylbenzidine (TMB)
Acid (H_2SO_4)	Diluted sulphuric acid solution
PBS tablet	Phosphate buffered saline
Tween 20 solution (10 %)	Concentrated detergent solution

SAMPLE COLLECTION AND TREATMENT

Blood samples can be drawn in dry or heparinized tubes. After sampling, serum or plasma can be stored between +2°C and +8°C for 24 hours, or frozen at -20°C for longer storage. The method and devices used for sample collection must comply with local regulation.

Serum and plasma PAP levels are equivalent.

Do not exceed 3 freeze/thaw cycles on samples.

Hemolysed or hyperlipemic samples of plasma or serum may interfere with the PAP assay.

CAUTION FOR USE

This kit must be used for research purposes only, by properly trained staff provided with suitable protective equipment.

Samples from patients (serum, plasma or whole blood) as well as biotinylated antibodies contain products of human or animal origin, respectively. 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, chromogenic substrate (TMB) and acid 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 patients) and follow it strictly to avoid switching samples.

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 reagents (standard range, internal control, biotinylated antibodies and avidin-peroxidase) must be put in solution at least 10 minutes before use, to ensure complete dissolution and adequate homogeneity of the reagent.

Dilution buffer (10X) supplied in the kit must be diluted 1/10 in distilled water before use to prepare the range and dilutions of samples from patients.

Do not expose chromogenic substrate TMB to air and light before use.

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

As physiological blood PAP concentration in adult generally ranges between 0 and 25 ng/mL, it is recommended to dilute samples from patients before the assay. The dilution to apply depends on PAP concentration in the blood of the patient and thus varies from one individual to another. It is suggested to perform a first series of assays by applying a minimal dilution (1/50), and then to re-assay in a second step the samples that are still too concentrated, by diluting them more (1/100 or 1/500, even 1/1000 if necessary).

To realize sample dilutions, use the dilution buffer provided in the kit, which is also used to prepare the standard range: this buffer must be diluted 1/10 in distilled water before use.

In case a sample contains a very low PAP concentration, it may be necessary to assay it pure, without dilution, to obtain an accurate measure.

PREPARATION OF REAGENTS

Microtitration plate: Packaged under vacuum, it must be equilibrated at room temperature before removal from its wrapping. Do not remove the plate from its wrapping until all dilutions of range and samples from patients are prepared and ready for deposit. 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 1 to 12) to avoid switching them in case they fall from their frame while flicking the plate during washing steps.

Washing buffer (PBS/0.1% Tween)***:*** Dissolve the PBS tablet in 1L of distilled water and add the whole content of the supplied Tween 20 (10%) vial. Homogenize.

PAP standard range: The range is prepared from recombinant human freeze-dried PAP. The lyophilisate is reconstituted with 1 mL of distilled water to obtain 1.5 ng/mL of PAP. This standard solution at 1.5 ng/mL is then used to prepare a reference range presenting concentrations from 0.250 to 0.015 ng/mL, by successive dilutions in the buffer provided for that purpose.

Internal control: The internal control is also prepared from recombinant human freeze-dried PAP. The lyophilisate is reconstituted with 800 µL of distilled water to obtain 0.07 ng/mL of PAP.

Dilution buffer: The vial contains 10 mL of 10X dilution buffer and must be diluted 1/10 in distilled water before use. For example, to prepare 100 mL of 1X dilution buffer, add the 10 mL of 10X dilution buffer provided to 90 mL of distilled water. This 1X dilution buffer is then used to prepare the standard range and dilute the samples.

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.

Avidin-peroxidase: The lyophilisate is dissolved in 11 mL of distilled water directly in the vial. It is ready to use after complete dissolving and homogenisation.

Chromogenic substrate (TMB): Ready to use solution after homogenization.

Acid (H_2SO_4): Ready to use solution after homogenization.

ASSAY PROCEDURE

A reference PAP range is prepared from the standard lyophilized solution of PAP previously reconstituted at 1.5 ng/mL with 1 mL of distilled water. The dilution buffer provided with the kit is used to prepare serial dilutions of this standard solution to obtain the following PAP concentrations: 0.250 / 0.125 / 0.062 / 0.031 / 0.015 ng/mL. Each dilution from 0.250 to 0.015 ng/mL will be assayed in duplicate (100 µL/well).

Preparation of the PAP reference range (example for one PAP range assayed in duplicate):

100 µL of standard solution at 1.5 ng/mL + 500 µL of dilution buffer = 0.250 ng/mL
then:
300 µL of dilution at 0.250 ng/mL + 300 µL of dilution buffer = 0.125 ng/mL
then:
300 µL of dilution at 0.125 ng/mL + 300 µL of dilution buffer = 0.062 ng/mL
then:
300 µL of dilution at 0.062 ng/mL + 300 µL of dilution buffer = 0.031 ng/mL
then:
300 µL of dilution at 0.031 ng/mL + 300 µL of dilution buffer = 0.015 ng/mL

Micropipettes tips have to be changed between each dilution of standard PAP range.

Range, background, internal control and samples to assay are deposited in the wells (100 µL/well, in duplicate).

During deposit, micropipettes tips have to be changed when necessary (between every point of the range, blank, internal control and each patient sample) but not between duplicate of a same sample.

The plate is covered with the adhesive supplied with the kit and incubated 3 hours at room temperature (+19°C to +22°C).

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 µL/well) and incubated for 30 minutes at room temperature, after covering the plate with the adhesive.

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

Then the solution of avidin-peroxidase conjugate is added to each well (100 µL/well) and incubated 15 minutes at room temperature), after covering the plate with the adhesive.

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

The chromogenic substrate (TMB) is then added (100 µL/well): after covering it with the adhesive, the plate is immediately placed in the dark and incubated 15 minutes at room temperature.

After this 15 minutes TMB incubation protected from light, a blue coloration of variable intensity has appeared in the wells: without washing the plate, add directly 100 µL/well of acid solution to stop the enzymatic reaction. This final addition brings the total volume to 200 µL/well and transforms the coloration from blue to yellow.

The absorbance of each well of the plate must be read within 30 minutes after the reaction is stopped, using a spectrophotometer equipped with a 450 nm filter.

Note: Some spectrophotometers are programmed to read a first time at 450nm and a second time with a reference filter at 630nm. The absorbance at 630nm is then subtracted from the one at 450nm to eliminate residual blue coloration intensity. However, the 630nm reference filter is not mandatory.

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 ng/mL). 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 control and samples before calculating the mean of duplicates.

The table hereunder gives an example of a PAP range generated with a mean background giving an optical density of 0.100 (results provided for information only):

	Absorbance (Optical Density at 450 nm)				
PAP (ng/mL)	**Replicate 1**	**Replicate 2**	**Replicate 1 - mean background**	**Replicate 2 - mean background**	**Mean**
0	0.102	0.098			
0.015	0.393	0.386	0.293	0.286	0.289
0.031	0.673	0.662	0.573	0.562	0.567
0.062	1.113	1.116	1.013	1.016	1.014
0.125	1.966	2.044	1.866	1.944	1.905
0.25	3.407	3.275	3.307	3.175	3.241

The standard curve is constructed using the function [PAP] = f(mean absorbance), by plotting the mean optical density measured for each point of the range versus the theoretical PAP concentrations and applying a 4-parameter logistic adjustment. The use of a computer program to define the parameters of this function from range values is recommended. The concentration of PAP in control and samples is determined by extrapolation from this function.

Quality control

Assaying internal control is recommended to ensure the quality of results. The control provided in the kit is expected at 0.07 ng/mL. This control must be included in each assay, like the standard range. If the run of the day involves several plates, control must be included in each plate. It is recommended that results obtained for control are within +/-20% from its theoretical value:

Control – Theoretical concentration	Lower limit	Higher limit
Internal control – 0.07 ng/mL	0.056 ng/mL	0.084 ng/mL

Results for samples can be validated only if control value 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 samples from patients

Calculation of PAP concentration in blood of patients: if the protocol described above is carefully followed, PAP blood concentration for each patient is directly deduced from the equation of the range [PAP] = f(mean absorbance) and then by multiplying the obtained value by the dilution factor applied to the sample before the assay.

LIMITS OF THE ASSAY

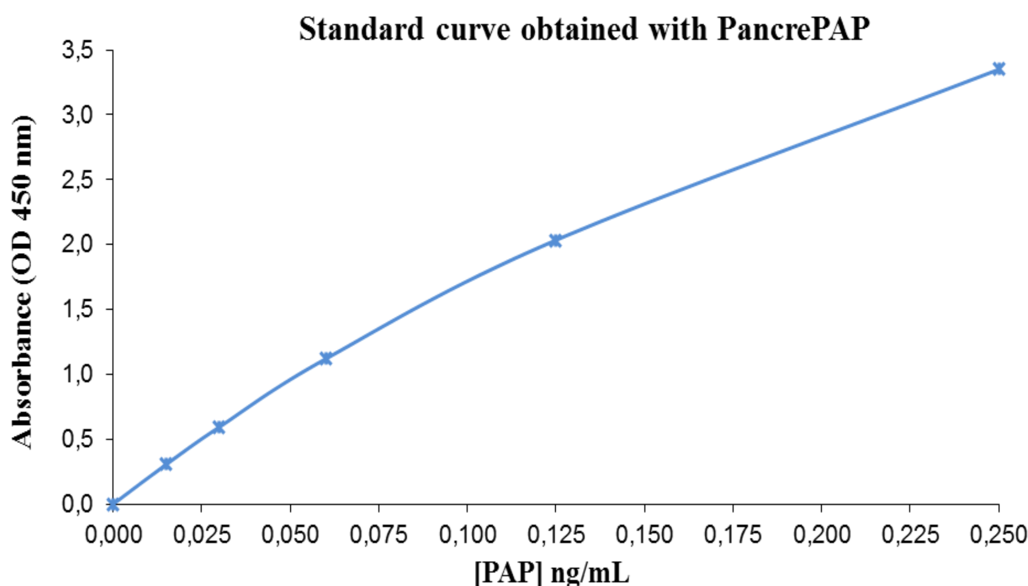
An adequate method of collection and storage of blood samples (serum or plasma) is essential to ensure good quality of results (see « Sample collection and treatment » section).

Hemolysed or hyperlipemic samples of plasma or serum may interfere with the PAP assay.

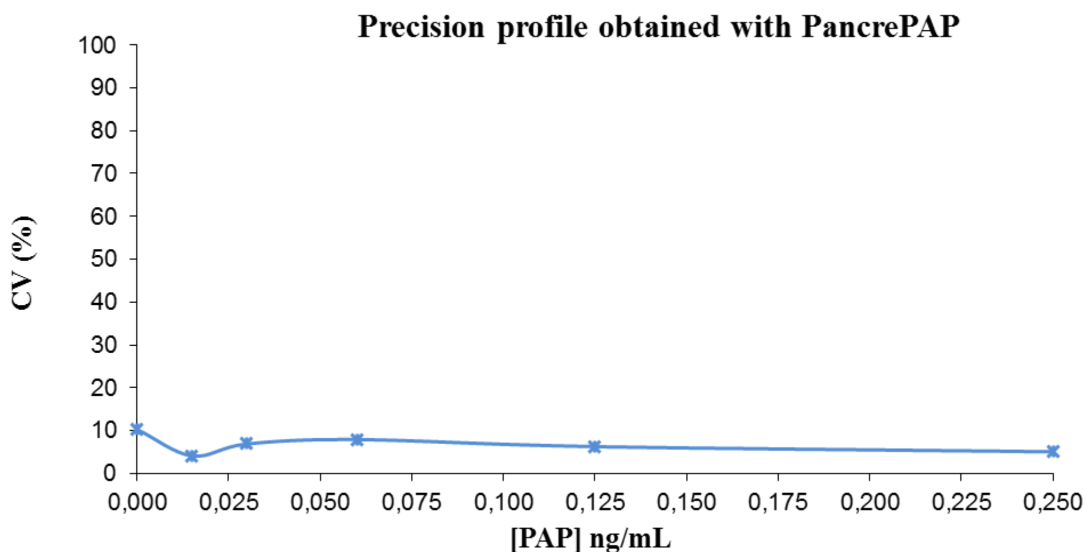
It is suggested to refer to sections « Caution for use » and « Recommendations for use ».

PERFORMANCES AND ANALYTICAL CHARACTERISTICS

Standard range: A typical standard range of the PancrePAP kit is shown below.



Precision profile: Precision profile of PancrePAP kit was established in duplicate using five different lots, thus on ten replicates for each point of the range. It is presented in the graph below.



Repeatability and reproducibility: Repeatability gives an estimate of the intra-lot variation and reproducibility an estimate of the inter-lot variation.

Repeatability and reproducibility of PancrePAP kit were established by assaying three different controls on five different lots. The controls used for this determination are:
- the internal control supplied with the kit, expected at 0.070 ng/mL, assayed on 4 replicates in each lot;
- an external control expected at 0.040 ng/mL, assayed on 10 replicates in each lot;
- another external control expected at 0.100 ng/mL, assayed on 10 replicates in each lot.

Results of repeatability and reproducibility study are presented in the following table:

Expected concentration of control (ng/mL)	Measured value (ng/mL)	Repeatability (%)	Reproducibility (%)
0.070	0.074	4.3	7.6
0.040	0.041	4.9	7.3
0.100	0.100	4.6	8.6

Detection and quantitation limits: Detection and quantitation limits of the PancrePAP assay (expressed as picograms of PAP per milliliter of blood) are respectively 3.0 pg/mL and 6.3 pg/mL, 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 PancrePAP assay with IL2, IL6, IFNγ, TNFα 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.

BIBLIOGRAPHY

1. Iovanna *et al.* Gastroenterology, 1994, 106:728-734.
2. Nigri *et al.* Cell. Mol. Life Sci., 2017, 74(22):4231-4243.

SUMMARY OF ASSAY

1. Warm up the assay plate to room temperature.
2. Prepare dilution buffer, range, control and samples from patients.
3. After room temperature is reached and all dilutions are prepared, remove the plate from its wrapping and deposit after homogenization the range, background, control, and samples (100 µL/well, in duplicate).
4. Incubate 3h at room temperature.
5. Prepare PBS/Tween wash buffer.
6. 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.
7. Distribute the biotinylated antibodies (100 µL/well).
8. Incubate 30 min at room temperature.
9. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
10. Distribute avidin-peroxidase conjugate (100 µL/well).
11. Incubate 15 min at room temperature.
12. Wash 5 times the wells with PBS/Tween and eliminate residual liquid by inverting the plate and tapping it on absorbent paper.
13. Distribute chromogenic substrate TMB (100 µL/well).
14. Incubate 15 minutes at room temperature in the dark.
15. Without washing, stop the reaction by adding H_2SO_4 (100 µL/well).
16. Read absorbance at 450 nm.

NOTES