

ZYMUTEST FPA

RK016A

Assay of human Fibrinogen Peptide A

(Complete competitive ELISA for the measurement of FPA)

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.



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INTENDED USE:

The ZYMUTEST FPA kit is a Competitive Enzyme Linked Immunosorbent Assay (CELIA) for measuring human FPA on bentonite adsorbed human plasma, or in any fluid where FPA can be present. Fibrinogen, when present, must be removed (i.e., bentonite adsorption of human plasma), as it cross-reacts with antibodies to FPA.

This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

FPA is measured on bentonite adsorbed human plasma, which is then fibrinogen free. In a first step, FPA calibrator or tested sample is preincubated with a constant and limited amount of affinity purified rabbit antibodies specific for human FPA. In a second step, the unreacted anti-FPA antibodies are then measured using a micro ELISA plate coated with synthetic FPA and stabilised. Free antibodies bind to immobilised FPA. Following a washing step, the immunoconjugate, which is a goat polyclonal antibody specific for rabbit IgGs and coupled to Horse-Radish-Peroxidase (HRP), is introduced into microwells and binds to immobilised anti-FPA. Following a new washing step, the peroxidase substrate, 3,3',5,5'-Tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H₂O₂), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with Sulfuric Acid. There is an indirect relationship between the colour developed and the concentration of FPA in the tested sample.

TEST SAMPLE:

- Human plasma, anticoagulated with the special anticoagulant mixture for FPA testing, and made fibrinogen free by a bentonite adsorption.
- Any biological fluid, fibrinogen free, where FPA must be measured.

REAGENTS:

1. **BS:** One vial of **50 mL of bentonite suspension**, ready to use.
2. **T20:** One vial of **5 mL of 2% Tween 20**, ready to use.
3. **COAT: Micro ELISA plate**, containing 12 strips of 8 wells, coated with synthetic human FPA, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
4. **SD:** One vial containing **50 mL of Sample Diluent**, ready to use.
5. **CAL:** Three vials of **FPA calibrator**, lyophilised.
Each vial, when restored with **2 mL of Sample diluent (SD)**, allows obtaining the FPA calibrator at a "**C**" (ng/mL) concentration (at about 50 ng/mL).

Note: The FPA concentration of the calibrator can vary according the lot used and is precisely indicated for each lot on the flyer provided with the kit.

6. **ABS:** Three vials of affinity purified **rabbit antibodies specific for human FPA**.
7. **IC:** Three vials of **Anti-rabbit IgG-HRP immunoconjugate**, a goat polyclonal antibody coupled to HRP, lyophilised.
8. **CD:** One vial of 25 mL of **Conjugate Diluent**, ready to use.
9. **WS:** One vial of 50 mL of 20 fold concentrated **Wash Solution**.
10. **TMB:** One vial of 25 mL of peroxidase substrate: **3,3',5,5'-Tetramethylbenzidine**, containing hydrogen peroxide, ready to use.
11. **ACS:** One vial of 20 mL of **special anticoagulant solution** for the assay of FPA.
12. **SA:** One vial of 6 mL of **0.45M Sulfuric acid (Stop Solution)**, ready to use.

Note: Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 50-300 μ L.
- **1-channel pipettes** at variable volumes from 0 to 20 μ L, 20 to 200 μ L and 200 to 1000 μ L.
- **Micro ELISA plate** washing equipment and shaker.
- Micro ELISA plate **reader** with a wavelength set up at 450 nm.
- Distilled water.
- Quality controls (ref SC015K).

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Bentonite suspension:** mix thoroughly before use. When open, it can be used for up to **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use. It contains **0.9 g/L sodium azide**.
2. **2% Tween 20:** it is ready to use. When open, it can be used for up to **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use. It contains **0.9 g/L sodium azide**.

Warning: Bentonite suspension and Tween 20 contain 0.9 g/L sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

3. **Micro ELISA plate:** open the plastic pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at **2-8°C** for **4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
4. **Sample Diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. Contains 0.05% Kathon CG as preservative.
5. **FPA calibrator:** restore each vial with **2 mL of Sample Diluent** in order to obtain the FPA calibrator "**C**" (ng/mL), ready to use. This solution is stable for at least **8 hours at room temperature**.

Warning: FPA calibrator is prepared with fibrinogen, extracted from normal human plasma. This latter was tested with registered methods and found negative for HIV 1 and 2 antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

6. **Affinity purified rabbit antibodies specific for human FPA:** restore each vial with **2 mL Sample-Diluent** in order to obtain the antibody ready to use. When open, it can be used for **1 week** stored at **2-8°C**, and provided that any bacterial contamination is avoided.
7. **Anti-rabbit IgG-HRP immunoconjugate:** each vial must be restored with **7.5 mL of Conjugate Diluent**. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least **24 hours at room temperature** or for at least **4 weeks at 2-8°C**.
8. **Conjugate diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. Contains 0.05% Kathon CG as preservative.
9. **Wash Solution:** Incubate the vial for 15-30 minutes in a water bath until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL contained in the vial allow to prepare 1 liter of Wash Solution). The Wash Solution must be stored at **2-8°C** in its original vial and used within **4 weeks** following opening. The diluted Wash Solution must be used within **7 days**, when protected from any contamination and stored at **2-8°C**. This reagent contains 0.05% Kathon CG.
10. **TMB Substrate:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2- 8°C**, and provided that any bacterial contamination is avoided during use.
11. **Special FPA anticoagulant solution:** it is ready to use. When open, it can be used for up to **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use. It contains **0.9 g/L sodium azide**.

Warning: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

12. **Stop Solution: 0.45M Sulfuric acid:** It is ready to use.

Cautions: Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulphuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

Note: Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

PROCEDURE:

Specimen collection:

Blood (9 vol.) must be collected by a net venipuncture on the special anticoagulant mixture (1 vol.) provided for the assay of FPA (contains Trisodium Citrate, Heparin, Hirudin, Aprotinin and sodium azide) and discarding the first drops. Blood must be rapidly mixed with the anticoagulant and centrifuged at 2,500 g for 20 min. Plasma supernatant must be decanted and is then ready for bentonite treatment. Plasma must be treated with bentonite within 8 hours after collection or deep frozen within 4 hours, and stored up to 1 month at -20°C or below. Just before use, it must be thawed for 30 min. at 37°C in a water-bath, and treated with bentonite.

Bentonite treatment:

Cross-reactive fibrinogen must be removed by bentonite adsorption. Mix thoroughly the bentonite suspension in order to make it homogeneous. To 1 mL of the anticoagulated plasma, add 0.5 mL of bentonite suspension. Mix and agitate for 10 min. using an end-over-end agitator. Centrifuge for 20 min. at 2,500 g and collect 1 mL supernatant.

Proceed to a new bentonite adsorption by adding again 0.5 mL of suspension to the 1 mL of supernatant, in a similar manner. The bentonite treated plasma is then fibrinogen free. It must be used within:

- 24 hours at room temperature or at $2-8^{\circ}\text{C}$.
- 1 month when stored frozen at -20°C or below.

Just before use, add 50 μL of 2% Tween 20 to 1 mL of bentonite adsorbed plasma.

Note: The bentonite treated plasma is two-fold diluted, and the measured FPA concentration must be multiplied by 2.

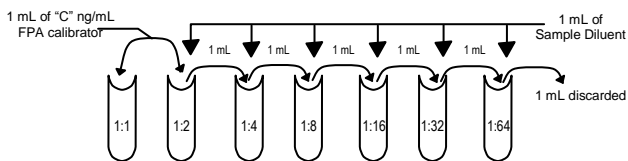
Preparation of tested samples and calibrators:

To 1 mL of the bentonite-adsorbed plasma containing Tween 20, add exactly 0.1 mL of affinity purified rabbit antibodies specific for FPA (anti-FPA) or to 0.5 mL of bentonite-adsorbed plasma containing Tween 20 add exactly 0.05 mL anti-FPA. Incubate the closed vial for 1 hour at 37°C (preferentially in incubator).

Quality controls: For controls I and II, refer to the corresponding insert (ref. SC015K).

Calibration: Prepare 1 mL of a serial two-step dilution of the FPA calibrator, at "C" (ng/mL), in Sample Diluent, from 1/1 to 1/64, as follows:

A calibration range of FPA, from C to C/64 (ng/mL) is obtained.



To 1 mL of each dilution, add exactly 0.1 mL of anti-FPA (reconstituted with 2 mL of Sample Diluent) or to 0.5 mL of each dilution, add exactly 0.05 mL of anti-FPA (reconstituted with 2 mL of Sample Diluent). Incubate the closed vial for 1 hour at 37°C (preferentially in incubator).

Note: The first condition allows tests in duplicate. The second condition allows tests in simplicate.

Warning: the calibration and controls should be treated under the same conditions as the samples.

Assay Procedure:

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Incubation mixture of FPA calibrator or of tested sample or of Sample Diluent (blank)	200 μL	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well.
Incubate for 1 hour at room temperature ($18-25^{\circ}\text{C}$)		
Wash Solution (20 fold diluted in distilled water)	300 μL	Proceed to 5 successive washings using the washing instrument (a).
Conjugate (anti-rabbit IgG polyclonal antibody coupled with peroxidase. Restored with 7.5 mL of Conjugate Diluent)	200 μL	Introduce the Anti-rabbit IgG-HRP immunoconjugate in the micro ELISA plate wells (a).
Incubate for 1 hour at room temperature ($18-25^{\circ}\text{C}$)		
Wash Solution (20 fold diluted in distilled water)	300 μL	Proceed to 5 successive washings using the washing instrument.
TMB / H_2O_2 Substrate	200 μL	Immediately after the washing, introduce the substrate into the wells. The substrate distribution, row by row, must be accurate and at exact time intervals (a, b).
Incubate for exactly 5 minutes at room temperature ($18-25^{\circ}\text{C}$) (c)		
0.45 M Sulfuric Acid (4)	50 μL	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid (b).
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450) (c, d).		

Note:

- Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
- For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-Elisa plate shaker can be used.
- For biochromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RESULTS:

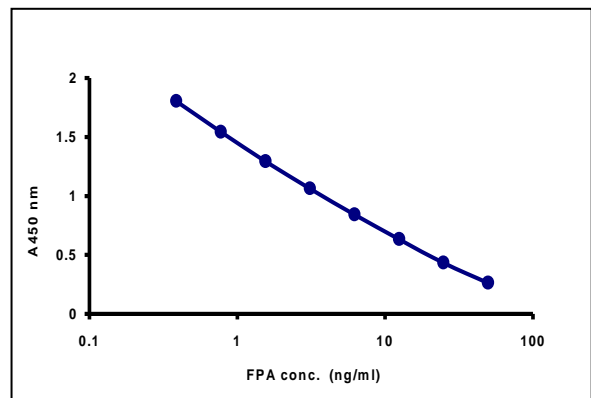
- On a semi-log graph paper plot the FPA concentration (ng/mL) on abscissae and the corresponding absorbance on ordinates (A450nm).
- From the curve obtained, deduce directly the FPA concentration in samples tested by multiplying the value obtained by 2 (for the two-fold dilution of plasma resulting from the bentonite treatment).
- For controls I and II, refer to the corresponding insert (ref. SC015K) and specific flyer for the lot.

Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only. Users must construct their own calibration curve obtained using their standard dilutions, according to the indicated FPA concentration "C" (ng/mL) of the calibrator used.



BIOCHEMISTRY:

- FPA is a 16 amino acid peptide, with a molecular weight of 1536, released from the amino terminal end of fibrinogen $\text{A}\alpha$ chains, upon the action of thrombin. Two molecules of FPA are released from one molecule of fibrinogen.
- The total FPA releasable from fibrinogen is then of 0.9% of the fibrinogen concentration. FPA has a very short half-life in body (<3 min.). The FPA concentration in normal human plasma is usually below 3 ng/mL.