ZYMUTEST™ FPA

REF RK016B

CELIA assay of FPA (Fibrino-Peptide A)

FOR RESEARCH USE ONLY.

DO NOT USE IN DIAGNOSTIC PROCEDURES

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INTENDED USE:

The ZYMUTEST™ FPA kit is a Competitive Enzyme Linked Immunosorbent Assay (CELIA) for the *in vitro* quantitative determination of human FPA, using on bentonite adsorbed human plasma, or in any fluid where FPA can be present. Fibrinogen, when present in the specimen, 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 must not be used for patient diagnosis or treatment.

SUMMARY AND EXPLANATION:

Technical:

FPA is a 16 amino acid peptide, with a molecular weight of about 1536 Da, released from the amino terminal end of fibrinogen Aα 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.

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 (H_2O_2), 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.

REAGENTS:

1. BS **Bentonite suspension :** 1 vial of 50 mL, ready to use. Contains small amounts of sodium azide (0.9 g/L).
2. T20 **2% Tween 20 :** 1 vial of 5 mL, ready to use. Contains small amounts of sodium azide (0.9 g/L).
3. COAT **ELISA microplate :** 12x8 containing 12 strips of 8 wells, coated with synthetic human FPA, stabilized and packed in an aluminium pouch in presence of a desiccant. Contains small amounts of sodium azide (0.9 g/L).
4. SD ELISA **Sample Diluent :** 1 vial of 50 mL, ready to use. Contains Proclin and BSA.
5. CAL **FPA calibrator :** 3 vials of 2 mL, lyophilized. Each vial should be reconstituted by 2 mL of SD ELISA to obtain a calibrator containing a concentration **"C"** (of about 50 ng/mL of FPA). Contains BSA.
6. CI **FPA Control I high :** 3 vials of 1 mL, lyophilized with high concentration of FPA. Contains BSA.
7. CII **FPA Control II low :** 3 vials of 1 mL, lyophilized with low concentration of FPA. Contains BSA.
8. ABS **Affinity purified rabbit antibodies specific for human FPA**: 3 vials of 2 mL. Contains BSA.
9. IC **Immunoconjugate :** 3 vials of 7.5 mL, a goat polyclonal antibody, specific for rabbit IgG and coupled to Horse-Radish-Peroxidase (HRP), lyophilized. Contains BSA.
10. CD ELISA **Conjugate diluent :** 1 vial of 25 mL, ready to use. Contains Proclin and BSA.
11. WS ELISA **Wash solution :** 1 vial of 50 mL, 20x 20 fold concentrated. Contains Proclin.
12. TMB **3,3',5,5'-Tetramethylbenzidine:** 1 vial of 25 mL, ready to use. Contains hydrogen peroxide.
13. ACS **Special anticoagulant solution for FPA assay :** 1 vial of 20 mL, ready to use. Contains small amounts of sodium azide (0.9 g/L).
14. Stop **0.45M Sulfuric acid:** 1 vial of 6 mL, ready to use.

The calibrator and controls concentrations may vary slightly from one batch to the next. For the assay, see the exact values provided on the flyer provided with the kit used.

The ZYMUTEST™ FPA kit is validated with the calibrator and controls included. Do not dissociate. If other controls must be used, each laboratory must define its acceptance ranges and verify the expected performance in its analytical system.

WARNINGS AND PRECAUTIONS:

- Some reagents provided in these kits contain materials of human and animal origin. Whenever human plasma is required for the preparation of these reagents, approved methods are used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV, and for hepatitis B surface antigen, and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- This device of *in vitro* use is intended for professional use in the laboratory.

REAGENT PREPARATION:

Allow the strips and reagents to stabilize for at least 30 min at room temperature before use. Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

COAT Open the aluminum pouch and take off the required amounts of strips for the test series. The strips must be used within 30 minutes.

Reconstitute the contents of each vial with exactly:

CI ➔ **1 mL of** SD ELISA. Let stabilize 15 min at room temperature. Shake gently until complete dissolution.

CII ➔ **1 mL of** SD ELISA. Let stabilize 15 min at room temperature. Shake gently until complete dissolution.

CAL ➔ **2 mL of** SD ELISA in order to obtain a ready to use calibrator solution, titrating "C" (ng/mL) of FPA. Shake vigorously until complete dissolution.

IC ➔ **7,5 mL of** CD ELISA at least 15 minutes before use. Shake gently until complete dissolution.

ABS ➔ **2 mL of** SD ELISA. Shake vigorously until complete dissolution.

SD ELISA | TMB | Stop | CD ELISA | BS | T20 | ACS
Reagent ready to use.

WS ELISA Shake the vial and dilute the wash solution **1:20 in distilled water** (the 50 mL of concentrated solution allow to prepare 1 liter of wash solution after dilution).
Incubate, if necessary, the vial in a water bath at **37°C**, until complete dissolution of solids.

STORAGE AND STABILITY:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

COAT Unused strips can be stored at 2-8°C **for 4 weeks** in their original aluminum pouch (hermetically closed, in presence of the desiccant), stored in the provided plastic microplate storage bag (minigrip), protected from any moisture.

Reagent stability after reconstitution, free from any contamination or evaporation, and stored closed, is of:

CAL ➔ **8 hours** at room temperature (18-25°C).

CI | CII ➔ **48 hours at** 2-8°C.
24 hours at room temperature (18-25°C).
2 months frozen at -20°C or less*

IC ➔ **4 weeks** at 2-8°C.
24 hours at room temperature (18-25°C).

ABS ➔ **1 week** at 2-8°C.

*Thaw only once, as rapidly as possible at 37°C and use immediately.

Reagent stability after opening, free from any contamination or evaporation, and stored closed, is of:

SD ELISA | CD ELISA | TMB | BS | T20 | ACS
➔ **4 weeks** at 2-8°C.

WS ELISA ➔ **4 weeks** at 2-8°C.
7 days at 2-8°C for the diluted solution.

Stop ➔ **8 weeks** at 2-8°C.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.

Materials:

- 8-channel or repeating pipette allowing dispensing volumes of 50-300 µL.
- Pipettes at variable volumes from 0 to 20 µL, 20 to 200 µL and 200 to 1000 µL.
- Micro ELISA plate washing equipment and shaker.
- Micro ELISA plate reader with a wavelength set up at 450 nm.

SPECIMEN COLLECTION AND PREPARATION:

The blood (9 volumes) should be carefully collected onto the ACS anticoagulant (1 volume) (trisodium citrate and inhibitors) by clean venipuncture. Discard the first tube. Promptly mix with ACS and centrifuge (eg 20 min at 2500g.) to collect the plasma supernatant thus ready for bentonite adsorption. Bentonite treatment should be promptly done, if not possible plasma should be promptly frozen at -20°C or below. Just before use, thaw plasma at 37°C and proceed to bentonite treatment.
Specimen should be promptly prepared and stored in accordance with applicable local recommendations (see references[1-5] for further information regarding specimen collection, handling and storage).

PROCEDURE:

Assay method:

1. Bentonite treatment:

Cross-reactive fibrinogen must be removed by bentonite adsorption. Mix thoroughly the **BS** in order to make it homogeneous. To 1 mL of the anticoagulated plasma, add 0.5 mL of **BS**. 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 **BS** to the 1 mL of supernatant, in a similar manner. The bentonite treated plasma is then fibrinogen free. It must be used[3,4]:

- As soon as possible, within 8 hours at room temperature or 24 hours at 2-8°C.
- Immediately if frozen beforehand.

Just before use, add **50 µL of T20** to 1 mL of bentonite adsorbed plasma.

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

2. Preparation of tested samples and calibrators:

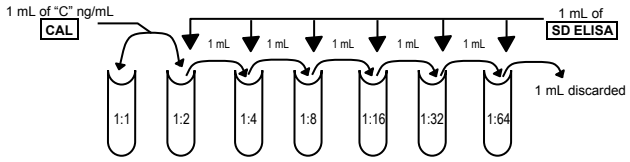
To **1 mL** of the bentonite-adsorbed plasma containing Tween 20, add exactly 0.1 mL of affinity purified rabbit antibodies specific for FPA (anti-FPA) or to 0.5 mL of bentonite-adsorbed plasma containing Tween 20 add exactly 0.05 mL anti-FPA. Incubate the closed vial for 1 hour at 37°C (preferentially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

3. Quality controls:

No bentonite treatment is required for these controls. To **1 mL** of each reconstituted control, add exactly **0.1 mL of affinity purified** rabbit antibodies specific for FPA. Incubate for 1 hour at 37°C.

4. Calibration:

Prepare **1 mL** of a serial two-step dilution of the **CAL**, at **"C"** (ng/mL), in **SD ELISA**, 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 **SD ELISA**) **or** to **0.5 mL** of each dilution, add exactly **0.05 mL of anti-FPA** (reconstituted with 2 mL of **SD ELISA**). Incubate the closed vial for 1 hour at 37°C (preferentially in incubator).

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

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

5. Put strips in the frame provided. Introduce the reagents in the micro ELISA plate wells and perform the assay as indicated on the following table:

Reagent	Volume	Procedure
Incubation mixture of: **CAL or** **CI or** **CII or** **Specimens to test diluted or** **SD ELISA (blank)**	**200µL**	Introduce the standard solutions or controls or the tested specimen in the corresponding micro ELISA plate well
Incubate for 1 hour at room temperature (18-25°C) (a)		
WS ELISA	**300µL**	Proceed to 5 successive washings **(b)**
IC	**200µL**	Introduce the **IC** in the micro ELISA plate wells **(b)**
Incubate for 1 hour at room temperature (18-25°C) (a)		
WS ELISA	**300µL**	Proceed to 5 successive washings **(b)**
TMB	**200µL**	Immediately after the washing, introduce the substrate into the wells **(b,c)**. **Nota:** The substrate distribution, raw by raw, must be accurate and at exact time intervals.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (a)		
Stop	**50µL**	Following exactly the same time intervals, raw to raw, than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid **(c)**.
Wait for 10 minutes in order to allow the colour to stabilize then measure absorbance at 450 nm. Subtract the blank values (d).		

(a) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro ELISA plate shaker can be used.

(b) 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 and reduce the reactivity plate. 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 damage coating and lower plate reactivity.

(c) For addition of the substrate, the time interval between each row must be accurate and exactly determined.

(d) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

QUALITY CONTROL:

Using quality controls allows validating the method compliance, as well as the homogeneous of assays for a same lot of reagents.

Quality control plasmas must be included in each series, as per good laboratory practice, in order to validate test results. A new calibration curve must be carried out for each test series.

Each laboratory can establish acceptance ranges and verify expected performances in its analytical system.

RESULTS:

- Plot the calibration curve log-lin (concentration-OD) with the OD 450 nm along the Y-axis and the FPA concentration in ng/mL, along the X-axis by choosing the "best fit" interpolation mode (refer to the flyer in the kit).
- The concentration of FPA (ng/mL) in the test specimen is directly inferred from the calibration curve, by multiplying the value obtained **by 2** (for the two-fold dilution of plasma resulting from the bentonite treatment).
- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor used.
- Alternatively, a specific software (i.e., Dynex, Biolise, etc...) can be used for the calculation of concentrations.
- For **CI** and **CII**, the concentrations are directly deduced from the calibration curve.

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

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- If the washing step is not correctly performed, the negative control can produce a high absorbance value. In order to avoid non-specific colour development, check that the washing step is performed efficiently.

REFERENCES:

1. CLSI Document GP44-A4: "Procedures for the handling and processing of blood specimens for common laboratory tests".
2. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". 2008.
3. Amiral J. *et al.* Development and Performance Characteristics of a Competitive Enzyme Immunoassay for Fibrinopeptide A. Seminars in Thrombosis and Hemostasis. 1984.
4. Kockum C. and Frebelius S. Rapid Radioimmunoassay of Human Fibrinopeptide A – Removal of Cross-reacting Fibrinogen with Bentonite. Thrombosis Research. 1980.
5. Soria J. *et al.* A Solid Phase Immuno Enzymological Assay for the Measurement of Human Fibrinopeptide A. Thrombosis Research. 1980.

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

SD ELISA **CD ELISA** **WS ELISA** H317 : May cause an allergic skin reaction.

Changes compared to the previous version.