HYPHEN BioMed
Sales and Support:
CoaChrom Diagnostica GmbH
www.coachrom.com | info@coachrom.com
Tel: +43-1-236 222 1 | Fax: +43-1-236 222 111
Toll-free contact for Germany:
Tel: 0800-24 66 33-0 | Fax: 0800-24 66 33-3

ZYMUTEST Fibrinogen

 # RK024A

(Complete ELISA kit for the assay of Fibrinogen)

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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INTENDED USE:

The ZYMUTEST Fibrinogen kit is a two site immuno-assay for measuring human Fibrinogen in plasma, or in any fluid where Fibrinogen can be present. This assay, developed with high affinity rabbit polyclonal antibodies, specific for human Fibrinogen, cross reacts with Fibrinogen from many species, and particularly with mouse Fibrinogen.
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

ZYMUTEST Fibrinogen is a two site ELISA, designed with rabbit polyclonal antibodies, specific for Fibrinogen.

First, the diluted tested sample is introduced into the microwells coated with rabbit polyclonal antibodies specific for Fibrinogen. When present, Fibrinogen binds onto the coated solid phase. Following a washing step, the immunoconjugate, which is a rabbit polyclonal antibody specific for Fibrinogen, coupled to horse radish peroxidase (HRP), is introduced and binds to Fibrinogen onto free epitopes. 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 color develops. When the reaction is stopped with Sulfuric Acid, a yellow color is obtained. The amount of color developed is directly proportional to the concentration of Fibrinogen in the tested sample.

TEST SAMPLE:

- Human plasma collected on trisodium citrate or Na_2 EDTA anticoagulant.
- Any biological fluid where Fibrinogen must be measured.
- Citrated or Na2 EDTA anticoagulated mouse plasma.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with a rabbit polyclonal antibody specific for Fibrinogen, then stabilized; the plate is packed in an aluminium pouch, hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 60 ml of **B2F-Sample Diluent**, ready to use, containing a Rheumatoid factor inhibitor.
3. **Std:** 3 vials of human **Fibrinogen Standard**, lyophilized. When restored with 2 ml of B2F-Sample Diluent (SD), a solution containing **"C"** ng/ml of human Fibrinogen is obtained (usually at about 50 ng/ml). The exact Fibrinogen concentration is indicated on the flyer provided in the kit.
4. **CI:** 1 vial of 1 ml **Fibrinogen Control I (High),** lyophilized.
5. **CII**: 1 vial of 1 ml **Fibrinogen Control II (Low),** lyophilized.

> ***Note:*** The Fibrinogen concentrations and acceptance ranges for calibrators and controls can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.

6. **IC:** 3 vials of **Anti-Fibrinogen HRP immunoconjugate**, a rabbit polyclonal antibody coupled to Horse-Radish-Peroxidase (HRP), lyophilized.
7. **CD:** 1 vial of 25 ml of **B2F-Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
9. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' **– Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
10. **SA:** 1 vial of 6 ml of **0.45M Sulfuric acid** (Stop solution). Ready to use.

> ***Note:*** Use only components from a same lot number of kits. Do not mix components from different lots of kits when running the assay.
>
> **NOTE: *Use of a Rheumatoid factor Inhibitor in the sample diluent avoids the interference of Rheumatoid Factor**

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 5-300 µl.
- **1-channel pipettes** at variable volumes from 0 to 20 µl, 20 to 200 µl and 100 to 1000 µl.
- **Micro ELISA plate** washing equipment and shaker.
- Micro ELISA plate **reader** with a wavelength set up at 450 nm.
- Distilled water.

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. **Micro ELISA plate:** open the aluminium 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 for up to **4 weeks at 2-8°C** 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).
2. **B2F-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. This reagent contains 0.05% Kathon CG.
3. **(h) Fibrinogen Standard:** restore each vial with **2 ml** B2F-Sample Diluent, in order to obtain a solution containing "**C" ng/ml** Fibrinogen. This solution is stable for at least **4 hours** at room temperature, and for **24 hours at 2-8°C**.
4. **Fibrinogen Control I (high)**: restore with **1 ml** B2F-Sample Diluent.
5. **Fibrinogen Control II (low)**: restore with **1 ml** B2F-Sample Diluent.

> ***Note:*** when restored, controls are stable for 4 hours at room temperature, **24 hours at 2-8°C** or **2 months** frozen **at –20°C** or below.
>
> ***Warning:*** Fibrinogen used for standard (3) and controls (4&5) is extracted from normal human plasma. This latter was tested with registered methods and found negative for HIV 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. **Anti-Fibrinogen-HRP- immunoconjugate**: each vial must be restored with **7.5 ml of B2F-Conjugate Diluent**. Let the pellet to be completely dissolved 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**.
7. **B2F-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. This reagent contains 0.05% Kathon CG.
8. **Wash Solution**: Incubate the vial for 15-30 minutes in a water bath, at **37°C**, until complete dissolution of solids. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow preparing 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**. Concentrated wash solution contains 0.05% Kathon CG.
9. **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.
10. **Stop solution:** It is ready to use.

> ***Cautions:*** Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric 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.
> The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

PROCEDURE:

Specimen collection:

Blood plasma (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within **24 hours** at 2-8°C, within **8 hours** at room temperature, or stored frozen at –20°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within **8 hours**.
EDTA collected plasma may also be used. Conditions of storage are the same than those for citrated plasma.

> ***Note***: Blood activation during specimen collection or plasma preparation, may induce clot formation. Measured Fibrinogen concentration is then underestimated.

Tested plasma or specimen

This assay is proposed for measuring Fibrinogen in biological fluids where it is present at trace amounts, such as cell culture supernatants. The tested specimen must be diluted in order to have an expected Fibrinogen concentration **< "C" or about 50 ng/ml,** in the tested dilution. If Fibrinogen must be tested on human plasma, as an example, this latter must be diluted 1:100,000 or 1:200,000 (or more) with the B2F-Sample Diluent.

Controls I & II, restored with 1ml of B2F-Sample Diluent, are tested "undiluted".

Calibration:

Using the **"C" ng/ml** Fibrinogen standard provided in the kit ("C" is indicated, for each lot of reagents, on the flyer provided in the kit), prepare the following standard solutions:

Fibrinogen concentration (ng/ml)	C	C/2	C/4	C/10	C/20	0
Vol. of standard at C ng/ml	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of B2F-Sample Diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenization.
The standard dilutions are stable for at least **8 hours** at room temperature.

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
Fibrinogen Standard or controls or tested sample or B2F-Sample Diluent (blank)	**200 µl**	Introduce the standard solutions or controls or the tested samples in the corresponding micro ELISA plate well
Mix gently on a plate shaker or manually and Incubate for 1 hour at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Empty the wells and proceed to 5 successive washings using the washing instrument. **(b)**
Anti-Fibrinogen HRP Immunoconjugate (Restored with 7.5 ml of B2F-Conjugate Diluent)	**200 µl**	Introduce the Anti-Fibrinogen HRP immunoconjugate into the micro ELISA plate wells
Mix gently on a plate shaker or manually and Incubate for 1 hour at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Empty the wells and proceed to 5 successive washings using the washing instrument **(b)**
TMB/H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. ***Note:*** The substrate distribution, row by row, must be accurate and at exact time intervals **(b, c)**.
Let the color develop for exactly 5 minutes at room temperature (18-25 °C) (c)		
0.45 M Sulfuric Acid (5)	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the color development by introducing the 0.45M sulfuric acid.
Wait for **10 minutes** in order to allow the color to stabilize and measure absorbance at **450 nm (A450)**. Subtract the blank value **(d)**.		

Note:

a. Avoid letting the plate in the bright sunlight during incubations and more particularly during color development. A micro-ELISA plate shaker can be used. Calibrators, controls and tested specimen must be distributed as rapidly as possible on the microElisa plate (within 10 min.) in order to allow obtaining homogeneous immunological kinetics for immunocapture.

b. Never let the plates empty between the additions 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 immobilised 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.

c. 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.

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

RESULTS:

- On a linear graph paper, plot the **Fibrinogen concentration** (in ng/ml) on abscissae and the corresponding absorbance (**A450**) on ordinates.
- Users must construct their own calibration curve obtained using their standard dilutions. From the curve obtained, deduce the Fibrinogen concentration for the tested dilution. For obtaining the Fibrinogen concentration in the tested sample, this value must be **multiplied by the dilution factor**. (See model on the flyer).
- For controls I and II, the concentrations measured is directly deduced from the curve.

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.

BIOCHEMISTRY OF FIBRINOGEN:

Fibrinogen is a 340 Kd glycoprotein, containing 6 peptidic chains, with a 2 to 2 symmetry, and linked by disulfide bridges (2 Aα, 2 Bβ and 2 γ chains). Thrombin clots fibrinogen and forms fibrin, which is stabilized by activated factor XIII in presence of calcium. Fibrinogen is lysed by plasmin to fragments X and Y, first, then D and E. Fibrinogen concentration in normal human plasma is in the range 1.5 to 5 mg/ml.

STANDARDISATION:

The Zymutest Fibrinogen kit is calibrated with human fibrinogen, highly purified (clotting ability> 99%), and which concentration is exactly determined with a protein assay.

ASSAY REACTIVITY:

The Zymutest Fibrinogen kit is designed for being reactive with all the fibrinogen material, including fibrinogen or fibrin degradation products.
The Zymutest Fibrinogen kit cross-reacts with fibrinogen from other species (different from rabbit), and more particularly with mouse fibrinogen. When this kit is used for testing fibrinogen from other species than human, calibrator concentration must be adjusted, according to the reactivity of the tested fibrinogen species. A specimen with an accurately known fibrinogen concentration, from the assayed species, must be tested. A ratio must be calculated between the measured concentration and the expected one. This ratio must be used for obtaining the actual fibrinogen concentration for the assayed species.

RECOMMENDATIONS:

When fibrinogen is measured on plasma, very high dilution factors are required (1:100,000 or more). For a better accuracy proceed to successive serial 1:10 dilutions, until the expected dilution is obtained.

HYPHEN BioMed | 155, rue d'Eragny | F 95000 Neuville-sur-Oise