

RAT FIBRINOGEN ELISA TEST KIT

Life Diagnostics, Inc., Catalog Number: 3200-2

Enzyme Immunoassay for the Quantitative Determination of Rat Fibrinogen in Plasma

INTRODUCTION

Fibrinogen is dimeric protein (mol wt, 340 kDa), that is synthesized in the liver and circulates in rat plasma at a concentration of approximately 3 mg/ml. It is significantly elevated during the acute phase response^{1,2} and therefore serves as a useful marker of infection, disease and inflammation.

PRINCIPLE OF THE TEST

The rat fibrinogen ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat fibrinogen antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat fibrinogen antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 30 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in fibrinogen molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of fibrinogen is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat fibrinogen antibody 96-well plate (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 µg/ml rat fibrinogen when reconstituted as detailed on the vial label
- 20x Wash Solution, 50 ml
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate washer
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8°C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.
3. Optimum results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

WASH SOLUTION PREPARATION

The wash solution is provided as a 20x stock. Prior to use dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The rat fibrinogen standard is provided as a lyophilized stock. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved to obtain a 2 µg/ml stock (***the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended.***)
2. Label 8 polypropylene or glass tubes as 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Dispense 525 µl of diluent into the tube labeled 250 ng/ml and 300 µl of diluent into the remaining tubes.
4. Pipette 75 µl of the 2 µg/ml fibrinogen standard into the tube labeled 250 ng/ml and mix. This provides the working 250 ng/ml fibrinogen standard.
5. Prepare a 125 ng/ml standard by diluting and mixing 300 µl of the 250 ng/ml standard with 300 µl of diluent in the tube labeled 125 ng/ml.
6. Similarly prepare the 62.5, 31.25, 15.6, 7.8 and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Fibrinogen is present in normal rat plasma at a concentration of ~ 3 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be

diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 μl and 497.5 μl of 1x diluent into separate tubes.
2. Pipette and mix 2 μl of the serum/plasma sample into the tube containing 998 μl of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 μl of the 500 fold diluted sample with the 497.5 μl of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 μl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution using a plate washer (400 μl /well). The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
7. Add 100 μl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
9. Wash as detailed in 4 to 6 above.
10. Dispense 100 μl of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
12. Stop the reaction by adding 100 μl of Stop Solution to each well.
13. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
14. Read the optical density at 450 nm with a microtiter plate reader *within 5 minutes.*

CALCULATION OF RESULTS

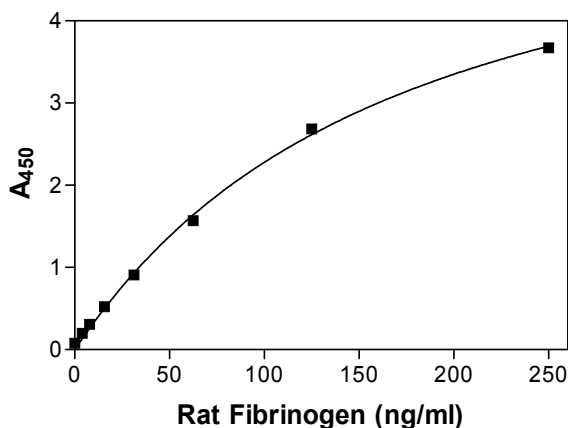
1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of fibrinogen in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of fibrinogen in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000 samples should be diluted appropriately and re-tested.

Typical Standard Curve

A typical standard curve with optical density readings at 450nm on the Y axis against fibrinogen concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Fibrinogen (ng/ml)	Absorbance (450 nm)
250.0	3.673
125.0	2.684
62.5	1.568
31.25	0.907
15.63	0.523
7.81	0.308
3.91	0.197
0.0	0.079

Typical Rat Fibrinogen Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. G Schreiber et. al., The acute phase response in the rodent. Ann N Y Acad Sci. 557:61-85 (1989)
2. A Larrson, J Bjork and C Lundberg. Nephelometric Determination of rat fibrinogen as a marker of inflammatory response. Vet Immunol Immunopathol 59:163-169 (1997)