



## ANTI-HUMAN FACTOR IX, MATCHED PAIR ANTIBODIES FOR EIA

**Product Reference: 5D-18113**

**For Research Use Only.  
Not for use in diagnostic procedures.  
For in vitro use only.**

**Store at -10 to -20°C  
5 x 96 Tests**

### DESCRIPTION OF FACTOR IX (FIX):

Factor IX (FIX, Christmas Factor) is a vitamin K-dependent glycoprotein produced in the liver. Plasma concentration of FIX is normally around 5 µg/ml (87 nM) in plasma. The biological importance of FIX is demonstrated in Haemophilia B (Christmas disease), an X-linked congenital bleeding disease resulting from a quantitative (low activity and low antigen) or qualitative (low activity and normal antigen) defect in FIX function.

In its zymogen form FIX is a single chain molecule of 55,000 daltons. It contains two EGF-like domains and an amino-terminal domain containing 12 γ-carboxy-glutamic acid (Gla) residues. These Gla residues allow FIX to bind divalent metal ions and participate in calcium-dependent binding interactions. The activation of FIX occurs by limited proteolysis in the presence of calcium by activated factor XI (FXI<sup>a</sup>) and/or by a complex of VII<sup>a</sup>/tissue factor/phospholipid and activated Factor X between residues Arg<sup>146</sup>-Ala<sup>147</sup> and between Arg<sup>180</sup>-Val<sup>181</sup>. The terminal activated product in either case is FIX<sup>a</sup>, a two-chain enzyme consisting of a heavy chain (28,000 daltons), a light chain (18,000 daltons) and an activation peptide product of 11,000 daltons. FIX can also be cleaved into inactive products by thrombin and by elastase. The activity of FIX<sup>a</sup> in plasma is inhibited by antithrombin and this inhibition is accelerated approximately 1000-fold in the presence of optimal concentrations of heparin<sup>1-3</sup>.

### PRINCIPLE OF SANDWICH-STYLE ELISA:

Affinity-purified antibody to FIX is coated onto the wells of a microtitre plate. The plates are washed and plasma or other fluids containing FIX are applied. The coated antibody will capture the FIX in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to FIX is added to the plate to bind to the captured FIX. After washing the plate to remove unbound conjugated antibody, the peroxidase activity is expressed by incubation with o-phenylenediamine (OPD). After a fixed development time the reaction is quenched with the addition of H<sub>2</sub>SO<sub>4</sub> and the colour produced is quantified using a microplate reader. The colour generated is proportional to the concentration of FIX present in the sample.

### REAGENTS:

Required Materials (provided); enough for 5x96 Tests:

**C: Capture Antibody (5D-18113-C).** 1 vial of 0.5 mL polyclonal affinity-purified antibody specific for human Factor IX. For coating plates. Supplied in a 50% v/v glycerol solution. Yellow cap.

**D: Detecting Antibody (5D-18113-D).** 1 vial of 0.5 mL polyclonal antibody specific for human Factor IX, coupled to peroxidase. For detecting captured Factor IX. Supplied in a 50% v/v glycerol solution. Red cap.

Note: Antibodies are provided in a glycerol solution (50% v/v) and should be stored at -10 to -20°C. Vials should be tightly capped. Do not store in frost-free freezers.

Required Materials (not provided):

Optimum performance can be obtained when the following solutions and assay conditions are used.

- **Coating Buffer.** 50 mM Carbonate. 1.59 g of Na<sub>2</sub>CO<sub>3</sub> and 2.93 g of NaHCO<sub>3</sub> up to 1 L. Adjust pH to 9.6. Store at 2-8°C up to 1 month.
- **Phosphate-Buffered Saline (PBS).** [Base for Wash Buffer]. 8.0 g NaCl, 1.15 g Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g KH<sub>2</sub>PO<sub>4</sub> and 0.2 g KCl, up to 1 L. Adjust pH to 7.4, if necessary. Store up to 1 month at 2-8°C, discard if there is evidence of microbial growth.
- **Wash Buffer:** PBS-Tween (0.1% v/v). To 1 L of PBS add 1.0 mL of Tween-20. Check that the pH is 7.4. Store at 2-8°C up to 1 week.
- **Sample Diluent:** HBS-BSA-EDTA-T20. 5.95 g HEPES (free acid), 1.46 g NaCl, **0.93 g Na<sub>2</sub>EDTA**, 2.5 g Bovine Serum Albumin (Sigma, RIA grade) dissolved in 200 mL H<sub>2</sub>O. Add 0.25 mL of Tween-20, check and adjust pH to 7.2 with NaOH; then make up to a final volume of 250 mL with H<sub>2</sub>O. Aliquot and store frozen at -20°C.
- **Substrate Buffer:** Citrate-Phosphate buffer pH 5.0. Dissolve 2.6 g Citric acid, monohydrate and 6.9 g Na<sub>2</sub>HPO<sub>4</sub> in 450 mL H<sub>2</sub>O. Adjust pH to 5.0 with phosphoric acid or NaOH, if necessary, then make up to a final volume of 500 mL with H<sub>2</sub>O. Store at 2-8°C up to 1 month.
- **OPD Substrate:** o-phenylenediamine.2HCl **☒ Toxic!** (5 mg tablets: Sigma #P-6912). Prepare immediately before use. Dissolve 5 mg OPD in 12 mL substrate buffer and then add 12 µL 30% H<sub>2</sub>O<sub>2</sub>. Do not store.
- **Stop Solution:** 2.5 M H<sub>2</sub>SO<sub>4</sub>. **☒ Caution: Very corrosive! Generates heat on dilution!** Handle with great care. Avoid any skin and eye contact. Wear protective glasses and gloves when handling. Where stock sulphuric acid is 18 Molar, add 13.9 mL to 86 mL H<sub>2</sub>O. Store at room temperature.
- **Other:**

Microplates, for example, 96-well Immulon 4-HBX  
Microplate washer (optional)  
Microplate reader.

## ASSAY PROCEDURE:

**1. Coating of plates:** Dilute the Capture Antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 µl to every well in the plate. Incubate for 2 hours at 22°C.

**2. Blocking:** Blocking is not required under the conditions described. Washing the plate with PBS-Tween is sufficient to block non-specific interactions. Wash plate X 3 with wash buffer.

**3. Samples:** Reference plasma is diluted 1/100 (100%) then serial 1/2's down to 1/3200 (3.13%). Sample plasmas are diluted 1/200, 1/400 and 1/800. All dilutions are made in HBS-BSA-EDTA-T20 sample diluent. Apply 100 µl/well and incubate plate at 22°C for 90 minutes. Wash plate X 3 with wash buffer.

**4. Detecting Antibody:** Dilute the detecting antibody 1/100 in HBS-BSA-EDTA-T20 sample diluent and apply 100 µl to each well. Incubate plate at 22°C for 90 minutes. Wash plate X 3 with wash buffer.

**5. OPD Substrate:** Apply 100 µl of freshly prepared OPD substrate to every well. Allow colour to develop for 5-10 minutes then stop colour reaction with the addition of 50 µl/well of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The plate can be read at wavelength of 490 nm.

## RESULTS:

From the constructed calibration curve, directly determine the Factor IX concentration and multiply by the appropriate dilution factor. The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit.<sup>4,5</sup> In general, the simplest model that defines the concentration-response relationship should be used.<sup>6</sup>

The "back-fit" test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits.<sup>6</sup> The coefficient of determination ( $R^2$ ) is a valuable indicator of the overall fit, but should not be used by itself in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone.<sup>5,6</sup>

In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed using serial dilutions of normal pooled plasma, will produce a correlation coefficient ( $R^2$ ) of at least 0.980 using a log-log fit, and an  $R^2$  of at least 0.990 using a 4-parameter logistic curve fit algorithm. However, the performance characteristics of in-house assays developed using this product in other laboratories may vary slightly from ours. Different curve fitting methods may be employed but we recommend that the back-fit test be applied as evidence that the fitting method is appropriate.

## ADDITIONAL NOTES:

- This paired antibody product is intended to facilitate the end user in establishing an in-house immunoassay for research purposes only. It must not be used for diagnostic applications. Assay validation is the responsibility of the end user and should be done according to user-defined protocols<sup>6</sup>.
- Reference calibrators should be of the same matrix and anticoagulant as the samples to be tested (example serum or plasma, citrate or EDTA)
- Do not use samples diluted less than 1/10, as falsely high readings may result.
- The optimal colour development time should be determined empirically as the time required to obtain an absorbance of at least 1.000 at 490 nm for the 100% reference point, not to exceed 20 minutes.
- Rheumatoid factor in samples may interfere in ELISA by binding to the capture and/or detecting antibodies.
- The wells should not be allowed to become dry. Keep plate covered or in a humid chamber during incubations.
- Antibodies are supplied in a 50% glycerol solution and can be centrifuged briefly in a micro-centrifuge to gather residual reagent from the cap and walls of the tube.

## SPECIES CROSS REACTIVITY:

(Relative reactivity compared to normal human reference plasma under conditions specified in assay protocol)

Human:	145.2%	Mouse:	<1%	Rat:	<1%
Rabbit:	5.4%	Pig:	2.8%	Dog:	<1%

## REFERENCES:

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