

# ZYMUTEST FREE PROTEIN S

# RK015A-RUO

Complete one step ELISA kit for the assay of Free Protein S

**FOR RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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**INTENDED USE:**

The ZYMUTEST Free Protein S kit is a one step, two-site immuno-assay, for measuring human Free Protein S (the Activated Protein C cofactor) in plasma, or in any fluid where Free Protein S can be present. **This assay is for research use only and should not be used for patient diagnosis or treatment.**

**ASSAY PRINCIPLE:**

First, the immunoconjugate, which is a monoclonal antibody specific for Free Protein S coupled to horse radish peroxidase (HRP), is introduced into the microwells coated with another monoclonal antibody specific for Free Protein S. Then, the diluted tested plasma or biological fluid is immediately introduced, and the immunological reaction starts. When present, the Free Protein S binds onto the monoclonal antibody coated solid phase through one epitope, and fixes the second monoclonal antibody coupled to HRP by another epitope. Only Free Protein S is bound while Protein S-C4b-BP (C4b Binding protein) complexes are not reactive in the assay. Following a washing step, the peroxidase substrate, 3,3',5,5' – Tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained. The amount of colour developed is directly proportional to the concentration of human Free Protein S in the tested sample.

**TEST SAMPLE:**

- Trisodium Citrate anticoagulated human plasma.
- Any biological fluid where Free Protein S must be measured.

**REAGENTS:**

- COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with a mouse monoclonal antibody specific for human Free Protein S, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
  - SD:** 2 vials containing 50 ml of Protein S Sample Diluent, ready to use (contains calcium).
  - Cal:** 3 vials of Plasma Protein S Calibrator, (normal plasma calibrated with a reference plasma pool), lyophilised, prediluted 1:50. Each vial, when restored with 2 ml of Protein S Sample Diluent, allows obtaining the plasma calibrator, already diluted 1:50. The exact Free Protein S concentration is indicated on the flyer provided in the kit.
  - CI:** 1 vial containing 0.5 ml of lyophilised Protein S Control I (plasma, high)
  - CII:** 1 vial containing 0.5 ml of lyophilised Protein S Control II (plasma, low)
- Note:** The Free Protein S concentrations and acceptancy ranges for control plasma I and II, and calibrator, can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.
- IC:** 3 vials of Anti-(h)-Free Protein S-HRP immunoconjugate, a mouse monoclonal antibody coupled to HRP, lyophilised.
  - CD:** 1 vial of 15 ml of Protein S Conjugate Diluent, ready to use.
  - WS:** 1 vial of 50 ml of 20 fold concentrated Protein S Wash Solution (contains calcium).
  - TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' – Tetramethylbenzidine, containing hydrogen peroxide. Ready to use.
  - SA:** 1 vial of 6 ml of 0.45 M Sulfuric Acid (Stop solution). Ready to use.

**Note:** Use only components from kits with the same lot number. Do not mix components from different lots of kits when running the assay.

**REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:**

- 8-channel or repeating pipette allowing dispensing 50-300 µl.
- 1-channel 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.
- 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.

- 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).
- Protein S 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. It contains 0.05% Kathon CG.
- Plasma Protein S Calibrator:** restore each vial with 2 ml of Protein S Sample Diluent, in order to obtain the calibrator plasma, containing the Free PS concentration "C%", **already diluted 50 fold**. This solution is stable for at least 8 hours at room temperature.
- Protein S Control I (human plasma, high):** restore with 0.5 ml distilled water.
- Protein S Control II (human plasma, low):** restore with 0.5 ml distilled water.

**Note:** When restored, Protein S controls are stable for 8 hours at room temperature, 24 hours at 2-8°C or 2 months frozen at -20°C or below.

**Warning:** Plasma Protein S calibrator (3) and controls (4&5) are prepared with 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.

- Anti-(h)-Free Protein S-HRP immunoconjugate:** each vial must be restored with 4 ml of Protein S Conjugate Diluent. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content. The restored conjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2-8°C.
- Protein S 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. It contains 0.05% Kathon CG.
- Protein S Wash Solution:** Incubate the vial for 15-30 minutes in a water bath at 37°C 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 preparing 1 liter of Wash Solution). The Protein S 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. It contains 0.05% Kathon CG. This Protein S Wash Solution contains calcium and must be used for Protein S assay.
- 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.
- 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 performed at 30°C show that the reagents keep their performances and can be shipped at room temperature without any damage.

**PROCEDURE:****Specimen collection:**

Blood (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 8 hours 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 4 hours.

**Tested plasma or sample:**

The sample must be tested diluted **fifty fold (1:50)** in the Protein S Sample Diluent. For expected Protein S concentrations > 100 %, plasma or samples must be tested at a higher dilution, i.e 1:100 or more. If the dilution factor is D, concentrations obtained must then be multiplied by the complementary dilution factor which is D:50 (i.e. x2 for 1/100 etc...). For low Protein S levels (<10%) the sample can be tested at a lower dilution D', and the concentration obtained must be divided by 50:D'.

Protein S controls I and II must be tested diluted fifty fold (1:50) with Protein S Sample Diluent.

### Calibration:

Free Protein S concentrations are expressed as % of a normal pooled human plasma. For the Free Protein S assay, the **100% concentration** corresponds to a normal pooled human plasma **diluted 1:50**, which is the standard assay dilution. Using the Protein S Calibrator provided in the kit (2 ml of plasma calibrator already prediluted **1:50** and with a Free Protein S concentration "**C**" indicated, for each lot of reagents, on the flyer provided in the kit), prepare the following standard solutions:

Free Protein S concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Protein S Calibrator	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of Protein S -Sample Diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenisation. The standard dilutions are stable for at least **4 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
Anti (h)-Free Protein S-HRP immunoconjugate (Restored with 4 ml of Protein S Conjugate Diluent)	100 µl	Introduce the Anti-(h)-Free Protein S- HRP immunoconjugate in the micro ELISA plate wells
Protein S Calibrator or tested sample or Protein S Sample Diluent (blank)	100 µl	Introduce <b>immediately</b> the standard solutions or the tested samples in the corresponding micro ELISA plate well
<b>Mix gently on a plate shaker or manually and incubate for 1 hour at room temperature (18-25°C)</b>		
Protein S Wash Solution (20 fold diluted in distilled water).	300 µl	Proceed to 5 successive washings using the washing instrument. <b>(a)</b>
TMB/H <sub>2</sub> O <sub>2</sub> Substrate	200 µl	Immediately after the washing, introduce the substrate into the wells. <b>Note</b> : The substrate distribution, row by row, must be accurate and at exact time intervals <b>(b, c)</b> .
<b>Incubate for exactly 5 minutes at room temperature (18-25 °C) (d)</b>		
0.45 M Sulfuric Acid (5)	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>(c)</b> .
<b>Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450). Subtract the blank value.</b>		

### Note:

Distribute calibrators, controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for antigen binding. A too long delay between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results

- Only the specific Protein S washing solution, which contains calcium, must be used, for this assay, as the monoclonal antibodies are calcium dependent.
- 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 bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

### RESULTS:

Users must construct their own calibration curve, obtained using their standard dilutions (see flyer).

On a linear graph paper plot the **Free Protein S concentrations (%)** on abscissa and the corresponding absorbances (**A450**) on ordinates.

From the curve obtained, deduce directly the Free Protein S concentration in samples tested at the standard **1:50 dilution**. When higher dilutions are used (i.e **D**), the Free Protein S concentration must be multiplied by the complementary dilution factor (i.e. **D:50**). When lower dilutions are used (i.e. **D'**), the concentration obtained must be divided by **50:D'**.

For **controls I and II**, the concentrations are directly deduced from the calibration 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:

- The Protein S concentration in normal human plasma is of about 25 µg/ml (1). About 40% (i.e. 10 µg/ml) is in the Free form and 60% (i.e. 15 µg/ml) circulates in blood as a non-covalent complex with C4b-BP. Only the Free form has an anticoagulant activity as the cofactor of Activated Protein C.
- Protein S is synthesized in liver. It is a vitamin K dependent glycoprotein, with a molecular weight of 80,000 daltons. The balance between the free form and the C4b-BP bound form of protein S plays an important role because only the Free Protein S is active.

### CHARACTERISTICS:

The ZYMUTEST Free Protein S assay is specific for the Free form of Protein S, and is designed with 2, calcium dependent, monoclonal antibodies unreactive with Protein S-C4b-BP complexes. It measures specifically the functional, native, and active form of Protein S.

- Dynamic range: 0 to about 100%.
- Detection threshold ≤ 5%.
- Intra-assay CV: 3-8%.
- Inter-assay CV: 5-10%.
- No significant interference of heparin up to 2 IU/ml, of bilirubin up to 0.1 mg/ml and of haemoglobin up to 10 mg/ml.
- Reference material: International Standard for Protein S (93/590) and normal plasma pools.

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