HYPHEN BioMed
155 rue d'Eragny, 95000 Neuville-sur-Oise, France
Tél : +33 (0)1 34 40 65 10
Fax : +33 (0)1 34 48 72 36
www.hyphen-biomed.com
info@hyphen-biomed.com

ZYMUTEST CRP

 # RK010A

Highly sensitive assay for Cross Reactive Protein

(Complete ELISA kit for the assay of CRP)

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Last revision: 17/11/2022

INTENDED USE:

The ZYMUTEST CRP kit is a highly sensitive two-site immuno-assay for measuring human CRP in plasma, serum, or in any fluid where CRP can be present.
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

ZYMUTEST CRP is a "one step" sandwich ELISA technique specific for human CRP (Cross Reactive Protein).

The highly sensitive ZYMUTEST CRP is performed with a one step method. First, the immunoconjugate, a goat polyclonal antibody, specific for human CRP, and coupled to horse-radish-peroxidase (HRP), is introduced into the microwell, coated with a polyclonal antibody ($F(ab')_2$ fragments*) specific for CRP. Then, the diluted test sample is introduced and the immunological reaction is initiated. When present, CRP binds to the coated microwell and reacts with the immunoconjugate, simultaneously. Following a washing step, the peroxidase substrate, tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H2O2), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with Sulfuric Acid. The amount of colour developed is directly proportional to the concentration of CRP in the tested sample.

TEST SAMPLE:

- Trisodium Citrate or Na_2 EDTA anticoagulated human plasma.
- Human serum
- Any biological fluid where CRP must be measured.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with $F(ab')_2$ fragments* from a goat polyclonal antibody specific for human CRP, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 ml of **Sample Diluent**, ready to use.
3. **Cal:** 3 vials of **Plasma CRP Calibrator**, (normal plasma calibrated and standardised with the NIBSC international standard), lyophilised, prediluted. Each vial, when restored with **2 ml of Sample Diluent (SD)**, allows obtaining the calibrator plasma, **already diluted 1:100,** with a CRP concentration of "**C**" ng/ml. The exact CRP concentration is indicated on the flyer provided in the kit. It is of about 100 ng/ml (corresponding to a plasma with a CRP concentration of 10µg/ml).
4. **CI:** 1 vial containing 0.5 ml of lyophilised **CRP control I** (human plasma, high).
5. **CII:** 1 vial containing 0.5 ml of lyophilised **CRP control II** (human plasma, low).

   The CRP concentrations and acceptancy ranges for calibrators and controls are indicated on the flyer provided in the kit.
6. **IC:** 3 vials of **Anti-(H)-CRP-HRP immunoconjugate**, a goat polyclonal antibody coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 ml of **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 **0.45 M Sulfuric Acid** (Stop Solution)**.** Ready to use.

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

***Note: Use of anti-CRP $F(ab')_2$ fragments for the pre-coated plates allows to avoid the interference of Rheumatoid Factor.**

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.

1. **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).
2. **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. **Plasma CRP Calibrator:** restore each vial with **2 ml** of Sample Diluent in order to obtain the calibrator plasma, already diluted **100 fold**. The CRP concentration is of **"C" ng/ml** in the restored vial. This solution is stable for at least **24 hours** at room temperature, or for **72 hours at 2-8°C**.
4. **CRP Control I** (human plasma, high): restore with **0.5 ml** distilled water.
5. **CRP Control II** (human plasma, low): restore with **0.5 ml** distilled water.

   Note: Following reconstitution, control plasma I and II are stable **24 hours** at Room Temperature (18-25°C), or **72 hours at 2-8°C**. They can be kept frozen at **–20°C** or colder for **up to 2 months**.

Warning: Plasma CRP 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.

6. **Anti-(H)-CRP-HRP immunoconjugate**: each vial must be restored with **7.5 ml of Conjugate Diluent**. Let the pellet completely dissolve 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. **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, 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 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**. This reagent 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.

Note: Before performing the assay, the reagents must equilibrate at room temperature (18-25°C), for at least 30 min. Using reagents at a too low temperature decreases the immunological kinetics.

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 24 hours or stored frozen at –20°C or colder 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 human plasma may also be used. The assay can also be performed with human serum. Conditions of storage are the same than those for citrated plasma.

Tested plasma or sample or controls:

The sample must be tested diluted **one hundred fold (1:100)** in the Sample Diluent. For expected CRP concentrations >10 µg/ml, plasma or samples can be tested at a higher dilution, (**i.e., 1:200, or 1:400, or more**). For low or very low CRP concentrations lower dilutions (**i.e., 1:50, 1:20 or 1:10**) can be used.

Controls must be diluted **one hundred fold (1:100)** as for plasmas.

Calibration:

CRP concentrations in plasma or serum are expressed in µg/ml (or mg/L).

Using the diluted Plasma CRP calibrator provided in the kit (2 ml of calibrator already prediluted 1:100), with a CRP concentration of **"C" ng/ml** (indicated for each lot of reagents on the flyer, usually in the range 85 to 115ng/ml), prepare the following standards for the calibration curve:

CRP concentration (ng/ml)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma CRP calibrator at C ng/ml	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of 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 solutions are stable for at least **8 hours at room temperature, or 24 hours at 2-8°C**.

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
Conjugate (anti CRP polyclonal antibody coupled with peroxidase). Restored with 7.5 ml of Conjugate Diluent.	**200 µl**	Introduce the Anti-(H)-CRP- HRP immunoconjugate in the micro ELISA plate wells.
Standard dilutions or tested sample diluted 1:100	**50µl**	Introduce the standard solutions or the tested sample in the corresponding micro ELISA wells **(a)**.
Mix gently to homogenize		
Incubate for 1 hour at R.T (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument **(c)**.
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 **(a, c)**.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (b)		
0.45 M Sulfuric Acid (4)	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45 M sulfuric Acid **(a).**
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **450 nm (A450).(d)**		

Note:

a) For addition of samples and 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.

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

c) 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.

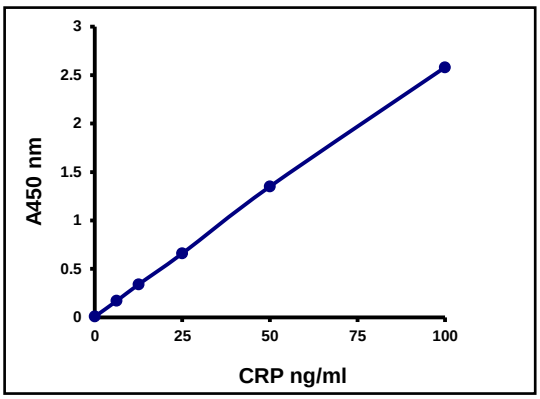
d) For dual wavelength analysis, 620 nm should be used as a referenced wavelength.

RESULTS:

On a linear graph paper plot the **CRP concentration** (in ng/ml) on abscissae and the corresponding absorbance (A450) on ordinates.
From the curve obtained, interpolate the CRP concentration in the diluted tested samples. The concentration measured must be multiplied by the dilution factor for obtaining the CRP concentration in the tested sample (i.e., x 100 when the recommended 1:100 dilution is used).
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.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only. Users must construct their own curve obtained using their standard dilutions.



ASSAY CHARACTERICS:

- The assay is calibrated with the NIBSC international standard for CRP (1st international standard for C-Reactive Protein, Human, 1986, Ref: 85/506), expressed in "milli International units" (mIU). One mIU/ml corresponds to about 1µg/ml.
- There is no interference of Rheumatoid Factor, as $F(ab')_2$ fragments are used for coating the micro-ELISA plate.
- No Prozone effect was observed for CRP concentrations up to 100 µg/ml, using the recommended protocol.

BIOCHEMISTRY:

Cross Reactive Protein (CRP) is a pentameric molecule with a molecular weight of 110 Kd, composed of 5 identical subunits. It is produced by hepatocytes. Its exact biological role remains unclear, but it can stimulate mononuclear cells to release tissue factor, it activates the complement pathway, and it neutralises platelet-activating factor. In recent studies, the CRP concentration in normal human plasma is usually between 0.2 and 10µg/ml. CRP is an acute phase reactant protein and is increased during inflammation.

Changes compared to the previous version.