

BIOPHEN Prothrombin

Ref 221605

Chromogenic assay for the measurement of Prothrombin in plasma

For in vitro research use only

HYPHEN BioMed

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INTENDED USE:

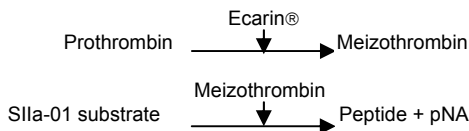
BIOPHEN Prothrombin kit is an in vitro assay for the quantitative determination of Prothrombin in human citrated plasma with a chromogenic assay, using a manual or automated protocol.

SUMMARY:

Congenital or acquired Prothrombin deficiency is a risk factor for a bleeding disorder. Elevated prothrombin concentration could be associated with an increased thrombotic risk.

ASSAY PRINCIPLE:

Using the BIOPHEN Prothrombin assay, Prothrombin is measured following its specific activation with Ecarin®, an enzyme extracted from snake venom (Echis Carinatus). Meizothrombin is formed and then it specifically cleaves the specific substrate S11a-01, releasing para-nitroaniline (pNA), which colour is measured at 405nm. There is a direct relationship between colour development and Prothrombin activity in the tested plasma.



REAGENTS:

R1: Reagent 1: Ecarin®.

Highly purified enzyme, extracted from the Echis carinatus snake venom, lyophilized in the presence of a fibrin polymerization inhibitor, and stabilized; Ecarin® can specifically activate prothrombin into meizothrombin:

4 vials containing about 2 U of Ecarin® (to be reconstituted with 2.5 mL of distilled water).

R2: Reagent 2: S11a-01 substrate

Chromogenic substrate, specific for thrombin (S11a-01), lyophilized:

4 vials containing about 5 mg of S11a-01 (to be reconstituted with 2.5 mL of distilled water).

R3: Reagent 3: TBSA buffer "10xconc."

Ten fold concentrated Tris-BSA buffer. Contains BSA and sodium azide. To be diluted ten fold with distilled water before use. 4 vials containing about 5 ml.

Precaution and warnings:

- Bovine serum albumin (BSA) was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no test may totally exclude the absence of infectious agents. As any product of bovine origin, reagents must be used with all the cautions required for handling a material potentially infectious.
- All the required cautions must be respected in order to avoid any risk of ingestion or accidental introduction of R1 (Ecarin®) or R2 in body. In case of skin contact, wash extensively with water. In case of contact with a wound, address to the appropriate medical service, and indicate the biological origin and the nature of the product.
- The TBSA buffer (R3) contains sodium azide, which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.
- Avoid contact with skin and eyes. Do not empty into drains. Wear suitable protective clothing.
- For in vitro research use.
- The Ecarin® concentration may present variations from lot to lot, but it is exactly adjusted for each new lot of reagent, for an optimized assay reactivity.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, preferentially sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Calibration and quality control plasmas, titrated for Prothrombin.

Material:

- Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm.
- Manual method: Water bath or incubator at 37°C, plastic tubes or microplates, stop watch.
- Calibrated pipettes.

STORAGE CONDITIONS:

BIOPHEN Prothrombin kits must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

PREPARATION AND STABILITY OF REAGENTS:

R1: Reagent 1: Ecarin®

Reconstitute each vial with exactly 2.5 ml of distilled water; homogenize the content. Let the reagent to stabilise for 30 min at Room Temperature, before use, while shaking from time to time. Homogenize before use.

Stability of reconstituted Ecarin®, kept in its original vial:

- 1 week at 2-8°C.
- 3 days at Room Temperature (18-25°C).
- Do not freeze.

R2: Reagent 2: Thrombin specific Chromogenic substrate (S11a-01)

Reconstitute each vial with 2.5 ml of distilled water; homogenize the content. Incubate at Room Temperature (18-25°C) for 30 min, before use, while shaking from time to time. Homogenize before use.

Stability of restored substrate, kept in its original vial:

- 1 month at 2-8°C.
- 3 days at Room Temperature (18-25°C).
- Do not freeze.

R3: Reagent 3: TBSA buffer "10xconc."

Ten fold concentrated TBSA buffer. Shake the vial and dilute the amount required 1:10 in distilled water (the 5 ml contained in the vial allow preparing 50ml of ready to use buffer). The buffer must be stored at 2-8°C in its original vial and used within 4 weeks following opening. The diluted buffer must be used within 7 days, when protected from any contamination and stored at 2-8°C.

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use (white caps for Ecarin®, yellow caps for S11a-01, white cap for the buffer).
- Reagents must be handled with care, in order to avoid any contamination during use.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- Incubating the reconstituted vials, for 30 min. at RT, allows stabilising the reagents, and obtaining a homogeneous reactivity over time.
- In order to avoid evaporation of reagents, limit the exchange surface by using, for example, a vial neck or an operculated cap.

Note:

- R1 and R2 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between R1 and R2 must be strictly respected.
- Use only reagents from kits with a same lot number. Do not mix reagents from kits with different lots when running the assay. Reagents R1, R2 and R3 are optimized for each lot of kits.
- The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

PREPARATION OF PLASMA (SPECIMEN COLLECTION):

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), with great care, in a silicon glass or a plastic tube. Sampling must be performed through a net venipuncture, avoiding any blood activation.

- Within 4 hours, blood must be centrifuged at 3,000 g for 20 min at 18-25°C or below, and plasma decanted into a plastic tube, using a plastic pipette. Separate carefully plasma from blood cells.
- Storage of plasma:
 - Up to 4 hours at Room Temperature (18-25°C).
 - Up to 24 hours at 2-8°C.
 - Up to 1 month frozen at -20°C or below (before use, thaw for 15 min. in a water bath at 37°C).

Refer to GEHT or NCCLS guidelines for further instructions on specimen collection, handling and storage.

TEST PROCEDURE:

BIOPHEN Prothrombin kit is designed for being used in kinetics methods, automated, but it can also be used for end point manual methods. Adaptations for the various automates are available upon request. The assay is performed at the controlled temperature of 37°C and the colour development is measured at 405 nm.

CALIBRATION:

Calibration is performed with a normal pooled citrated plasma (made with plasmas from at least 30 normal individuals, males or females, aged between 18 and 55 years, and free of any medication or disease), with the assigned value of 100 % Prothrombin. The assay includes a standard plasma dilution of 1:50. By definition, this latter dilution of the pool represents the 100 % Prothrombin activity. The dynamic range is from 0 to 200 % Prothrombin. The 200 % Prothrombin activity is then the 1:25 dilution of the plasma pool (in the ten fold diluted Tris-BSA buffer (R3)).

Or calibration is performed with a commercially available plasma calibrator, with a known Prothrombin concentration (C). The 1:50 dilution corresponds to the indicated Prothrombin concentration, and the 1:25 to twice this concentration. Using a plasma calibrator with a Prothrombin concentration of C, the 200% Prothrombin concentration is obtained (in the assay conditions) by using the following dilution factor: 25 x C : 100.)

The calibration curve can then be prepared as follows from the preparation already adjusted at 200% Factor II:

% Prothrombin	200% FII Calibrator (µl)	Diluted buffer (R3) (µl)
0	0	500
50	125	375
100	250	250
200	500	0

ASSAY PROTOCOL:

Manual Method:

Dilute the tested samples, and the controls 1:50 with TBSA buffer (R3, prediluted 1:10 in distilled water).

In a microplate well, or in a **plastic** tube preincubated at 37°C, introduce:

Reagents	Microplate	Test Tube
Calibrators; Controls or tested plasmas diluted 1:50	50 µL	200 µL
Incubate for 2 min at 37°C, then introduce:		
R1 : Ecarin® preincubated at 37°C	50 µL	200 µL
Mix and Incubate for 3 min at 37°C, then introduce:		
R2 : SIIa-01 Substrate preincubated at 37°C	50 µL	200 µL
Mix and Incubate for 2 min at 37°C, exactly		
Stop the reaction by introducing:		
Citric Acid (20g/L)	100 µL	400 µL
Mix and measure the optical density at 405nm against the sample blank.		

The yellow colour obtained is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the opposite order from that of the test i.e.: Citric Acid (20 g/L), SIIa substrate, Ecarin®, diluted plasma.

Measure the Absorbance at 405 nm (A405). Subtract the sample blank from the A405 obtained for the assay.

Automated methods:

Detailed instrument settings including instructions for the preparation of the reagents for a variety of automated instruments are available upon request.

Note:

- If higher or lower reactive volumes are required for the method used, the same respective proportions for each reagent concentration, and for the overall reactive volume, must be strictly respected, in order to keep the assay performances.
- Do always a sample blank in presence of highly lipemic, icteric or hemolysed plasmas, or if plasma has a "colour" different from the usual one.

QUALITY CONTROL:

Use of quality control plasmas, titrated for Prothrombin, allows validating the calibration curve, as well as the homogeneous reactivity of the BIOPHEN Prothrombin assay from run to run, and from series to series, when using a same lot of reagents.

Controls should be analyzed at least every 8 hours shift in accordance with good laboratory practice.

LIMITATIONS OF THE PROCEDURE:

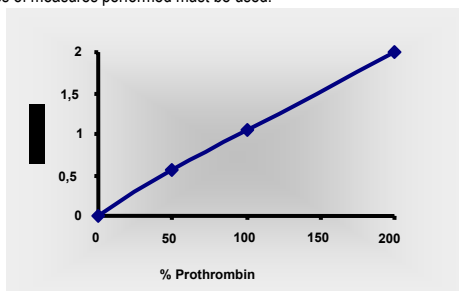
- The assay is not sensitive to the presence of heparin in plasma up to a concentration of at least 2 IU/ml.
- Presence of anti-human Prothrombin antibodies in plasma, may inhibit meizothrombin amidolytic activity when performing the assay.
- Patients receiving a dicoumarol therapy should not be analyzed with this method, which yields higher factor II concentrations than a clotting method.
- In order to get the optimal performances of the assay, the procedural instructions must be strictly adhered to.
- For in vitro research use.

RESULTS:

- For the end point method, using a linear graph paper, plot on abscissa the Prothrombin concentration (%) and on ordinates the corresponding absorbance (**A405**).
- The Prothrombin concentration in the tested sample is directly obtained on the calibration curve. Results are expressed as % of Prothrombin.
- Using automated methods, the Prothrombin concentrations are directly calculated by the analyzer, respectively to the calibration curve.
- The dynamic range is from 5 to 200 %.
- When the assay dilution is 1:50, the Prothrombin concentration is directly read on the calibration curve. When different dilutions are used, the results must be multiplied by the dilution factor "D", divided by 50, i.e. D/50.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is indicated as an example only. Only the calibration curve generated for the series of measures performed must be used.



VALIDATION OF CALIBRATION CURVE:

The calibration curve is acceptable when the concentrations measured for the Control Plasmas are within the acceptance range.

PERFORMANCES AND CHARACTERISTICS:

- The detection threshold is calculated by measuring the "apparent" A405 obtained for a Prothrombin deficient sample plus 3 standard deviations (SD). This detection threshold is $\leq 5\%$.
- The assay is linear up to 200% Prothrombin.
- Example of Intra-Assay reproducibilities obtained for samples with variable Prothrombin concentrations (water bath):

Samples	Prothrombin concentrations (%)	Intra-Assay CV%	N
Sample 1	91	3.4	8
Sample 2	52	4.0	8

- Correlation: The BIOPHEN Prothrombin assay shows good correlation with a clotting based assay for prothrombin activity, performed by manual method: $Y = 1.02 X$ $n=48$ $r = 0.99$

EXPECTED VALUES:

By definition, the 100 % Prothrombin concentration corresponds to the concentration in a normal human citrated plasma pool, obtained by pooling plasmas from healthy males or females aged from 18 to 55 years, and out of any medication.

The Prothrombin concentration in adults is usually between 70 and 130%.

BIOCHEMISTRY:

Prothrombin, also called Factor II, is a vitamin K dependant coagulation factor, glycoprotein of about 72 kD, synthesized in the liver. Prothrombin is usually present at about 100µg/ml in plasma.

Prothrombin is a zymogen converted to thrombin by the action of factor Xa and factor V in the presence of phospholipids and calcium. Meizothrombin is an intermediate formed during this conversion of prothrombin, which is active toward synthetic specific peptide substrate.

CLINICAL INFORMATIONS:

- Various prothrombin variants have been identified. Among them, the mutation G20210A on the prothrombin gene constitutes a risk factor for venous thromboembolism (VTE). Prothrombin plasma activity for carriers of the G20210A mutation is increased, and associated with an increased risk of venous thrombosis (5) or myocardial infarction. G20210A mutation is involved in the pathogenesis of thrombophilia (4).
 - Elevated prothrombin is a risk factor for thrombosis and cerebral arterial ischemia in young adults (1), even in the absence of G20210A mutation.
 - Prothrombin deficiency is a congenital bleeding disorder characterized by 2 phenotypes:
 - Hypoprothrombinemia, type I (reduced level of coagulant activity and antigen)
 - Dysprothrombinemia, type II (decreased activity, but borderline or normal for antigen levels).
- Type I prothrombin deficiency (homozygous) is characterized by severe bleeding manifestations.
- Acquired factor II deficiency is common and results from vitamin K deficiency, severe liver disease, and therapeutic use of anticoagulant drugs.
 - Patients under anticoagulant therapy with AVK drugs should not be analyzed with this method, which yields higher factor II concentrations than a clotting method.

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