



Manufactured By: HYPHEN BioMed

BIOPHEN Prothrombin Technical File

Ref. A221605

**Chromogenic assay for the quantitative
determination of Prothrombin activity
in human citrated plasma.**

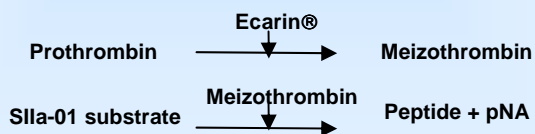
Assay range: 0 - 200% Factor II

Apr 2006

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Assay principle

- 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.
- 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 form generated during this conversion of prothrombin, which is active towards synthetic specific peptide substrates.
- 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 SIIa-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.



Intended use: For in vitro research use only

- Diagnosis of congenital or acquired Prothrombin deficiency as a risk factor for bleeding disorders.
- Measurement of elevated prothrombin concentrations, which could be associated with an increased thrombotic risk (especially in presence of the G20210A mutation).

Kit presentation: 4 x 50 tests (microplate)

R1: Reagent 1: Ecarin® : Highly purified enzyme, lyophilized with a fibrin polymerization inhibitor, and stabilized; Ecarin® can specifically activate prothrombin into meizothrombin (4 vials).

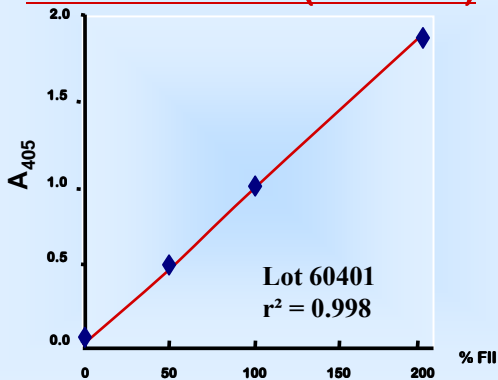
R2: Reagent 2: SIIa-01 chromogenic substrate, lyophilised (4 vials).

R3: Reagent 3: Tris BSA buffer "10xconc.": Contains BSA and sodium azide. Dilute ten fold with distilled water before use (4 vials).

Procedure

- Specimen: citrated human plasma.
- Plasma Dilution: 1:50.
- Calibration: prothrombin calibrator, plasma pool or standard.
- End-point method or kinetics protocols.

Calibration curve (0 to 200%)

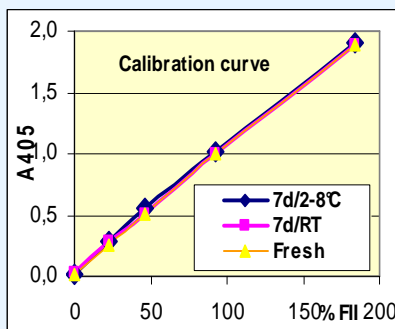


The assay has a dynamic range from 0 to 200% of Prothrombin.

Assay Characteristics

- Total assay time : **10 minutes** or below
- Assay range : **0 to 200 %** of Prothrombin in plasma (corresponds to about 0 to 2µg/ml of Prothrombin in the assayed dilution).
- Reproducibility: **≤ 2 %** (N≥10 R1 or R2, tested by manual method on the point 100% FII)
- Detection limit (blank+3SD, N≥10): **< 0.5 %** (specification ≤5%)
- Specificity: FII deficient plasma **< 0.5 %** (specification ≤5%)
- Can be used with: manual, automated, and microplate methods.

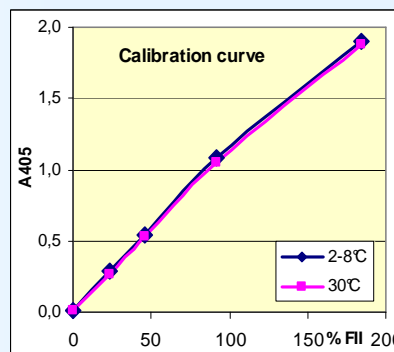
Stability of reconstituted reagents



Excellent preservation of performances of reconstituted reagents stored at 2-8°C or at RT for 7 days, compared with freshly reconstituted vials.

| Measured %FII | Fresh | 7d/2-8°C | 7d/RT |
|------------------|-------|----------|-------|
| Normal control | 92% | 94% | 91% |
| Abnormal control | 50% | 50% | 49% |

Overheating study



Excellent performances preservation of freshly reconstituted reagents, after storage of lyophilised products for 3 weeks at 30°C or 2-8°C.

Kits can be shipped at RT for a short period without damage.

| Measured %FII | 2-8°C | 30°C |
|------------------|-------|------|
| Normal control | 95% | 95% |
| Abnormal control | 52% | 53% |

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Recovery and accuracy

Goal: To validate the accurate recovery of prothrombin measurement using the BIOPHEN Prothrombin assay; recovery is evaluated by spiking various concentrations of a normal plasma pool or of purified human prothrombin into a prothrombin deficient plasma.

Material:

- Biophen Prothrombin (# 221605): lot 60401
- Biophen Plasma Calibrator: lot 51501-1(Rec. with 1 ml dist. water) (indicative value: 92% prothrombin), used to establish the calibration curve.
- Human Prothrombin 1mg: lot 051228B Exp : 2009-12. (Rec. with 1 ml dist. water)
- Prothrombin deficient Plasma: lot 040720B Exp : 2007-01 (Rec. with 1 ml dist. water)
- Human Plasma Pool (Precision Biologics): Lot A1012.

Preparation: preparation of prothrombin deficient plasma, supplemented with variable amounts of Prothrombin by addition of various volumes of the normal plasma pool, or of various volumes of human purified prothrombin (note: 100µg/ml correspond by definition to about 100% Prothrombin in plasma), in order to obtain the expected concentrations ranging from 0 to 100% Prothrombin. Each point is then diluted 1:50 in buffer (R3) for the assay.

Protocol: according to the device insert (manual method)

Results:

| Calibration curve | | |
|-------------------|-------|-------|
| % FII | Dil. | A405 |
| 184 | 1:25 | 1.979 |
| 92 | 1:50 | 1.050 |
| 46 | 1:100 | 0.526 |
| 23 | 1:200 | 0.271 |
| 0 | 0 | 0.012 |

| | Expected Prothrombin (%) | Measured Prothrombin (%) |
|-------------------------------------|--------------------------|--------------------------|
| Addition of purified (h)Prothrombin | 100µg (100%) | 98% |
| | 50µg (50%) | 53% |
| | 10µg (10%) | 13% |
| | 5 µg (5%) | 6% |
| | 1 µg (1%) | 3% |
| Addition of the plasma pool | 100% (86%) | 86% |
| | 90% (78%) | 84% |
| | 70% (61%) | 66% |
| | 50% (43%) | 43% |
| | 20% (17%) | 18% |
| | 10% (9%) | 12% |
| | 0% (0%) | 1% |

Conclusion: Using BIOPHEN Prothrombin assay, there is an excellent recovery of purified prothrombin or of prothrombin in a normal pooled plasma spiked into Prothrombin deficient plasma.

Heparin interference

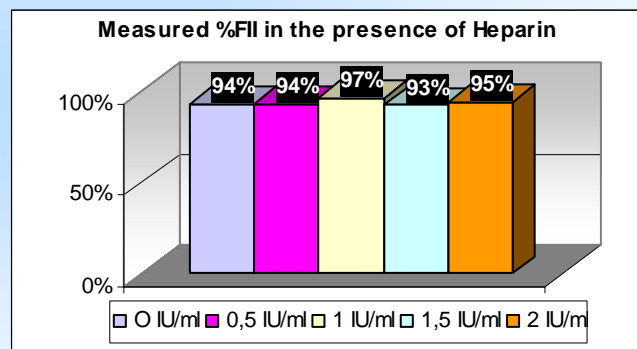
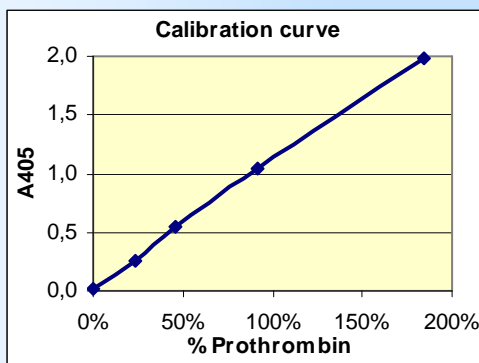
Goal: To check that there is no significant interference of heparin concentrations in plasma, up to 2IU/ml , on the prothrombin measurement using the BIOPHEN Prothrombin assay (thanks to the presence of polybren in the reaction buffer).

Material:

- Biophen Prothrombin: lot 60401
- Biophen Plasma Calibrator: 51501-1 (Rec. with 1 ml dist. water) (assigned value: 92% FII), used to establish the calibration curve

Preparation of tested samples: normal plasma pool with heparin addition (LMWH, Lovenox) in the range 0 to 2IU/ml..

Results:



Conclusion: No significant interference of heparin up to 2 IU/ml in plasma.

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Comparison of the BIOPHEN Prothrombin assay (chromogenic assay) with a clotting based assay for the prothrombin measurement; (normal range, specificity and reproducibility).

Goal: Comparison of the BIOPHEN Prothrombin assay performances with those of a conventional clotting assay for prothrombin measurement (prothrombin deficient plasma and calcium thromboplastin reagent).

Material:

- Chromogenic assay: Biophen Prothrombin lot 60401.
- Clotting assay : FII deficient plasma lot 040720B ; Stago Neoplastin (calcium thromboplastin).
- Calibration curve: established with the NIBSC 2nd international standard (SSC/ISTH lot 2) (assigned to 89% FII).

Samples:

- Quality controls: Biophen Plasma Calibrator, Normal Control and Abnormal Control.
- Normal plasmas: Precision Biologics (men and women).
- Pathological samples: dicoumarol therapy, FII Deficient plasma (lot 040720B).

Protocol for the chromogenic assay (Biophen Prothrombin, manual method):

- Calibration curve: SSC/ISTH lot 2 standard (89% FII) diluted 1:25 (178%); 1:50 (89%) ; 1:100 (44.5%); 1:200 (22.25%) ; and 0 (0%) in the ten fold diluted R3 buffer.
- Working dilution: each sample is tested at the standard 1:50 dilution in the ten fold diluted R3 buffer. (note: plasma calibrator, normal an abnormal control are tested at the 1:50 and 1:100 dilutions).

Protocol for the prothrombin clotting assay (manual method):

- Calibration curve: SSC/ISTH lot 2 standard (89% FII) diluted 1:10 (89%); 1:20 (44.5%); 1:40 (22.25%); 1:80 (11.12%); 1:160 (5.56%) in Owren Koller buffer.
- Working dilution: each sample is tested at the 1:20 dilution (note: the standard dilution for the tests is 1:10; plasma calibrator, normal an abnormal control are tested at the 1:10 and 1:20 dilution), and the measured prothrombin concentration is multiplied by 2.
- Protocol: 100µl FII deficient plasma
 100µl standard or diluted sample
 1 min at 37°C
 200µl Neoplastin preincubated at 37°C
 Measure clotting time (CT, in sec)

Results : * Calibration curves:

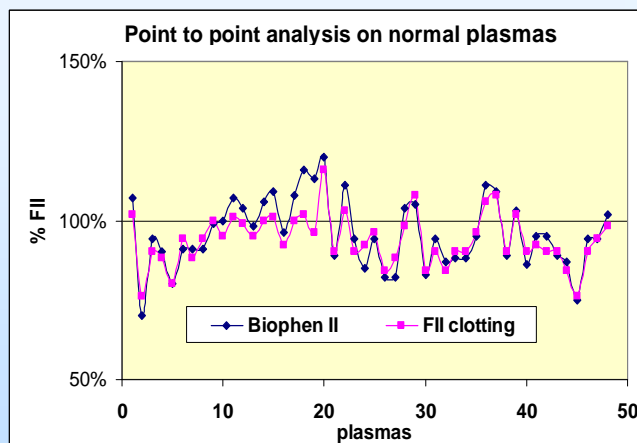
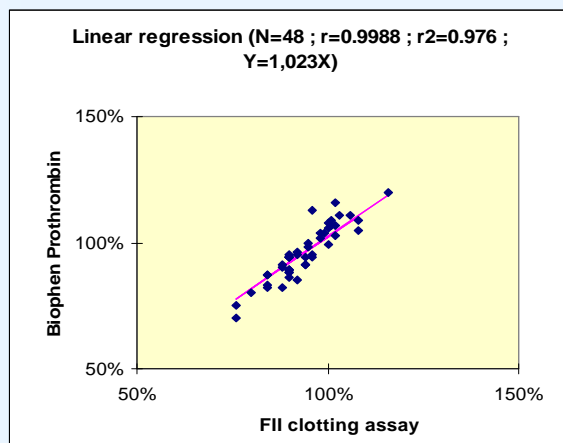
| Clotting assay | | | Biophen Prothrombin | | |
|----------------|-------|----------|---------------------|-------|-------|
| % FII | Dil. | CT (sec) | % FII | Dil. | A405 |
| 89 | 1:10 | 22.6 | 178 | 1:25 | 1.805 |
| 45 | 1:20 | 30.5 | 89 | 1:50 | 0.952 |
| 22 | 1:40 | 43.0 | 44.5 | 1:100 | 0.508 |
| 11,1 | 1:80 | 60.7 | 22.3 | 1:200 | 0.259 |
| 5.6 | 1:160 | 91.9 | 0 | 0 | 0.012 |

* Prothrombin measurement in normal samples: the expected normal range of about 70-130% is confirmed :

| «Normal samples» | Clotting assay | Biophen Prothrombin |
|------------------|----------------|---------------------|
| N | 48 | 48 |
| Mean | 94% | 96% |
| Median | 93% | 94% |
| SD | 8 | 11 |
| Min | 76% | 70% |
| Max | 116% | 120% |

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* Comparison of Prothrombin measurements in normal plasma samples:



* Prothrombin measurement on plasmas from dicoumarol treated patients, and prothrombin deficient plasma:

| Pathological samples | Clotting assay | Biophen Prothrombin |
|-----------------------------|----------------|---------------------|
| Dicou. 7068 | 15% | 36% |
| Dicou. 77402 | 22% | 48% |
| Dicou. 7274 | 33% | 79% |
| Dicou. 7360 | 22% | 46% |
| Dicou. 6877 | 14% | 39% |
| FII Deficient Plasma | < 1% | <0.1% |

* Prothrombin measurement in normal and abnormal controls, and calibrator: reproducibility data:

| % FII | Clotting assay | Biophen Prothrombin | |
|----------------|----------------|---------------------|-----------|
| | | Mean | CV (%) |
| Sample 1 (Cal) | 90% | 94% | 2.4 (N=5) |
| Sample 2 (NC) | 92% | 91% | 3.4 (N=8) |
| Sample 3 (AC) | 47% | 52% | 4.0 (N=8) |

Conclusions : There is a good correlation between the 2 methods; the expected normal range for prothrombin, of about 70-130%, is confirmed. For “dicoumarol treated” patient samples, measured prothrombin values with the BIOPHEN Prothrombin assay are slightly higher than those obtained with the clotting assay (which means that hypo- or acarboxy prothrombin is, at least partly, measured with the BIOPHEN Prothrombin kit). There is an excellent specificity (FII deficient plasma is measured at a concentration <0.1% FII). Reproducibility values obtained for plasma calibrator and control plasmas are in compliance with those expected.

Clinical applications

- The human prothrombin G20210A polymorphism results in elevated plasma prothrombin levels, and could be associated with an increased risk of venous (or arterial?) thrombosis (1,5,6).
- Elevated prothrombin levels is a risk factor for thrombosis and cerebral arterial ischemia in young adults, even in the absence of G20210A mutation (2,3). Higher prothrombin activity was reported in patients with severe coronary artery disease (4).
- Diagnosis of congenital or acquired prothrombin deficiency.

References:

- 1) « The relationship between the prothrombin upstream sequence element and the G20210A polymorphism: the influence of a competitive environment for mRNA3'-end formation », Sachchithanathan M., Stasinopoulos SJ, Wilusz J, Medcalf RL, Nucleic Acid Research, 33(3):1011-1020, 2005.
- 2) « Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk », Wolberg AS, Monroe DM, Roberts HR, Hoffman M, Blood, 101(8):3008-303, 2003.
- 3) “Elevated prothrombin is a risk factor for cerebral arterial ischemia in young adults”, Gomez Garcia EB, van Goor MP, Leebeek FW, Brouwers GJ, Koudstaal PJ, Dippel DW, Clin Neurol Neurosurg, 104(4):285-8, 2002.
- 4) « G20210A prothrombin gene polymorphism and prothrombin activity in subjects with or without angiographically documented coronary artery disease », Russo C, Girelli D, Olivieri O, Guarini P, Manzato F, Pizzolo F, Zaia B, Mazzucco A, Corrocher R, Circulation, 103:2436-2440, 2001.
- 5) « Prothrombin activity and concentrations in healthy subjects with and without the Prothrombin G20210A mutation », von Ahnen N, Lewczuk P, Schütz E, Oellerich M, Ehrenreich H, Thrombosis Research, 99:549-556, 2000.
- 6) « Prothrombotic genetic risk factors in young survivors of myocardial infarction », Ardissino D, Mannucci PM, Merlini PA, Duca F, Fetiveau R, Tagliabue L, Tubaro M, Galvani M, Ottani F, Ferrario M, Corral J, Margaglione M, Blood, 94(1): 46-51, 1999.