BIOPHEN™ DTI

REF 220202-RUO

R1 R1 2 x 2.5 mL, R3 2 x 25 mL

Chromogenic method for Direct Thrombin Inhibitors (DTI) assay.

FOR RESEARCH USE ONLY.

DO NOT USE IN DIAGNOSTIC PROCEDURES.

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English, last revision: 05-2018

INTENDED USE:

The BIOPHEN™ DTI kit is a chromogenic method for *in vitro* quantitative determination of Dabigatran and other Direct Thrombin (FIIa) Inhibitors like Hirudin, on human citrated plasma using anti-IIa method.

This kit is for research use only and must not be used for patient diagnosis or treatment.

PRINCIPLE:

BIOPHEN™ DTI is a chromogenic method based on the inhibition of a constant and in excess amount of thrombin (FIIa), by the tested Dabigatran or DTI. The hydrolysis of a thrombin specific chromogenic substrate (CS-01(81)), by the residual thrombin, releases the pNA from the substrate. The amount of pNA released (measured at 405 nm) is proportional to the residual thrombin activity. There is an inverse linear relationship between the concentration of Dabigatran (or DTI) and color development.

[DTI] + [FIIa (excess)] ➔ [FIIa-DTI] + [residual FIIa]
[FIIa (residual)] + Substrate ➔ Peptide + pNA

REAGENTS:

R1 **Thrombin specific chromogenic substrate (CS-01(81)),** lyophilized in presence of stabilizers, of an heparin neutralizing substance, and of a fibrin polymerization inhibitor.
2 vials of 2.5 mL.
R2 **Human thrombin**, lyophilized, in presence of stabilizers. Contains BSA.
2 vials of 2.5 mL.
R3 **Tris-BSA Buffer.** Tris NaCl reaction Buffer. Ready to use. Contains BSA and sodium azide.
2 vials of 25 mL.

Reagent R3 contains small amounts of sodium azide (0.9 g/L), see WARNINGS AND PRECAUTIONS.

WARNINGS AND PRECAUTIONS:

- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- The human plasma used to prepare the thrombin has been tested by approved methods and found negative for HIV 1/2 antibodies, HCV and HBs antigen.
- The bovine plasma used to prepare the BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- In contact with lead or copper pipes, sodium azide can generate explosive compounds.
- A yellow color indicates a contaminated substrate. Discard the vial and use a new one.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits. Do not mix reagents from different kit batches when performing an assay; they are optimized for each batch of kits.
- Handle the reagents with care to avoid contamination during use. If possible, avoid reagent evaporation during use by limiting the liquid-air exchange surface. Evaporation reduces the reagent's stability in the analyzer.
- To preserve reagent stability, seal the vials after use with their respective caps.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- This assay was optimized for minimizing any possible interference for heparinized samples.
- For *in vitro* use.

REAGENT PREPARATION AND STABILITY:

The reagents are lyophilized under vacuum in their vials. To avoid any product loss when opening the vial of lyophilized reagents, gently remove the freeze-drying stopper.

R1 **Reagent 1**: **Thrombin specific chromogenic substrate (CS-01(81))**
Reconstitute the contents of each vial with exactly **2.5 mL distilled water**, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.
Homogenize the reagent prior to use.
Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:

- **4 weeks** at 2-8°C.
- **24 hours** at room temperature (18-25°C).
- **2 months** frozen at -20°C or less*

R2 **Reagent 2**: **Human thrombin**
Reconstitute the contents of each vial with exactly **2.5 mL distilled water**, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.
Homogenize the reagent prior to use.
Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:

- **4 weeks** at 2-8°C.
- **24 hours** at room temperature (18-25°C).
- **2 months** frozen at -20°C or less*

*Thaw only once, as rapidly as possible at 37°C, adapting the incubation period to the volume of reagent**.** The stability of the thawed reagent should be checked under laboratory work conditions.

R3 **Reagent 3**: **Tris-BSA Buffer**
Ready to use. Allow to stabilize for 30 minutes at room temperature (18-25°C), before use.
Homogenize the reagent prior to use.
Reagent stability after opening, excluding any contamination or evaporation, and stored in the original vial, is of:

- **8 weeks** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **Do not freeze.**

STORAGE CONDITIONS:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.
- 20% acetic acid or 2% citric acid (end point method).
- Reference material for the DTI to be assayed (international or internal, pharmaceutical preparation…) or specific calibrators and controls with known titration, such as:

Calibrators	BIOPHEN™ Dabigatran Plasma Calibrator / Calibrator Low	Plasma Hirudin Standard Low / High
Reference	222801-RUO / 222901-RUO	SC020K-RUO / SC020L-RUO
Controls	BIOPHEN™ Dabigatran Control Plasma / Low	Plasma Hirudin Control
Reference	224701-RUO / 225001-RUO	SC025K-RUO

Materials:

- Spectrophotometer or automatic instrument for chromogenic assays.
- Stopwatch; Calibrated pipettes; plastic test tubes.

SPECIMEN COLLECTION AND PREPARATION:

Specimens should be prepared and stored in accordance with applicable local guidelines.

- Specimens:

Platelet poor human plasma obtained from anticoagulated blood (trisodium citrate).

- Collection:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) by clean venipuncture. Discard the first tube. The delay between the collections and the tests is ideally 1 to 2 hours and should not exceed 4 hours.

- Centrifugation:

Within 2 hours, use a laboratory-validated method to obtain platelet-poor plasma, for example at least 15 minutes at 2500g at room temperature (18-25°C) and allow the plasma to settle in a plastic tube.

- Plasma storage[1]:
  - 4 hours at room temperature (18-25°C).
  - 2 months at -20°C.

*Frozen plasma specimens should be thawed rapidly at 37°C, then shaken thoroughly and tested immediately. Resuspend any precipitate by shaking vigorously immediately after thawing and before use.

PROCEDURE:

The kit can be used for kinetics, automated or manual (endpoint) methods. Perform the test at **37°C** and read color intensity at **405nm**.

Automated methods:
Applications for the various analyzers are available on request. **See the specific application and specific precautions for each analyzer.**

Assay method (Dabigatran assay):
1. Reconstitute the calibrators as indicated in the specific instructions. Calibrators plasmas should be diluted in R3 buffer as described in the table below:

Calibrator	Reference	Dilution in R3
BIOPHEN™ Dabigatran Plasma Calibrator	222801-RUO	**1:10**
BIOPHEN™ Dabigatran Calibrator Low	222901-RUO	**1:2**

2. Dilute the specimens and controls in R3 buffer, as described in the table below:

Specimens	Reference	Dilution in R3
BIOPHEN™ Dabigatran Control Plasma	224701-RUO	**1:10**
BIOPHEN™ Dabigatran Control Low	225001-RUO	**1:2**
Samples	NA	**1:10 (standard range)** **1:2 (low range)**

Establish the calibration curve and test it with the quality controls within 1 hour for optimal assay performances. If stored at room temperature (18-25°C), test the diluted specimens within 1 hour. The exact calibrator and control concentrations for each batch are indicated on the flyer provided with the kit.

3. Dispense the following to a plastic test tube incubated at **37°C**:

	Volume
Specimen, calibrator or control diluted	200 µL
R1 **Thrombin specific chromogenic substrate**	200 µL
Mix and incubate **at 37°C** for **2 minutes**, then add the following:	
R2 **Human thrombin**. Pre-incubated at **37°C**	200 µL
Mix and incubate at **37°C** for **2 minutes exactly**	
Stop the reaction by adding:	
Citric acid (2%)*	400 µL
Mix and measure the optical density at **405nm** against the corresponding blank.	

*Or acetic acid (20%). The yellow color is stable for 2 hours.
The specimen blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R2, R1, diluted specimen**.**
Measure the optical density at **405 nm**. Subtract the measured blank value from the absorbance measured for the corresponding test.
Create a plasma blank if sample is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.

If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

Assay method (Hirudin assay):

1. Prepare the calibration curve according to the specific instructions indicated on the calibrator insert (SC020K-RUO/SC020L-RUO). Alternatively, a normal plasma pool supplemented with a known amount of Hirudin can be used. Calibrator plasmas should be prepared and then diluted in R3 buffer as described in the table below.

Hirudin Low range (SC020K-RUO)	µg/mL	0	0.5	1	1.5	2
Normal plasma at 2 µg/mL of Hirudin (µL)		0	25	50	75	100
Normal Plasma (µL)		100	75	50	25	0
R3 buffer		900	900	900	900	900

Hirudin High range (SC020L-RUO)	µg/mL	0	1.25	2.50	3.75	5
Normal plasma at 5 µg/mL of hirudin (µL)		0	25	50	75	100
Normal Plasma (µL)		100	75	50	25	0
R3 buffer		2400	2400	2400	2400	2400

2. Dilute the specimens and controls in R3 buffer, as described in the table below:

Specimens	Reference	Dilution in R3	
		High range	Low range
Plasma Hirudin Control	SC025K-RUO	1:25 (100µL + 2400µL of R3)	1:10 (100µL + 900µL of R3)
Samples	NA	1:25	1:10

Establish the calibration curve and test it with the quality controls within 1 hour for optimal assay performances. If stored at room temperature (18-25°C), test the diluted specimens within 1 hour. The exact calibrator and control concentrations for each batch are indicated on the flyer provided with the kit.

3. Dispense the following to a microplate or plastic test tube incubated at **37°C**:

	Microplate	Volume
Specimen, calibrator or control diluted	50 µL	200 µL
R1 **Thrombin specific chromogenic substrate**	50 µL	200 µL
Mix and incubate **at 37°C** for **2 minutes**, then add the following:		
R2 **Human thrombin**. Pre-incubated at **37°C**	50 µL	200 µL
Mix and incubate at **37°C** for **2 minutes exactly**		
Stop the reaction by adding:		
Citric acid (2%)*	100 µL	400 µL
Mix and measure the optical density at **405nm** against the corresponding blank.		

*Or acetic acid (20%). The yellow color is stable for 2 hours.

The specimen blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R2, R1, diluted specimen**.**

Measure the optical density at **405 nm**. Subtract the measured blank value from the absorbance measured for the corresponding test.

Create a plasma blank if sample is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.

If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

Kinetics mode:

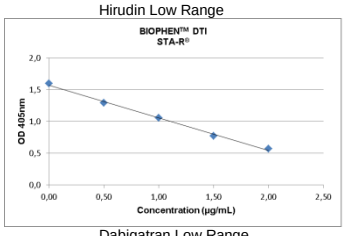
The assay can be run using a kinetics mode. In this case the change in absorbance is recorded from 10 to 100 seconds following the addition of substrate. There is then no need to subtract the sample blank, or to stop the reaction. The results are obtained using the change in absorbance (ΔA405) for calibrators and tested specimen.

CALIBRATION:

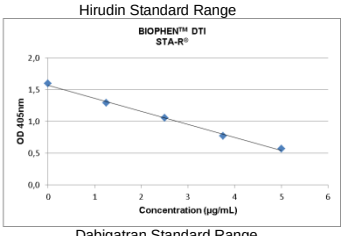
The BIOPHEN™ DTI assay can be calibrated for the assay of Direct Thrombin Inhibitors (DTI) such as Dabigatran and Hirudin. The plasma calibrators covering the calibration range are available from HYPHEN BioMed (see the REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED paragraph) and can be used to establish the calibration curve specific of the assayed analyte.

The calibration curves shown below, obtained with calibrators on STA-R® analyzer, are given by way of example only. The calibration curve established for the assay series must be used.

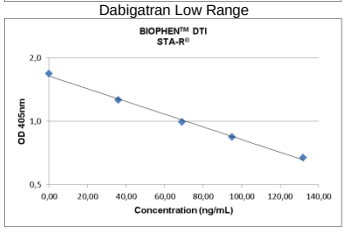
Hirudin Low Range



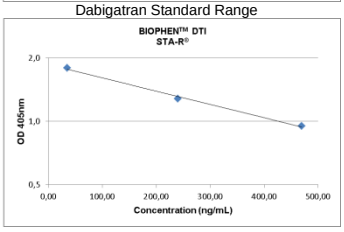
Hirudin Standard Range



Dabigatran Low Range



Dabigatran Standard Range



QUALITY CONTROL:

The use of quality controls serves to validate method compliance, along with between-test assay homogeneity for a given batch of reagents.

Include the quality controls with each series, as per good laboratory practice, in order to validate the test. A new calibration curve should be defined, preferably for each test series, and at least for each new reagent batch, or after analyzer maintenance, or when the measured quality control values fall outside the acceptance range for the method.

Each laboratory must define its acceptance ranges and verify the expected performance in its analytical system.

RESULTS:

- For the manual endpoint method, plot the calibration curve, with the OD 405 nm along the Y-axis and the analyte concentration along the X-axis:
  - **Dabigatran**, use a **Log-Lin** scale (ng/mL – OD).
  - **Hirudin,** use a **Lin-Lin** scale (µg/mL – OD).
- When employing the kinetic method, use ΔOD 405 instead of OD 405.
- The concentration of DTI in the test specimen is directly inferred from the calibration curve, when the standard dilution is used.
- Results are expressed, for example, in ng/mL for Dabigatran and µg/mL for Hirudin.
- If other dilutions are used, the level obtained is the measured level, multiplied by the additional dilution factor used

The results obtained should be for research use only and must not be used for patient diagnosis or treatment.

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully. The laboratory is responsible for validating any changes made to these instructions for use.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any plasma displaying a coagulum or showing signs of contamination must be rejected.
- Blood activation, during specimen collection and plasma preparation, may interfere in the assay. Discard any sample presenting an unusual aspect (icteric, haemolysed, lipaemic…). No significant interference of excess or deficiency of other plasma factors was identified, in compliance with the test principle using diluted test plasma and a substrate plasma in excess. However special caution is recommended for plasmas presenting a constitutional or acquired hypocoagulability. Each laboratory should establish and verify its own working range, expected values and acceptance ranges, as well as performances, in the exact laboratory working conditions (combination of assayed DTI, reagents lots and instruments used), and for its specific application.
- For the possible influence of interferences, refer to specific application for the analyzer used (no significant effect is observed on Sysmex CS-5100 for Heparin concentration up to 2 IU/mL, bilirubin concentration up to 10 mg/dL, hemoglobin concentration up to 50 mg/dL and intralipids concentration up to 150 mg/dL, by plasma overload tests).

PERFORMANCES:

- **The lower limit and the measurement range are defined by the analytical system used.**
- For the standard range, the calibration range is about **0 to 500 ng/mL Dabigatran.**
- For the low range, the calibration range is about **0 to 110 ng/mL Dabigatran and about 0 to 2 µg/mL Hirudin.**
- For the high range, the calibration range is about **0 to 5 µg/mL Hirudin.**
- The measuring range of Dabigatran depends on the analytical system used (about 15 to 120 ng/mL (low range) or 20 to 500 ng/mL (standard range) on STA-R®-series or CS-series.
- The measuring range of Hirudin depends on the analytical system used (about 0.15 to 2 µg/mL (low range) or 0.30 to 5 µg/mL (high range) on STA-R®-series.
- BIOPHEN™ DTI reagent **contains a heparin neutralizing substance:** the assay can be run on heparinized samples (no significant interference is observed up to 2 IU/mL UFH or LMWH in plasma).
- The influence of progressive thrombin inhibitors can be neglected because of the short incubation time.
- Performance studies were conducted internally on 1 batch of reagent using a Sysmex CS-series and STA-R®-series. Performance was assessed using laboratory controls. The following results were obtained:

	Intra assay			Inter assays		
Echantillons	n	Mean	CV%	N	Mean	CV%
Dabigatran low range level 1	6	28,8 ng/mL	5,3	8	23,4 ng/mL	10,7
Dabigatran low range level 2	6	86,2 ng/mL	4,1	8	79,4 ng/mL	3,0
Dabigatran high range level 1	6	111,2 ng/mL	1,4	6	110,8 ng/mL	7,1
Dabigatran high range level 2	6	280,5 ng/mL	2,4	6	281,8 ng/mL	2,4
Hirudin level 1	10	1,00 µg/mL	4,8	4	1,26 µg/mL	<5%
Hirudin level 2	10	2,00 µg/mL	1,5	4	2,16 µg/mL	<5%

REFERENCES:

1. Woodhams B *et al*. Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.