

CE HEMOCLOT THROMBIN INHIBITORS

CK002K

Clotting assay for the quantitative measurement of hirudin and other direct thrombin inhibitors in plasma

For in vitro diagnostic use only

HYPHEN BioMed

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Last revision: 17/12/2010

Intended use:

HEMOCLOT® THROMBIN INHIBITORS is an in-vitro diagnostic device intended to be used for the quantitative measurement of direct thrombin inhibitors (DTI), such as hirudin, Argatroban®, and dabigatran in human citrated plasma, with a clotting method based on the inhibition of a constant and defined concentration of thrombin. It is intended for prescription use. Measuring DTI concentrations in patient's plasma may be used as an aid in the management of patients receiving DTIs who are suspected of having excess anticoagulant activity.

Specimen:

Plasma prepared from citrated anticoagulated blood, where hirudin or any other DTI activity must be measured.

Assay principle:

For measuring hirudin or any other DTI in plasma, first, the diluted tested plasma is mixed with a normal pooled human plasma (R1). Clotting is then initiated by adding a constant amount of highly purified human thrombin, in the α form (R2). The clotting time measured is directly related to the concentration of hirudin or assayed DTI in the tested plasma.

Reagents:

Each kit contains:

- **R1 (Reagent 1)** : 3 vials of 1 mL of normal pooled citrated plasma, lyophilized.
- **R2 (Reagent 2)** : 3 vials of 1 mL of highly purified human calcium thrombin (in the α form), stabilised with additives, and lyophilized.

Warning: Thrombin (R2) is prepared by activation of purified prothrombin extracted from human plasma. Human plasmas used for the pool (R1) and thrombin preparation were tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. Bovine Serum Albumin (BSA) (R2) was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.

Reagents and material required, but not supplied

Reagents:

- Distilled water, preferentially sterile.
- Dilution buffer: 0.15M NaCl physiological saline solution, or Owren Koller type buffer (eg #AR003A/K). The same diluent must be used for all the tests performed.
- Normal plasma (or plasma pool) and reference material for Hirudin or other assayed DTI, or calibration and quality control plasmas titrated for the assayed DTI. The following references are available:

	Hirudin (Lepirudin/ Refludan®)	Argatroban®	Dabigatran
Calibrators	SC020K (usual low range) or SC020L (high range) (2 calibrators to prepare 5 levels, per kit)	SC030K (5 levels, ready to use)	222801 (3 levels, ready to use)
Controls	SC025K	SC035K	224701

Material:

- Pipettes with dispensing volumes of 20 μ L, 50 μ L and 100 μ L.
- Pipette with a variable dispensing volume from 50 μ L to 1,000 μ L.
- Semi-automatic or automatic coagulation instrument, or fibrometer or electromagnetic water bath and stop watch.

Storage conditions

Reagents must be stored at 2-8°C, in their original packaging box. They are then stable, before any use, until the expiration date printed on the box.

Note: Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

Preparation and stability of reagents:

- **R1: Normal pooled plasma:**
Reconstitute each vial with exactly 1 mL of distilled water. Shake until complete dissolution of the content (vortex). Let to homogenize for 15 min. at room temperature (18-25°C) while shaking the vial from time to time.
Homogenize before each use. .

- **R2: Human calcium Thrombin:**

Reconstitute each vial with exactly 1 mL of distilled water. Shake until complete dissolution of the content (vortex). Let to homogenize for 15 min. at room temperature (18-25°C) while shaking the vial from time to time.

Homogenize before each use.

Stability of restored reagents, provided that any contamination or evaporation is avoided, kept in the original vial or in a plastic tube, is at least:

- 8 hours at room temperature (18-25°C).
- 24 hours at 2-8°C.
- 2 months frozen in the original vial or in a plastic tube at -20°C or below (before use thaw in a water bath at 37°C for at least 15 min.).

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use. .
- Reagents must be handled with care, in order to avoid any contamination or evaporation during use.
- Reagents are closed under vacuum. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.
- Incubating the reconstituted vials at RT allows stabilizing the reagents, and obtaining a homogeneous reactivity.
- Take care to limit as much as possible any evaporation of the reagents during use, eg. by using chimneys.
- Use only reagents from kits with the same lot number.
- Do not mix reagents from kits with different lots when running the assay. Reagents are optimized for each lot of kits.

- Refer to each specific adaptation.

Sample collection and preparation:

Blood (9 vol.) must be collected on 0.109M trisodium citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma must be tested within:

- 8 hours when stored at room temperature (18-25°C)
- 24 hours if kept at 2-8°C
- frozen at -20°C or below, for up to 6 months. Just before use, the plasma must be thawed for 15 min. in a water bath at 37°C. Thawed plasma must be used within 4 hours, at room temperature (18-25°C).

Note: Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (icteric, haemolysed, lipaemic aspect....).

Tested plasma

Tested plasma must be used diluted in 0.15 M NaCl physiological saline solution or in Owren Koller buffer, according to the assay variant used:

Low range protocol (usual one) : 1:8 dilution
High Hirudin range protocol : 1:20 dilution

Procedure:

The assay is calibrated with the DTI used. The kit is currently validated for assaying Hirudin (Lepirudin/Refludan®), dabigatran, and Argatroban®.

The assay working ranges are:

Low range protocol (usual one) :

Hirudin (usual posology): 0 to 2 μ g/ml
Dabigatran: 0.05 to 0.5 μ g/ml
Argatroban®: 0 to 2 μ g/ml

High Hirudin range protocol :

Hirudin (elevated concentrations): 0 to 5 μ g/ml

The kit can also be used with other DTIs, but for current research use only, as associated commercial calibrators are still not available yet. When required, the protocol must be adjusted to the DTI used : a calibration curve can be prepared by spiking the assayed inhibitor into normal plasma. Alternatively, inhibition can be expressed as "hirudin equivalent".

1. Usual Low Range :

Calibration curve:

Prepare the calibration curve for the assayed DTI according to the specific instructions indicated on each calibrator insert (Hirudin low range #SC020K, Argatroban® #SC030K, or dabigatran #222801). Consider the exact concentrations ("C") indicated for each lot on the flyer provided within the kit.

Alternatively, if a homemade calibration is used, prepare a normal citrated plasma (or plasma pool) containing 2 µg/mL of hirudin (using preferably the hirudin used for patient's treatment) or Argatroban®, or 0.50 µg/ml of dabigatran.

Then prepare the indicative following calibration curve in normal plasma, according to the DTI used:

µg/mL (Hir. or Argatroban®)	0	0.5 or C:4	1 or C:2	1.5 or 3C:4	2 or C
µg/mL (dabigatran)	0.05 or C:10	0.25 or C:2	0.50 or C		

These calibration plasmas must then be diluted 1:8 in the diluent, for the test (ie 100µl of point + 700µl of diluent). OR Refer to each specific adaptation.

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

Tested plasmas or controls:

Tested plasmas or controls must be diluted 1:8 in the diluent, for the test (ie 100µl of point + 700µl of diluent). The diluted samples must be tested within 1 hour.

2. High Range for hirudin :

Used for hirudin concentrations in plasma of about 2 to 4 µg/mL (eg: ECC).

Calibration curve:

Prepare the calibration curve according to the specific instructions indicated on the calibrator insert (Hirudin High range #SC020L). Consider the exact concentrations ("C") indicated for each lot on the flyer provided within the kit.

Alternatively, if a homemade calibration is used, prepare a normal citrated plasma (or plasma pool) containing 5 µg/mL of hirudin (using preferably the hirudin used for patient's treatment).

Then prepare the indicative following calibration curve in normal plasma:

µg/mL (Hirudin)	0	1.25 or C:4	2.50 or C:2	3.75 or 3C:4	5 or C
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These calibration plasmas must then be diluted 1:20 in the diluent, for the test (ie 100µl of point + 1900µl of diluent). OR Refer to each specific adaptation.

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

Tested plasma or controls:

Tested plasmas or controls must be diluted 1:20 in the diluent, for the test (ie 100µl of point + 1900µl of diluent). The diluted samples must be tested within 1 hour.

3. Assay protocol :

Note: Testing in duplicate is recommended for all calibrators, controls and samples.

Manual method:

Preincubate thrombin at 37°C.

In a test tube or in a cuvette at 37°C introduce:

- 100 µL of normal pooled plasma (R1)
- 50 µL of calibration solution or of tested plasma, diluted 1:8 (low range) or 1:20 (high range)

Incubate for 1 Min. at 37°C, then introduce:

- 100 µL of thrombin (R2), preincubated at 37°C, starting the stop watch.

Record the clotting time (in seconds).

Note: The assay is suitable for testing other DTIs, but for research purposes only. Users should prepare their own calibration curve according to the expected therapeutic levels, assay dynamic range for the DTI used, and adjust the working dilution when required.

Automated methods:

Adaptations to various analysers are available upon request. Refer to each specific DTI, adaptation and specific cautions for each instrument.

Quality control:

Using suitable commercially available quality control plasmas, titrated for the assayed DTI, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents. The calibration curve is acceptable when linearity ($r^2 \geq 0.98$) and the concentrations measured for controls are within the acceptance range. Various control plasmas are available:

Hirudin (Lepirudin)	SC025K (C1 more representative for low range, and C2 for high range)
Argatroban®	SC035K
Dabigatran	224701

Each laboratory should verify (and adjust if required) its own target values and acceptance ranges, in the exact working conditions, for each new lot of reagents used.

Note: Include at least one quality control at each level in each series, as per good laboratory practice. A new calibration curve must be carried out preferentially for each test series, and at least for each new lot of reagents, after each important maintenance of the analyzer, or when measured values for the quality controls are out of the acceptance range determined for the method (after checking all other parameters of the system). Each laboratory should establish and verify its own target values, acceptance ranges and expected performances, according to the combination of assayed DTI, reagents lots, instruments and protocols used, and in its exact working conditions.

Expression of results

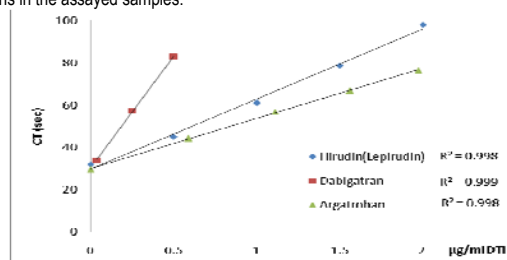
On a linear graph paper, plot on abscissae the assayed DTI concentrations (eg in µg/ml) and on ordinates the corresponding clotting times (CT in seconds). On the calibration curve obtained, interpolate directly the corresponding DTI concentration for the tested plasma (when the standard dilution is used for the assay).

Using automated methods, the DTI concentrations are directly calculated by the analyzer, respectively to the calibration curve, and the sample dilution used.

The measured DTI concentration must be analyzed considering the posology used and the clinical context for the patient. In case of unexpected result, the concentration must be verified by performing a new testing, and if required by using another method to evaluate the hypocoagulability state of the patient.

Example of calibration curve:

The calibration curve below is given as an example only, using the STAR (low range). Only the calibration curve generated for the series of assays performed must be used for calculating the concentrations in the assayed samples.



Performances and characteristics, Interferences:

- The HEMOCLOT THROMBIN INHIBITORS reagents do not contain heparin inhibitors. Presence of heparin or of other anti-thrombin substances, different from the one to be tested, may interfere in the assay and prolong the clotting time. Therefore, any anti-thrombin activity present in the tested plasma is not masked and this allows avoiding any underestimation of an existing hypocoagulability, as the result from the presence of an anti-thrombin substance.
- Normal plasmas (without treatment) do not contain Thrombin Inhibitors (≤ 0.05 to 0.10 µg/ml) using the low range protocol.
- Example of reproducibility data using STAR instrument (low range) and lyophilized calibrators:

Lyophilized sample	µg/ml	Intra Assay CV%	Inter Assay CV%
Hirudin	1.15	2.8% (N=10)	5.0% (N=6)
Dabigatran	0.12	2.2% (N=20)	5.3% (N=9)
Argatroban®	1.25	2.3% (N=10)	2.2% (N=5)

Limitations of the procedure:

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. In order to get the optimal assay performances, the working instructions must be carefully observed. 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 reagents lots and instrument used), and for its specific application.

Complementary Information :

The assay is optimised for hirudin concentration, expressed in µg/mL. The specific activity for hirudin drugs can vary from product to product or from lot to lot (from $< 10,000$ ATU*/mg to $> 15,000$ ATU*/mg). The curves are constructed respectively to the hirudin concentration. If a calibration by hirudin activity, expressed in ATU*/mL, is needed, or when a different thrombin inhibitor is used, the user must take into account the specific anti-thrombin activity of the preparation used.

*ATU: Anti-Thrombin Unit

References:

- Greinacher A., Warkentin T., "The direct thrombin inhibitor hirudin", Thromb Haemost 2008; 99:819-829.
- "Landmarks in Anti-Thrombin Drug Development: The Argatroban Story", Seminars in Thrombosis and Hemostasis, Vol 34, Suppl 1, Oct 2008.
- J Stangier et al, "Measurement of the Pharmacodynamic Effect of Dabigatran Etexilate: Thrombin Clotting Time", Poster PP-TH-134, ISTH 2009.
- van Ryn et al, "Dabigatran etexilate – a novel, reversible, oral direct thrombin inhibitor: Interpretation of coagulation assays and reversal of anticoagulant activity", Thromb Haemost 2010; 103: 1116–1127.