

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

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Intended use

The HEMOCLOT THROMBIN INHIBITORS kit is proposed as an aid for the quantitative measurement of hirudin and other Direct Thrombin Inhibitors (DTIs) (eg: Argatroban®) in plasma, with a clotting method based on the inhibition of a constant and defined concentration of thrombin.

Specimen

Plasma prepared from citrated anticoagulated blood, where hirudin (or any other direct thrombin inhibitor, eg Argatroban®) activity must be measured.

Assay principle

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

Reagents

Each kit contains:

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

Warning: Thrombin (R2) is prepared by activation of purified prothrombin extracted from human plasma. Human plasmas used for the pool preparation (R1) and prothrombin 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.

Note: Use only reagents from a same kit lot. Do not mix reagents from kits with different lots.

Reagents and material required, but not supplied

Reagents:

- Distilled water, preferentially sterile.
- 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 normal pooled plasma) and reference material for Hirudin (or other DTI to be assayed), or calibration kits for hirudin testing (Lepirudin/Refludan® eg #SC020K or SC020L) or Argatroban® testing (eg #SC030K).
- Quality control plasma titrated for the assayed inhibitor (eg Lepirudin #SC025K or Argatroban® #SC035K).

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 the content before each use.

This restored plasma is stable for:

- 8 hours at room temperature (18-25°C).
- 24 hours at 2-8°C.
- 2 months, frozen in its 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.).

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 the content before each use.

This restored human calcium thrombin is stable for at least:

- 8 hours at room temperature (18-25°C).
- 24 hours at 2-8°C.
- 2 months frozen in its 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 lost of powder when opening the vials.
- 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.

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), or can be used within 24 hours if kept at 2-8°C, or it can be 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 **1:8** or **1:20** in 0.15 M NaCl physiological saline solution, or in Owren Koller buffer, according to the assay variant used:

- **1:8** for the low concentration range of Hirudin or Argatroban® (0 to 2 μ g/ml)
- **1:20** for the high Hirudin concentration range (0 to 5 μ g/ml)

Procedure:

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

The assay working ranges are:

Hirudin (usual posology):	0 to 2 μg/ml	(low range protocol).
Argatroban®:	0 to 2 μg/ml	(low range protocol).
Hirudin (elevated concentrations):	0 to 5 μg/ml	(high range protocol).

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 range: Low range calibration for Hirudin or Argatroban®

Calibration curve:

Prepare the calibration curve for Hirudin or Argatroban® according to the instructions indicated on each specific calibration kit insert (eg #SC020K for Hirudin low range, or SC030K for Argatroban®).

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

Using this preparation, or with the calibration kit (considering the concentrations indicated for each lot), prepare the following calibration curve:

µg/mL (assayed DTI: Hirudin or Argatroban®)	0	0.5 or C:4	1 or C:2	1.5 or 3C:4	2 or C
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The calibration plasmas must be diluted **1:8** in physiological saline or Owren Koller buffer for the test.

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

Tested plasma or controls:

Tested plasma or control must be diluted **1:8** (i.e. 100 µL of plasma and 700 µL of diluent) in Owen Koller type buffer or in a physiological saline solution (0.15M sodium chloride). The diluted samples must be tested within 1 hour.

Assay protocol :

Preincubate thrombin at 37°C.

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

- **100 µL** of normal pooled plasma (**Reagent 1**)
- **50 µL** of calibration solution or of tested plasma diluted **1:8**.

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

- **100 µL** of thrombin (**Reagent 2**), 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 (eg: refer to PP-TH-134 ISTD 2009)

2. High hirudin concentration range:

This protocol is used for hirudin concentrations in plasma of about 2 to 4 µg/mL (eg: ECC).

Calibration curve:

Prepare the calibration curve for Hirudin according to the instructions indicated on the specific calibration kit insert (eg #SC020L for Hirudin high range).

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

Using this preparation, or with the calibration kit (considering the concentrations indicated for each lot), prepare the following calibration curve:

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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The calibration plasmas must be diluted **1:20** in physiological saline or Owren Koller buffer for the test.

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

Tested plasma or controls:

Tested plasma or control must be diluted **1:20** (i.e. 100 µL of plasma and 1900 µL of diluent) in Owren Koller type buffer or in a physiological saline solution (0.15M sodium chloride). The diluted samples must be tested within 1 hour.

Assay protocol:

Preincubate thrombin at 37°C.

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

- **100 µL** of normal pooled plasma (**R 1**)
- **50 µL** of calibration solution or of tested plasma diluted **1:20**.

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

- **100 µL** of thrombin (**R 2**), preincubated at 37°C, starting the stop watch.

Record the clotting time (in seconds).

Automated methods:

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

Quality control:

Using suitable commercially available quality control plasmas, titrated for Hirudin or Argatroban® (or other 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 the linearity ($r^2 \geq 0.98$) and the concentrations measured for controls are within the acceptance range.

Various control plasmas are available:

Plasma Hirudin Control (#SC025K) (C1 is representative for the low range protocol, and C2 is representative for the high range protocol), or **Argatroban® Control Plasma (#SC035K)**. Each laboratory should verify its own target value and acceptance ranges, in the exact working conditions, for each new lot of reagents used.

Note :

- Include at least one quality control 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.
- Each laboratory should establish and verify its own target values, acceptance ranges and performances, according to the combination of reagents lots, instruments and protocols used.

Expression of results

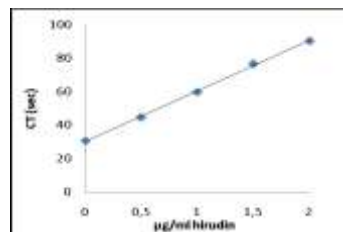
On a linear graph paper, plot on abscissae the assayed DTI concentrations (eg in µg/ml) and on ordinates the corresponding clotting times (CT in seconds). On the calibration curve obtained, interpolate directly the corresponding DTI concentration for the tested plasma.

Using automated methods, the DTI concentrations are directly calculated by the analyser, 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 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 manual method for Hirudin (low range). Obtained CT are expected slightly shorter for Argatroban®. 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 (≤ 0.10 µg/ml) using the low range protocol.

- Example of reproducibility results obtained using the STAR instrument and lyophilized calibrators:

Lyophilized sample	µg/ml	Intra Assay CV%	Inter Assay CV%
Hirudin (low range)	1.15	2.8% (N=10)	5.0% (N=6)
Argatroban® (low range)	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:

1. Greinacher A, Warkentin T., "The direct thrombin inhibitor hirudin", Thromb Haemost 2008; 99:819-829.
2. "Landmarks in Anti-Thrombin Drug Development: The Argatroban Story", Seminars in Thrombosis and Hemostasis, Vol 34, Suppl 1, Oct 2008.
3. J Stangier et al, "Measurement of the Pharmacodynamic Effect of Dabigatran Etexilate: Thrombin Clotting Time", Poster PP-TH-134, ISTD 2009.