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 plasma used for the pool (R1) and thrombin preparation were tested with registered methods and found negative for HV antibodies, Hbs Ag and Hbc 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:
- Distilled water, preferentially sterile.
- Pipettes with a variable dispensing volume from 50 μL to 200 μL.
- Pipette with a variable dispensing volume from 50 μL to 1,000 μL.
- Semi-automatic or automatic coagulation instrument, or fibrrometer 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.

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 vial (vortex). Let to homogenize for 15 min. at room temperature (18-25°C) while shaking the vial from time to time.

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
- 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.).

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.).

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”.

Preparation:
Reconstitute each vial with exactly 1 mL of distilled water. Shake until complete dissolution of the vial (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.

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

**Hirudin (Lepirudin/Refludan®)**
- Low range protocol (usual one): 1:8 dilution
- High Hirudin range protocol: 1:20 dilution

**Argatroban®**
- Low range protocol (usual one): 0 to 2 µg/ml
- High Argatroban®: 0 to 2 µg/ml

**Dabigatran**
- Low range protocol (usual one): 0 to 5 µg/ml
- High Dabigatran: 0 to 5 µg/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”.

Last revision: 17/09/2013
### 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 (\(^\circ\)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 500 ng/mL of dabigatran.

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

<table>
<thead>
<tr>
<th>µg/mL (Hir. or Argatroban)</th>
<th>0</th>
<th>0.5 or C:4</th>
<th>1 or C:2</th>
<th>1.5 or 3C:4</th>
<th>2 or C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/mL (dabigatran)</td>
<td>50 or C:10</td>
<td>250 or C:2</td>
<td>500 or C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These calibration plasmas must then 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.

#### 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 (\(^\circ\)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:

<table>
<thead>
<tr>
<th>µg/mL (Hirudin)</th>
<th>0</th>
<th>1.25 or C:4</th>
<th>2.50 or C:2</th>
<th>3.75 or 3C:4</th>
<th>5 or C</th>
</tr>
</thead>
</table>

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 plasmas 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 linearly (R2>0.98) and the concentrations measured for controls are within the acceptance range. Various control plasmas are available:

- Hirudin (Leprudin) #SC025K (C1 more representative for low range, and C2 for high range)
- Argatroban® #SC030K
- Dabigatran #222801

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.

### Expression of results:
On a linear graph paper, plot on abscissa the assayed DTI concentrations (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 physiopathology 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 hypoocoagulability 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 hypoocoagulability, 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:

### 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 hypoocoagulability. 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 calibration of the 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: