Recommended methodology
for using WHO International Reference Preparations for Thromboplastin

The International Reference Preparations for Thromboplastin are held and distributed by:
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1. General

The WHO International Reference Preparations (IRP’s) shall be used by National Reference Laboratories for the calibration of thromboplastins in the laboratory control of oral anticoagulant therapy. The value of the International Sensitivity Index (ISI) as indicated on the leaflets attached to the IRP’s was obtained by expert laboratories which tested on each day two fresh normal and six fresh patient plasmas. The majority of the laboratories performed these tests on ten different occasions. This number of normal and patient specimens is required because of a large inter-individual variation in respect to the coumarin induced coagulation defect. Repeating the investigation on a significant number of occasions largely averages:

a. the influence of both the inter-vial variation of the IRP’s and
b. the inter-assay variation in the prothrombin time determination.

The variation of prothrombin times, as shown by the scatter around the correlation line is highest when thromboplastins of different species and of different composition are tested against each other. Depending on the composition of the preparation, thromboplastins are being referred to as thromboplastin plain or thromboplastin combined:

- Thromboplastin plain: a suspension of tissue extract in saline or buffer, with phenol added in case of BCT/253.
- Thromboplastin combined: a suspension of tissue extract in saline or buffer, with a suitable concentration of fibrinogen, factor V and calciumchloride.

The IRP human (BCT/253) and the IRP rabbit (RBT/79) are thromboplastins plain, the IRP bovine (OBT/79) is a thromboplastin combined.

The calibration of a working thromboplastin preparation should be done in a two step procedure.
In the first step, a National or Working Reference Preparation (WRP), i.e., a representative batch of the working preparation, will be calibrated with the most similar IRP.
In case of rabbit or mixed rabbit-monkey thromboplastin plain this is RBT/79, in case of human brain and of human placenta thromboplastin plain this is BCT/253, and in case of a combined thromboplastin this is OBT/79.
Calibration of the WRP with the most similar IRP shall be done according to the following protocol. In doing so, the ISI value and the standard error (SE) of the ISI value for the National or Working Reference Preparation can be calculated according to the method given in Appendix 1.
The second step is calibration of the working preparations against the WRP. This batch-to-batch calibration may be performed with frozen or lyophilized instead of fresh plasmas, in accordance with WHO requirements (WHO Technical Report Series 687, 1983).
Laboratories with access to a large number of patients are requested to select the 6 patient samples displaying the widest possible variety of levels of anticoagulation, with International Normalized Ratios between 1.5 and 5. (this range is equivalent to prothrombin time ratio’s from 1.2 to 2.2 times normal with most of the common commercially available rabbit thromboplastins having ISI values of about 2.1) Laboratories having only a limited number of patients available should take 6 samples irrespective of their level of anticoagulation. Take a different set of 6 patients on each day. To avoid unwanted bias, all results obtained with the chosen samples must be recorded.

2.1.2 Blood samples

Blood samples shall be obtained by venepuncture, avoiding haemolysis and contamination with tissue fluids. It shall be drawn either with a plastic syringe and transferred to a plastic tube, or with other non-contact activation equipment. Nine volumes of blood shall be decalcified with one volume of 100-136 mmol/l trisodium citrate solution (sterile). Alternatively, if measured as a final concentration of citrate in plasma, the value shall not exceed 25 mmol/l. The same concentration of citrate shall be used for all the samples in a given calibration. The sample shall be centrifuged as soon as received, and the plasma kept undisturbed in a narrow, stoppered, non-contact tube. The sample shall be tested without delay and in any event within six hours. Frozen plasma samples shall not be used. The plasma need not be taken off the red cell layer, but the centrifugation should be such that the plasma is rendered platelet-poor (at least 5 min at 800 g).

2.1.3 IRP of Thromboplastin

The appropriate International Reference Preparations of Thromboplastin (human, rabbit, or bovine) shall be reconstituted as instructed and the contents of the ampoules transferred to a container in sufficient volume for all tests on a single calibration occasion.

2.1.4 Number of IRP's (ampoules) needed per occasion

For human (BCT/253): 2 ampoules
For bovine (OBT/79): 2 ampoules
For rabbit (RBT/79): 2 ampoules

2.1.5 Handling the IRP's

For IRP human (BCT/253): reconstitute 2 ampoules with 0.5 ml phenolized water each (phenolized water is provided and stored at + 4°C). Shake gently and keep at room temperature until use. This thromboplastin does not contain calcium.

For IRP bovine (OBT/79): reconstitute 2 ampoules with 2.2 ml of 3.2
Pipettes, test tubes, dispensers and thermostats customarily used in the laboratory are allowed if they fulfil all the above mentioned preconditions. For plasma fresh pipetting tips shall be used for each test.

2.1.8 Actual testing

The coagulation endpoint shall be determined either by hand and eye (tilt-tube or Kolle-Hook technique) or by means of a semi-automatic coagulation endpoint reading machine. If the tilt-tube technique is used, test tubes must be kept as deep as possible under water in order to maintain optimal temperature (5 cm appears to be optimal, but 3 cm should do equally well for the investigator using small test tubes). The use of an illuminated water-bath avoids the necessity for removal of the tube from the water during tilting.

The sequence of testing is indicated in the following scheme by Roman numerals.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IRP</th>
<th>WRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal 1</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>patient 1</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>patient 2</td>
<td>V</td>
<td>VI</td>
</tr>
<tr>
<td>patient 3</td>
<td>VII</td>
<td>VIII</td>
</tr>
<tr>
<td>patient 4</td>
<td>IX</td>
<td>X</td>
</tr>
<tr>
<td>patient 5</td>
<td>XI</td>
<td>XII</td>
</tr>
<tr>
<td>patient 6</td>
<td>XII</td>
<td>XIV</td>
</tr>
<tr>
<td>normal 2</td>
<td>XV</td>
<td>XVI</td>
</tr>
</tbody>
</table>

Proceed as follows: Place 2 glass test tubes for at least 5 minutes in the waterbath for testing each plasma sample according to the order indicated in Roman numerals as given in the scheme.

a. Testing shall not be done in duplicate but in single determinations. The main reasons for using duplicate determinations would be outlier detection and estimation of residual errors. However, in the thromboplastin calibration series outliers will be detected in a plot of IRP clotting times against WRP times. Furthermore, the variation between duplicates would only a relatively small contribution to the total variability in the data. Single determinations are also preferable to duplicate determinations, because it is important to minimize the total duration of the testing procedure in order to avoid bias due to time-dependent changes of the materials.

b. The two determinations (one with each thromboplastin) on each plasma (normal or patient) shall be performed immediately after each other. Thus, both determinations on one plasma should be completed before starting the new series of two determinations with the next subject’s plasma. This order of testing means that determinations on each plasma will be connected in time as closely as possible.

For IRP bovine (OBT/79) containing calcium:

a. transfer 0.4 ml thromboplastin to the test tubes;

b. incubate for 2 min;
The final preparation shall be platelet-poor plasma, which has been freeze-dried or frozen (at —30°C or below) in suitable containers. After reconstitution or thawing, the pH shall not exceed 7.8, and the preparation shall not show any shortening or prolongation of clotting times for at least 2 hours when held at ambient temperature.

The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma should not show a prolongation of prothrombin time of over 5% after 4 weeks at 37°C.

The factor V activity shall be between 70% and 140% of the average activity of fresh normal plasma.

To permit the determination of the endpoint by photoelectric coagulometers, the turbidity at 320 nm shall be less than 2.0.

2.2.1.2 Properties of pooled coumarin plasma

Pooled coumarin plasma is obtained from at least 10 different patients who have been "stabilized" on long-term oral anticoagulants for at least 6 weeks.

In order to ensure that a patient has become "stabilized", the clotting times of two samples taken no longer than 14 days apart should not differ by more than 20% in terms of the INR.

Plasma shall not be obtained from donors with a history of jaundice or from those with plasma lipid abnormalities.

The collection of plasma, and the stability of the freeze-dried pools are described in section 2.2.1.1.

The International Normalized Ratio of the pooled plasma shall be stated. At least 2 different plasma pools having an International Normalized Ratio between 2.0 and 4.0 are necessary for the calibration procedure.

The factor V activity, turbidity, and citrate concentration shall comply with the requirements for normal plasma.

2.2.2 Freedom from infectious agents

The plasma shall be shown to be free from hepatitis B virus (HBV).

Preferably the test should be done by a highly sensitive method, such as radioimmunoassay (RIA), reserved passive haemagglutination (RPHA), or enzyme-linked immunosorbent assay (ELISA).

It is preferable for plasma pools to be sterile.

2.2.3 The test

The test shall be carried out by the same procedure as described for procedure 1 and an example of the protocol for the recording of the results is given in Appendix 2. It is recommended to use at least 15 independent prothrombin time assessments with each thromboplastin.

The freeze-dried plasma samples shall be reconstituted 15 min before pooling individual vials or ampoules in a non-contact container. Plasma that has been frozen and subsequently thawed, or reconstituted freeze-
Appendix 1

Example of use of suggested method for reporting the data for the calibration of a Working Reference Preparation of thromboplastin against an International Reference Preparation.

In this example the test results are used which are obtained on only one of the ten occasions on which a calibration procedure exists. The real calculations are done with the pooled data obtained at all ten occasions.

Date: 2.6.1983

Water-bath temperature 37.1°C

Thromboplastin:
2. International Reference Preparation of Thromboplastin Rabbit, Plain, RBT/79.

Endpoint recording: tilt tube technique (waterbath)

Time started: 10 h 15

Time finished: 11 h 00

Table 1: Suggested protocol for the recording of clotting times in the calibration of a Working Reference Preparation of rabbit thromboplastin.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Order of testing</th>
<th>Clotting time (seconds)</th>
<th>Order of testing</th>
<th>Clotting time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 1</td>
<td>1</td>
<td>15.9</td>
<td>2</td>
<td>13.8</td>
</tr>
<tr>
<td>Patient 1</td>
<td>3</td>
<td>21.4</td>
<td>4</td>
<td>19.6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>5</td>
<td>35.6</td>
<td>6</td>
<td>30.8</td>
</tr>
<tr>
<td>Patient 3</td>
<td>7</td>
<td>29.1</td>
<td>8</td>
<td>28.6</td>
</tr>
<tr>
<td>Patient 4</td>
<td>9</td>
<td>21.8</td>
<td>10</td>
<td>19.6</td>
</tr>
<tr>
<td>Patient 5</td>
<td>11</td>
<td>41.1</td>
<td>12</td>
<td>33.6</td>
</tr>
<tr>
<td>Patient 6</td>
<td>13</td>
<td>37.9</td>
<td>14</td>
<td>38.1</td>
</tr>
<tr>
<td>Normal 2</td>
<td>15</td>
<td>17.1</td>
<td>16</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Calculation

The International Sensitivity Index of the working preparation (ISI_{WRP}) is obtained by plotting the prothrombin times using the two thromboplastins on logarithmic axes as shown in Fig. 1, fitting a straight line and estimating the slope. Any suitable statistical procedure for fitting the line and for estimating the slope and standard error of the slope may be used. However, the technique of orthogonal regression may be preferred. With this technique, the slope, C_{IRP WRP}, can be calculated from the following formulae:
Fig. 1. Double logarithmic plot of prothrombin times obtained on one out of ten different occasions for determination of the International Sensitivity Index.

Example

Using the data from Table 1, the calculated value for $C_{IRP, WRP}$ is 0.968. The International Sensitivity Index for RBT/79 is 1.4. Thus, the International Sensitivity Index for the working reference preparation is estimated as $0.968 \times 1.4 = 1.355$.

The standard error for $C_{IRP, WRP}$ is calculated as 0.072. Thus, S.E. (ISI$_{WRP}$) $= 0.072 \times 1.4 = 0.100$. The coefficient of variation for the ISI of the working reference preparation is $100 \times 0.100/1.355 = 7.4\%$. The coefficient of variation is large because Table 1 contains data from only eight plasmas, i.e., one day's tests. In practice, tests would be conducted on several days and the coefficient of variation using the combined data would be smaller.
Example

Using the data from Table 2, the calculated value for $C_{\text{WRP, b}}$ is 0.996. The International Sensitivity Index for the working reference preparation is given as 1.05. Thus, the ISI for the batch is estimated as $1.05 \times 0.996 = 1.046$.

The standard error for $C_{\text{WRP, b}}$ is 0.008. If the standard error of the ISI of the working reference preparation, determined at the time of its calibration, is 0.018, the total standard error of the ISI of the batch taking account of the imprecision of both $\text{ISI}_{\text{WRP}}$ and $C_{\text{WRP, b}}$ is

$$[(1.05 \times 0.008)^2 + (0.996 \times 0.018)^2]^{1/2} = 0.020.$$  

The coefficient of variation for the ISI of the batch is $100 \times 0.020/1.046 = 1.9\%$.

Appendix 3

Calculation of International Normalized Ratio (INR) for a given patient's clotting time.

Example

<table>
<thead>
<tr>
<th>Thromboplastin: rabbit, International Sensitivity Index = 1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient's prothrombin time = 28 seconds</td>
</tr>
<tr>
<td>Normal prothrombin time: = 12 seconds (geometric mean)</td>
</tr>
<tr>
<td>Prothrombin time ratio, $R = 28/12 = 2.33$</td>
</tr>
</tbody>
</table>

To calculate the INR, the following formula is used:

$$\text{INR} = R^{\text{ISI}}$$

where ISI is the International Sensitivity Index of the thromboplastin used to measure the prothrombin time ratio $R$.

Thus the INR is $2.33^{1.5} = 3.56$.

If the facility to calculate fractional powers is not available, the alternative formula $\text{INR} = \text{antilogarithm} (\text{ISI} \times \log R)$ may be used, i.e., $\text{INR} = \text{antilog} (1.5 \times \log 2.33) = 3.56$. The two formulae for INR are exactly equivalent, and the results will be identical apart from possible rounding error. If a normal plasma is used with a prothrombin time deviating from the prothrombin time of normal reference plasma, the prothrombin-time ratio should be corrected for this deviation. The magnitude of the deviation depends on the thromboplastin used, but it should not exceed 10\%.