# Clotting Assay for Measuring Tissue Factor Activity

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http://tf7.org/mlabprotocols.htm

**Background:** The integral membrane protein, tissue factor (TF), initiates the extrinsic pathway of blood clotting. In this protocol, TF activity is measured by its ability to shorten the clotting time of plasma. The TF activity of unknown samples can be measured by reference to a standard curve. Since there is no internationally agreed-upon unit definition for TF, we define one unit of TF activity as that amount of TF that gives a 50 sec clotting time with normal pooled plasma[1-3].

## 1. TF Standards

A. *Using a preparation of relipidated TF in which you know the TF concentration* – Prepare serial ten-fold dilutions of relipidated TF[4] in HBSA/PCPS to give a range of TF concentrations from about 0.1 ng/ml to 100 ng/ml.

B. *Using a source of TF in which you do not know the TF concentration* – Prepare serial ten-fold dilutions of TF reagent (e.g., a commercial thromboplastin) in HBSA/PCPS to give clotting times ranging from about 15 sec to about 200 sec.

## 2. Sample Preparation: Cell Lysates

A. Rapidly rinse cells twice with ice-cold, isotonic phosphate-buffered saline.

B. Add a measured amount of ice-cold HBSA to the dish (typically, just enough to cover the monolayers).

C. Freeze at –80°C (the frozen cells can be stored at –80°C).

D. Thaw at 37°C.

E. Repeat steps C & D until the cells have been through three cycles of freeze/thaw lysis.

F. Rapidly pipet the lysates up & down to resuspend the cell debris. Transfer the lysates to Eppendorf tubes and store on ice until assayed. Dilute samples in HBSA/PCPS to get clotting times within the ~linear range of the standard curve.

## 3. Clotting Assay

A. Pipet 50 µl TF Standards or Cell Lysates into a pre-warmed coagulometer cuvette.

B. Add 50 µl pooled normal plasma.

C. Incubate 2 min at 37°C (to allow the mixture to reach proper temperature).

D. Add 50 µl pre-warmed 25 mM $CaCl_2$.

E. Measure the time to clot formation from the moment of adding the $CaCl_2$ solution. (Typically, in duplicate.)

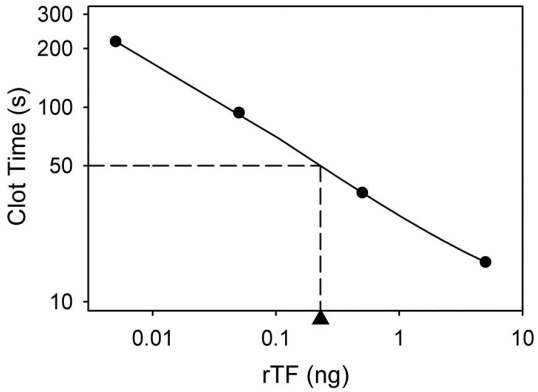


**Figure 1.** Standard curve for a typical relipidated TF preparation. The amount of TF in each 50 µl test sample is indicated on the *x*-axis. (Multiply by 20 to obtain TF concentrations in ng/ml.) One unit of tissue factor activity (upward arrowhead) is arbitrarily defined as that amount of tissue factor (in the 50 µl test sample) which yields a 50 sec clotting time with pooled normal plasma. In this example, a 50 sec clotting time corresponded to 0.230 ng TF. So, the the specific activity of this relipidated TF preparation was the reciprocal of 0.230 (i.e., 4.35 units/ng).

## 4. Standard Curve

A. Prepare a log-log plot of the data (clotting times on the *y*-axis; TF concentration on the *x*-axis). If you know the TF concentration, plot the *x*-axis in ng TF (or ng/ml TF). If you do not know the TF concentration, plot the *x*-axis in arbitrary concentration units (e.g., 1x, 0.1x, 0.01x, etc.)

B. Fit a line or polynomial to the data as appropriate. Results of a typical clotting assay are shown in Fig. 1.

C. Determine the TF activity in your unknown samples by reference to the standard curve. (Aim for clotting times in the part of the standard curve that is approximately linear.) You can report your results in units of TF activity or in ng of TF. Be advised, however, that specific activities of TF preparations can vary widely, depending upon a number of factors. So it is probably more appropriate to report your findings in units of TF activity. In the example in Fig. 1, we can multiply all of our apparent TF concentrations by 4.35 to obtain units of TF activity.

## Materials & Solutions

- **PCPS vesicles –** We typically use phospholipid vesicles composed of 80% phosphatidylcholine (PC) and 20% phosphatidylserine (PS). The vesicles can be prepared by extrusion, sonication, detergent/dialysis or detergent/Bio-Bead methods. See http://tf7.org/mlabprotocols.htm for suitable protocols for preparing these vesicles. Commercial cephalin preparations may also be suitable, as long as they lack any activator of clotting (i.e., do not have TF or an activator of the contact pathway).
- **HBS** 20 mM HEPES-NaOH buffer, pH 7.5<br>100 mM NaCl<br>0.02% $NaN_3$
- **HBSA –** HBS containing 1 mg/ml bovine serum albumin
- **HBSA/PCPS –** HBSA containing 50 µM PCPS vesicles
- **Relipidated TF (for Standard Curves) –** Recombinant human TF can be reconsituted into PCPS vesicles using the rapid Bio-Bead method[4]. This and other protocols for TF relipidation can be found at http://tf7.org/mlabprotocols.htm. (Use the membrane-anchored form of TF, not a soluble, truncated form.) Or, you can use a commercial thromboplastin reagent suitable for use in Prothrombin Time (PT) assays.
- **Pooled Normal Plasma –** Citrated plasma pooled from a number of human donors (at least 20). You can purchase citrated, pooled normal plasma from George King Bio-Medical (Overland Park, KS) or other suppliers. Use only plasma that is anticoagulated with citrate.
- **Calcium Chloride –** The stock solution is 1 M $CaCl_2$ in water. Dilute with water to 25 mM on the day of use. (Do not store 25 mM $CaCl_2$, as it will grow microbes.)

## Notes

- You can substitute 50 mM Tris pH 7.5 for the 20 mM HEPES.
- Almost any coagulometer, fibrometer, etc., can be used to measure clotting times. Or, you can use the manual, tilt-tube method in which clotting times are performed in test tubes in a 37°C water bath and clotting times are estimated visually. (If so, increase the aliquot sizes from 50 to 100 µl.)
- We include phospholipid vesicles in our sample diluent to minimize the effect of differences in phospholipid content of samples on clotting time.
- Rodent TF binds poorly to human factor VII[5-7]. If you want to measure rat or mouse TF activity, use a mixture of 10% rat plasma/90% human plasma in this assay. (The 10% rat plasma provides sufficient rodent factor VII.)
- You can lyse cells by sonication or dounce homogenizer instead of freeze-thaw lysis. You can also measure TF activity in unlysed cells, but the specific activity will be much lower.
- It is best to wash cultured cells before lysis to remove any serum that might be present in the culture medium. (Serum could alter the clotting times.) If you can't do this, then include the same final concentration of culture medium in all of the standards as well.
- If you extend the standard curve to lower or higher TF concentrations, the shape of the curve will become sigmoidal (i.e., flattened at high & low [TF]).

## References


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