Optimization of Plasma Fluorogenic Thrombin-Generation Assays

Wayne L. Chandler, MD, and Mikhail Roshal, MD, PhD

Key Words: Thrombin generation; Fluorogenic; Tissue factor; Plasma; Coagulation

Abstract

We optimized fluorogenic thrombin-generation assays with regard to sample volume, calibration, analytic corrections, and activation reagents. Lower sample volumes (40 vs 80 µL) were associated with better recovery of thrombin activity, lower interference due to absorbance of light, and higher total thrombin generation (area under the curve), even using internal standards to calibrate plasma samples. With lower sample volumes, there was no advantage to internal calibration of samples without obvious interference (hemolysis). Previously developed corrections for measured vs expected fluorescence units, residual thrombin–α2-macroglobulin activity, and hemolysis improved the analytic accuracy of the assay. An optimized assay with a 40-µL sample volume, analytic corrections, and a corn trypsin inhibitor to block contact activation showed that 0.6 pmol/L tissue factor activator was better than 5 pmol/L at differentiating healthy subjects from patients with sepsis while demonstrating good reproducibility (area under the curve, 4% within-run and 7% between-run coefficient of variation).

Thrombin-generation assays measure the amount of active thrombin produced in plasma or whole blood after recalcification. Thrombin generation can be initiated by tissue factor–bearing microparticles or other substances in the sample itself (native thrombin generation) or by addition of exogenous activators such as tissue factor and phospholipids. Older methods measured the formation of thrombin-antithrombin complexes or other thrombin markers as a measure of thrombin generation. This method worked but was time-consuming, manually intensive, and not suited for the evaluation of large numbers of clinical samples. Subsequently, methods based on thrombin-sensitive fluorogenic or chromogenic peptide substrates have been developed that offer greater applicability to clinical studies. Fluorogenic assays have the advantages that they do not require addition of fibrin polymerization inhibitors and can be calibrated to absolute thrombin-generation rates (nmol/L of active thrombin per minute) rather than a percentage of normal plasma. Prospective studies have shown that these assays have the potential for predicting the risk of thrombosis.1,2

Currently, there are 2 commercially available fluorogenic thrombin-generation assays, the Calibrated Automated Thrombogram, developed by Hemker et al3 and marketed by Thrombinoscope, Maastricht, the Netherlands, and the Technothrombin TGA method marketed by Technoclone, Vienna, Austria. Although similar in overall design, the details of these 2 methods are different, and many modifications have been described in the literature.2,8 The purpose of this study was to optimize fluorogenic thrombin-generation assays with regard to sample volume, calibration, activation reagents, and corrections for hemolysis and other problems. We did not attempt to repeat all prior evaluations of these assays. When an extensive evaluation had already been done,
we confirmed the results and evaluated whether it should be incorporated in an optimized method.

Materials and Methods

Human Subjects

The study was approved by the University of Washington (Seattle) Human Subjects Review Committee. Informed consent was obtained from subjects. Blood samples were anticoagulated with 0.109 mol/L citrate. Citrated blood was centrifuged at 4,400g for 2 minutes at room temperature. Platelet-poor plasma was decanted and frozen at –80°C.

Materials

Lipidated recombinant human tissue factor (RecombiPlasTin) was obtained from Instrumentation Lab, Bedford, MA. Corn trypsin inhibitor was obtained from EMD Biosciences, San Diego, CA. L-α-phosphatidylcholine and L-α-phosphatidylserine were obtained from Avanti Polar Lipids, Alabaster, AL. Phospholipid vesicles composed of 0.8 mmol/L phosphatidylcholine and 0.2 mmol/L phosphatidylserine in 20 mmol/L HEPES, 100 mmol/L sodium chloride, 0.2 g/L sodium azide, pH 7.5, were prepared by drying the phospholipids under dry nitrogen, then strong vacuum, followed by suspension in HEPES buffer and sonication. Pooled normal plasma anticoagulated with 0.109 mol/L citrate was obtained from Precision Biologics, Dartmouth, Canada. Thrombin calibrator (865 nmol/L) and thrombin-sensitive fluorogenic substrate (Z-Gly-Gly-Arg-AMC) calcium reagent were obtained from Technoclone through Diagnostica Stago, Parsippany, NJ.

Thrombin-Generation Assay

Both fluorogenic thrombin-generation assays combine 3 solutions, a plasma sample, an activation reagent, and a combined fluorogenic substrate and calcium solution to start the reaction. When only buffer is added as the activation reagent (termed native thrombin generation), initiation of thrombin generation is dependent on procoagulants present in the plasma.

All samples and reagents were warmed to 37°C before use. The activation reagent consisted of thrombin-generation buffer (20 mmol/L HEPES, 140 mmol/L sodium chloride, 5 mg/mL bovine serum albumin, 0.02% sodium azide, pH 7.35) containing variable amounts of lipidated tissue factor, phospholipid vesicles, and corn trypsin inhibitor to block contact activation. In the Technoclone assay, 40 µL of plasma was added to 10 µL of activation reagent followed by 50 µL of calcium–fluorogenic substrate reagent (7.5 mmol/L calcium and 0.5 mmol/L substrate final reaction concentrations) to start the reaction. In the Thrombinscope assay, 80 µL of plasma was added to 20 µL of activation reagent followed by 20 µL of calcium–fluorogenic substrate reagent (16.7 mmol/L calcium and 0.42 mmol/L substrate final reaction concentrations). Fluorescence units were monitored at 37°C at 1-minute intervals for 90 minutes using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT) set at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Results included determination of lag time in minutes, defined as the interval from the start of substrate/calcium addition until formation of 10 nmol/L of thrombin, peak height for thrombin generation in nmol/L, peak height time in minutes, velocity index or peak rate of thrombin generation [peak thrombin/(peak time – lag time)], and area under the curve (AUC), defined as the sum of thrombin concentrations from 1 to 90 minutes (nmol/L × min). The AUC has also been referred to as the endogenous thrombin potential (ETP). Correction of measured fluorescence units for the inner filter effect and substrate exhaustion and correction for residual thrombin–α₂-macroglobulin complex formed during the reaction were as previously described. Absorbance in plasma at 360 and 460 nm was determined in a clear microtiter plate using the same sample volume used in the thrombin-generation assay.

Statistics

Results are given as the mean ± SD unless otherwise indicated. Statistical comparisons used paired t tests for paired samples and 2-way unpaired t tests for unpaired samples.

Results

Comparison of the Technoclone and Thrombinscope Methods

Figure 1 shows a comparison of the AUC results for the Technoclone method run per manufacturer’s recommendations (thrombin calibrator in buffer, no fluorescence unit correction, no residual thrombin–α₂-macroglobulin correction) vs the Thrombinscope method run per manufacturer’s recommendations (thrombin–α₂-macroglobulin calibrator added to plasma samples, correction for inner filter effect, correction for residual thrombin–α₂-macroglobulin) in samples without obvious hemolysis or other interference. On average, the Technoclone method gave higher AUC results for most samples.
Effect of Plasma Volume

The 2 assays use different amounts of plasma in their final reactions. The Technoclone assay uses 40 µL of plasma in a 100-µL total volume (40% plasma), whereas the Thrombinoscope assay uses 80 µL of plasma in a 120-µL total volume (67% plasma). Figure 2 shows that as the percentage of plasma volume in the assay decreases, thrombin generation goes up, peaking when the plasma volume is about 20% to 30% and then falling for lower plasma volumes. The pattern was similar for substrates from either company.

Calibration of Thrombin Generation

Table 1 shows a comparison of the purified active thrombin calibrator from Technoclone with the thrombin–α2-macroglobulin complex calibrator from Thrombinoscope. The specific activity of the 2 calibrators was similar in both assays, but each company’s calibrator showed slightly more activity in its own assay. In the Thrombinoscope assay, the Thrombinoscope calibrator in buffer had a specific activity 6% higher than the Technoclone calibrator in buffer. In the Technoclone assay, the Thrombinoscope calibrator in buffer had a specific activity 20% lower than the Technoclone calibrator in buffer.

Effect of Corrections

The Thrombinoscope assay uses 3 corrections to improve the accuracy of estimated active thrombin concentrations generated in plasma that are not used in the Technoclone method: (1) a correction for inner filter effect and substrate exhaustion, (2) a correction for residual thrombin–α2-macroglobulin activity in the sample, and (3) a correction for hemolysis and other interfering factors. We evaluated the effect of these corrections on the Technoclone method.

When thrombin generation is high, measured fluorescence unit levels may be underestimated owing to a combination of substrate exhaustion and the inner filter effect. We found that as substrate was consumed, measured fluorescence units underestimated expected fluorescence in the Technoclone assay Figure 3. Based on this observation, we determined the fluorescence unit correction for our fluorescence reader and confirmed that the correction was a function of the

Table 1
Technothrombin vs Thrombinoscope Calibrator

<table>
<thead>
<tr>
<th>Assay</th>
<th>Technoclone</th>
<th>Thrombinoscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technoclone: purified thrombin in buffer</td>
<td>7.41 ± 0.66</td>
<td>8.80 ± 0.78</td>
</tr>
<tr>
<td>Thrombinoscope: thrombin–α2-macroglobulin in buffer</td>
<td>7.86 ± 0.46</td>
<td>7.05 ± 0.43</td>
</tr>
</tbody>
</table>

* Specific activity of each calibrator given as mean ± SD fluorescence units per minute per nanomole per liter of thrombin in the final reaction mixture based on the reported equivalent active thrombin concentration in the calibrator.
measured fluorescence units only, not the thrombin concentration. Figure 3 shows that on average, the peak thrombin generation was 22% ± 10% higher when the fluorescence unit correction was applied.

During the thrombin-generation assay, part of the thrombin formed binds to α2-macroglobulin in plasma and accumulates as thrombin–α2-macroglobulin complexes that remain active against the fluorogenic substrate but do not activate coagulation, leading to a stable level of apparent residual thrombin activity at the end of the reaction. We determined that this residual thrombin–α2-macroglobulin activity was present in the Technoclone assay (Figure 4), leading to an overestimate of the AUC. The correction for residual thrombin–α2-macroglobulin activity had only a minor effect on peak thrombin generation, producing results that were on average 2% lower than the uncorrected values with high correlation ($r^2 = 0.99$), but had a greater effect on the AUC, which was, on average, 26% ± 7% lower ($r^2 = 0.91$).

**Correction for Hemolysis**

Hemolysis, icterus, and other factors that increase the absorbance in the sample can result in a falsely low AUC owing to absorbance of the excitation and emission light in the sample. Hemker et al proposed a method for correcting absorbance interference to the AUC or ETP by adding a known amount of exogenous thrombin–α2-macroglobulin to each sample as an internal calibrator. The Thrombinscope method recommends calibrating every sample using exogenous thrombin–α2-macroglobulin, even the samples showing no evidence of hemolysis or other interference.
In the Thrombinoscope assay, background absorbance was twice as high as in the Technoclone assay (80- vs 40-µL sample volumes), resulting in more interference in that assay, even in samples with no apparent hemolysis or other problems. In the Thrombinoscope assay (80-µL sample volume), the thrombin–α2-macroglobulin calibrator showed 71% ± 5% as much activity in normal plasma as it did in buffer (n = 12). In the Technoclone assay (40-µL sample volume), the thrombin–α2-macroglobulin calibrator showed 96% ± 4% as much activity in normal plasma as it did in buffer (n = 12). In the Technoclone assay using pooled normal plasma (PNP), the within-run coefficient of variation was 5% for the AUC and 5% for the thrombin–α2-macroglobulin calibrator slope but 8% for the AUC calibrated using thrombin–α2-macroglobulin added to each plasma sample (n = 8 for each).

In plasma samples without evidence of hemolysis, icterus, or other color changes, calibration in each sample with exogenous thrombin–α2-macroglobulin did not improve the precision and had only minor effects on the accuracy of AUC measurements using the Technoclone assay with a 40-µL sample volume but was useful in the Thrombinoscope assay to improve calibration accuracy.

To evaluate the effect of hemolysis on the assay, increasing amounts of hemoglobin up to 700 mg/dL were added to normal plasma. The AUC was reduced in direct proportion to the concentration of hemoglobin in the sample. Addition of thrombin–α2-macroglobulin as an internal calibrator in the hemolyzed plasma samples corrected the AUC on average to 98% ± 5% of the original unhemolyzed value (n = 6).

We compared the correction predicted by calibration with
thrombin–α₂-macroglobulin with absorbance measurements at the excitation (360 nm) and emission (460 nm) wavelengths in 56 samples containing varying levels of plasma hemoglobin and bilirubin. Sample absorbance at 360 nm (Abs 360 nm) and 460 nm (Abs 460 nm) showed correlations of r² = 0.72 and 0.80, respectively, with the correction predicted by the thrombin–α₂-macroglobulin. The best correlation with the thrombin–α₂-macroglobulin correction was with what we termed the absorbance ratio (r² = 0.92) Figure 5.

Figure 5 Correcting the area under the curve in 56 samples with varying levels of hemolysis and icterus. Comparison of correction based on individual sample calibration using exogenous thrombin–α₂-macroglobulin vs absorbance ratio described in the text using sample absorbance measurements at 360 and 460 nm.

Absorbance Ratio = \frac{\text{Sample Abs 360 nm}}{\text{PNP Abs 360 nm}} + \frac{\text{Sample Abs 460 nm}}{\text{PNP Abs 460 nm}}

By using the best fit between the absorbance ratio and the thrombin–α₂-macroglobulin correction, we estimated an absorbance-based correction. In hemolyzed samples, the absorbance correction adjusted the AUC to 100% ± 6% of the original unhemolyzed value (n = 6).

Sample Activation

Both companies offer a variety of activation reagents ranging from physiologic buffer only for native thrombin generation to increasing amounts of tissue factor and phospholipid. Prior studies have shown that contact activation is occurring in the Thrombinoscope assay; it can be blocked by using corn trypsin inhibitor, and in the presence of corn trypsin inhibitor, healthy subjects showed little or no spontaneous thrombin generation.8

We evaluated the effect of contact system inhibition with corn trypsin inhibitor on the Technoclone assay. Addition of corn trypsin inhibitor to plasma to block contact activation during native thrombin generation (no tissue factor or phospholipids added) prolonged the lag time and reduced the peak thrombin generation (0 vs 40 µg/mL; P = .012; paired t test) Figure 6. The amount of peak thrombin generation due to contact activation (as estimated by percentage of activity blocked by corn trypsin inhibitor) varied widely among different healthy subjects, ranging from 16% to nearly 100%. With corn trypsin inhibitor present, little or no native thrombin generation occurred in normal plasma. When relatively high concentrations of tissue factor were added, the effect of contact system inhibition decreased as the tissue factor in the activator became...
the primary initiator of thrombin generation. Owing to the variable nature of contact activation, at intermediate levels of tissue factor and phospholipid, it became difficult to separate whether the tissue factor was the primary activator or the contact system. The effect of adding phospholipid was highly dependent on whether contact activation was blocked. Peak thrombin generation increased nearly 6-fold as phospholipid was increased from 0 to 4 µmol/L when no corn trypsin inhibitor was present and contact activation was occurring, but when corn trypsin inhibitor was present and contact activation blocked, addition of up to 4 µmol/L exogenous phospholipids increased peak thrombin generation only 88%. Figure 7I.

Based on the preceding results, we selected a candidate assay for further testing of tissue factor and phospholipid: 40-µL sample volume to increase thrombin generation and reduce interference, correction for inner filter effect and residual thrombin–α2-macroglobulin to improve accuracy, and addition of 40 µg/mL corn trypsin inhibitor to block contact activation. With this candidate assay, peak thrombin generation rose as more tissue factor was added to the reaction Figure 8I. The most rapid change in peak thrombin generation occurred between 0 and 1 pmol/L tissue factor in the final reaction, but peak thrombin generation continued to rise even at the maximum tissue factor concentration tested, 6 pmol/L. Similarly the AUC rose as more tissue factor was added, but the AUC appeared to plateau at about 1 pmol/L tissue factor with only a slow rise with higher tissue factor concentrations. With corn trypsin inhibitor present, addition of phospholipids increased peak thrombin generation only at the highest tissue factor concentrations. The maximum effect occurred at a phospholipid concentration of approximately 1 µmol/L.

By using 5 normal samples and 5 samples from patients with sepsis, we evaluated the effect on thrombin generation of 3 tissue factor–phospholipid concentrations (with corn trypsin inhibitor present): (1) no tissue factor or phospholipid (native thrombin generation), (2) 0.6 pmol/L tissue factor and 1 µmol/L phospholipid, and (3) 5 pmol/L tissue factor and 4 µmol/L phospholipid Table 2I. Peak thrombin generation and the AUC were not significantly different between healthy subjects and patients with sepsis when relatively low concentrations of tissue factor (0.6 pmol/L) and phospholipid (1 µmol/L) were used to activate thrombin generation. Peak thrombin generation and AUC were not significantly different between healthy subjects and patients with sepsis when a relatively high concentration of activator was used (5 pmol/L tissue factor and 4 µmol/L phospholipid). Based on these results, we selected 2 options for further thrombin-generation testing: native thrombin generation with no activator and tissue factor–stimulated thrombin generation with relatively low levels of tissue factor (0.6 pmol/L) and phospholipid (1 µmol/L).

Reference Range and Imprecision

Native thrombin generation and tissue factor–stimulated thrombin generation were evaluated in samples from 25 healthy subjects Table 3I. The majority of healthy subjects showed little or no native thrombin generation with corn trypsin inhibitor present. Table 4I shows the within-run and between-run imprecision for tissue factor–stimulated peak thrombin generation and the AUC.

Discussion

Thrombin-generation assays have multiple potential uses, including estimation of hemorrhagic and thrombotic risk, monitoring of therapy, and detection of circulating procoagulants and microparticles. Different protocols may be needed depending on the specific use. Two types of thrombin-generation assays are currently available. Chromogenic thrombin-generation assays use high concentrations of tissue factor or partial thromboplastin time reagents as activators, produce empiric results reported as a percentage of normal, require fibrin polymerization inhibitors, and have other disadvantages. Fluorogenic thrombin-generation assays use lower levels of tissue factor activator and are calibrated to give an estimate of the actual concentration of active thrombin formed in the assay. We optimized fluorogenic thrombin-generation...
assays with regard to sample volume, calibration, activation reagents, and corrections for thrombin-generation assays with regard to hemolysis and other problems.

Thrombin-generation assays can be calibrated by using purified thrombin standards in buffer or thrombin–α₂-macroglobulin standards added to plasma. The apparent specific activity of the 2 commercial thrombin-generation calibrators in buffer was similar but not identical. Substantial between-batch variability in calibrators has been reported. Further work is needed to define an international standard for calibration of thrombin generation. The thrombin–α₂-macroglobulin calibrator has the advantage that it can be used as a calibrator and as an internal standard for samples with absorbance interference problems, as discussed later.

Figure S1 Effect of tissue factor (TF) and phospholipid (PL) concentrations on peak thrombin generation and area under the curve (AUC). All reactions contained corn trypsin inhibitor, 40 µg/mL final concentration. The first column shows TF on the x-axis, and curves show PL concentrations. The second column shows PL concentration on the x-axis, and curves show TF concentrations.
Lower sample volumes in both assays were associated with increased total thrombin generation (AUC or ETP), down to a plasma volume of about 20% to 30% of the total reaction volume. This effect has been reported in another study and has been attributed to lower thrombin inhibition due to reduced antithrombin levels as the plasma volume in the assay is reduced.12

Prior studies reported that owing to absorbance of the excitation and emission light by substances in plasma, recovery of thrombin–α2-macroglobulin calibrator added to plasma was approximately 30% lower than calibrator added to buffer and was highly variable between different samples, even in the absence of hemolysis.13 Based on this observation, it was suggested that thrombin generation must be calibrated in each plasma sample analyzed using a separate reaction with thrombin–α2-macroglobulin calibrator added to plasma and that each plasma sample and plasma plus calibrator be run in duplicate to quadruplicate to reduce variability, resulting in 4 to 8 reactions per sample analyzed.3 We determined that when using an 80-µL sample volume in samples without obvious interference (hemolysis), the recovery of standard in normal pooled plasma was indeed reduced approximately 30% and showed up to 18% variability between different samples. However when a 40-µL volume was used in samples without obvious interference, calibration was similar in buffer or plasma with less variability between plasma samples. Thrombin-generation assays using higher plasma volumes require calibration in each sample, whereas lower plasma volume assays may use a single assay calibrator if the sample does not show absorbance interference (eg, hemolysis or icterus). Running the sample in duplicate is still required because occasional samples showed aberrant curves owing to bubbles or other problems.3

As expected, use of higher sample volumes (80 µL) in the Thrombinoscope assay was associated with greater interference when hemolysis was present. We determined that hemolysis affects the Technoclone assay and that the

<table>
<thead>
<tr>
<th>Peak Thrombin Generation (nmol/L)</th>
<th>Area Under the Curve (nmol/L × min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run (n = 8)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>454</td>
</tr>
<tr>
<td>SD</td>
<td>18</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4</td>
</tr>
<tr>
<td>Between-run (n = 15)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>404</td>
</tr>
<tr>
<td>SD</td>
<td>38</td>
</tr>
<tr>
<td>CV (%)</td>
<td>9</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.

* Tissue factor–stimulated thrombin-generation run with final reaction concentrations of 0.6 pmol/L recombinant human tissue factor, 1.0 µmol/L phospholipid, and 40 µg/mL corn trypsin inhibitor. Area under the curve determined over 90 minutes.

**Table 2**

Effect of Activator Concentrations on Thrombin Generation in Clinical Samples*

<table>
<thead>
<tr>
<th>Tissue Factor (pmol/L)</th>
<th>Phospholipid (µmol/L)</th>
<th>Peak Thrombin Generation (nmol/L)</th>
<th>Area Under the Curve (nmol/L × min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Healthy Subjects</td>
<td>Patients With Sepsis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>58 ± 22</td>
<td>276 ± 90</td>
</tr>
<tr>
<td>0.6</td>
<td>1.0</td>
<td>198 ± 34</td>
<td>362 ± 107</td>
</tr>
<tr>
<td>5.0</td>
<td>4.0</td>
<td>534 ± 46</td>
<td>504 ± 132</td>
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<td></td>
<td></td>
<td>534 ± 46</td>
<td>504 ± 132</td>
</tr>
</tbody>
</table>

NS, not significant.

† Unpaired t test, healthy subjects vs patients with sepsis; n = 5 for each group.

**Table 3**

Reference Ranges*

<table>
<thead>
<tr>
<th></th>
<th>Native TG</th>
<th>TF-Stimulated TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak thrombin generation (nmol/L)</td>
<td>12 (1-72)</td>
<td>240 (143-369)</td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>79 (32-90)</td>
<td>8 (6-10)</td>
</tr>
<tr>
<td>Peak time (min)</td>
<td>89 (49-90)</td>
<td>16 (13-22)</td>
</tr>
<tr>
<td>Velocity index (nmol/L/min)</td>
<td>2 (0-5)</td>
<td>29 (14-51)</td>
</tr>
<tr>
<td>Area under the curve (nmol/L × min)</td>
<td>179 (12-2,075)</td>
<td>4,522 (3,229-5,641)</td>
</tr>
</tbody>
</table>

* Range based on 25 healthy donors (12 women and 13 men; mean ± SD age, 32 ± 10 years; range, 19-56 years). Native thrombin generation (TG) run with corn trypsin inhibitor (40 µg/mL), Tissue factor (TF)-stimulated TG run with 0.6 pmol/L recombinant human tissue factor, 1.0 µmol/L phospholipid, and 40 µg/mL corn trypsin inhibitor. Data are shown as the median followed by the 4th to 96th percentile of the distribution. Area under the curve determined over 90 minutes.
thrombin–α₂-macroglobulin calibrator added to plasma could be used to correct the AUC, similar to prior reports for the Thrombinscope assay. In addition, we developed a sample absorbance ratio that could be used in place of an internal standard to provide a similar correction to the AUC in hemolyzed and icteric samples.

We determined that inner filter effect, substrate exhaustion, and residual thrombin–α₂-macroglobulin activity occur in the Technoclone assay, as previously described for the Thrombinscope assay. We further determined that corrections for measured vs expected fluorescence units and residual thrombin–α₂-macroglobulin activity can be used in the Technoclone assay to improve analytic accuracy.

Native thrombin generation is potentially useful for detecting circulating procoagulants in plasma. Variable levels of contact activation occurred in samples during thrombin generation in the Technoclone assay and have been reported in the Thrombinscope assay. To assess native thrombin generation, contact activation must be blocked, which has typically been done by using corn trypsin inhibitor at 40 µg/mL. In the Technoclone assay, healthy subjects showed little or no thrombin generation in the presence of corn trypsin inhibitor.

Because most patients show little or no native thrombin generation, it cannot be used to assess the overall response of the system to a given stimulus. Most studies have used a fixed amount of activator, usually tissue factor and phospholipid, to stimulate thrombin generation. The optimal tissue factor–phospholipid concentration for clinical studies is still being evaluated; higher tissue factor concentrations produced less variability in the AUC and less sensitivity to contact activation, whereas lower concentrations were more sensitive to changes in thrombin generation. Prior studies reported no difference in the plasma AUC and lower peak thrombin generation in control subjects vs patients with sepsis using 5 pmol/L tissue factor and no corn trypsin inhibitor. By using corn trypsin inhibitor, we found that native and low-level tissue factor (0.6 pmol/L)–stimulated thrombin generation could be used to differentiate healthy subjects from patients with sepsis, but higher levels of tissue factor stimulation (5 pmol/L), which overwhelmed differences in normal vs septic samples, could not be used. Others have reported that corn trypsin inhibitor plus low tissue factor stimulation could be used to differentiate pathologic from normal samples. At present, tissue factor preparations are highly variable in source material and specific activity. International standards are needed for tissue factor activators.

Both of the commercial assays had disadvantages. Based on the results, we developed an optimized assay. We selected a sample volume of 40 µL to increase thrombin generation and reduce interference and still allow use of commercial fluorogenic substrate–calcium reagents. The optimized assay uses corrections for inner filter effect and residual thrombin–α₂-macroglobulin to improve accuracy and addition of 40 µg/mL corn trypsin inhibitor to block contact activation. Calibration with thrombin–α₂-macroglobulin has the advantage that it can be used as an internal standard for samples with absorbance interference problems. As an alternative, the AUC can be corrected by using an absorbance ratio. Like others, we found that in the presence of corn trypsin inhibitor and lower dose tissue factor activator seemed to be more sensitive to differences in thrombin generation between plasma samples. Tissue factor and phospholipid concentrations can be adjusted to suit specific clinical questions.

This assay depends on fluorescence measurements that are less standardized than absorbance measurements. Fluorescence can vary with the light source and detectors used in the instrument. An important question is, What aspects of this study apply to other microtiter plate fluorescence instruments? In setting up this assay on any instrument from any company, including the model used in this study, it is most important to calibrate thrombin generation using an active thrombin standard. If inner filter effect and substrate exhaustion corrections will be used, the correction factors will need to be determined. The correction for residual thrombin–α₂macroglobulin activity is independent of the fluorescence instrument used. If exogenous thrombin–α₂-macroglobulin calibrator is used to correct the AUC for hemolysis or other interference, this is also independent of the fluorescence instrument. If an absorbance-based hemolysis correction is used, the correction will need to be determined for the instrument used because it is dependent on the original fluorescence measurements. Once all of these have been established, reference range and imprecision should be determined based on the final reagents and instrument selected.

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References


