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RECOMMENDATIONS AND GUIDELINES

Recommendations for the measurement of thrombin generation: Communication from the ISTH SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies

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Abstract

Thrombin generation (TG) assay is an overall assay to assess the functionality of the hemostatic system and may be a useful tool in diagnosing patients with hyper- and hypocoagulability. Lack of standardization in performing the assays contributes largely to poor correlation between assays and study results. The current lack of standardization remains a major issue in the setting of TG, as illustrated in a recent survey of the ISTH/SSC indicating differences in pre-, analytical, and post-analytical factors among users. These factors may considerably affect the between-laboratory reproducibility of results. Based on the results of the survey and a current review of the literature, along with insights and strong consensus of key investigators in the field, we present guidance for measurement of TG in a clinical setting. Recommendations on blood drawing, handling, processing, and sample storage; reagent concentration and source; analytical conditions on dilution of samples and temperature; calibration and replicate testing; calculation and interpretation of results; and reference values are addressed to help in reducing interlaboratory variation. These recommendations aim at harmonization between methods and laboratories to support the application of TG in patient diagnosis and management.

KEYWORDS
clinical laboratory tests, standardization, thrombin, thrombin generation assay, thrombography

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1 | INTRODUCTION

Thrombin generation (TG) has been used since the 1950s,[1] and the assay principle has been modified over the years (1980s and 1990s).[2,3] These modifications resulted in an assay that can be performed in a semi- or fully automated way and is widely applied in the field of thrombosis and hemostasis.[4-7] The TG assay mirrors a significant part of the coagulation system and provides more information than the clotting time-based assays.[8,9] TG assays monitor the hemostatic system in platelet poor plasma (PPP), platelet rich plasma (PRP), and whole blood.[10-12] It is used to investigate the contribution of natural pro- and anticoagulants, or to study the impact of drugs on hemostasis.[11,13,14] Although attempts have been made,[15-17] daily practice illustrates that standardization has not yet been achieved.[17,18]

Currently there are several commercial assays available. One of their major differences is the type of substrate used, which is either fluorogenic or chromogenic. The TG assays using a fluorogenic substrate are the Calibrated Automated Thrombinography (CAT; Diagnostica Stago), ST Genesia (Diagnostica Stago), Technothrombin (Technoclone), and Ceveron alpha TGA (Technoclone).[10] Alternatively, the Innovance ETP assay (Siemens Healthcare Diagnostics), HaemoScan Thrombin Generation Assay (HemoScan), and Pefakit TDT (Pentapharm) are performed using a chromogenic substrate. A few groups have compared some of the TG assays in the past, and concluded that there was a poor correlation between the assays.[19,20] It is accepted that part of the poor correlation is due to lack of standardization.

The current lack of standardization remains a major issue in the setting of TG.[21-23] In 2018 a working party of the Scientific and Standardization Committee for Lupus Anticoagulant/Antiphospholipid Antibodies of the International Society on Thrombosis and Haemostasis (ISTH-SSC LA/aPL) organized a survey to investigate how laboratories measure TG in practice.[24] The ISTH SSC LA/aPL aims to contribute to standardization, as TG assays are also used in patients with antiphospholipid syndrome.[25-28] TG depends on the presence of negatively charged phospholipids, and may provide more accurate information than the multitude of clotting assays currently used for LA testing.[29] However, TG assays are not yet robust enough to use for routine testing.[30] The information obtained from the survey included preanalytical variables, type of method, and reagents and samples used. The survey showed that individual laboratories use their own protocols, software versions, and different sources and concentrations of reagents such as tissue factor (TF), phospholipids, thrombomodulin, and substrate, resulting in numerous variations of the TG assay. While there was good agreement on the preanalytical phase (e.g., blood collection, anticoagulant, and discard tube), other factors (e.g., centrifugation, sample storage, reagent composition, and results normalization) lacked agreement.[17] The concern is that these factors may considerably affect the between-laboratory reproducibility of results.[31] Based on the ISTH/SSC survey and a current review of the literature, along with insights and strong consensus of key investigators in the field, we hereby present guidance for measurement of TG in a clinical setting. Our main aim is to reach a reduced interlaboratory variation by following this guidance protocol, which will be helpful to further integrate TG assay for patient management. Used in the context of antiphospholipid antibody testing, TG can be applied beyond this field, as a suitable assay to investigate hypo- or hypercoagulability.[32-34]

2 | PREANALYTICAL FACTORS

Different guidelines have to be applied for measuring TG in the setting of PPP, PRP, and whole blood, to account for the (pre)analytical aspects.[35] Here we focus on the measurement of TG in PPP and PRP.

2.1 | Blood drawing

Three different types of blood collection methods are used in clinical practice. Most frequently the butterfly or straight needle are used, but sometimes blood is drawn from a catheter.[17] The use of a straight needle is preferred over the butterfly needle, as the latter may induce contact activation and hemolysis.[31] When you use a butterfly needle, we advise using short connecting tubes. When the assay is performed with a high TF concentration, contact activation is negligible. As applied by the majority of participants,[17] independently of the needle type, the first tube of blood should be discarded and blood should flow without intermediate stopping. For practical reasons, especially in the pediatric population, we advise discarding only the first milliliter(s) of blood. The use of a tourniquet is discouraged as it can lead to blood cell activation and hemolysis,[36,37] and its use is only tolerated for locating the vein, with a gentle tourniquet pressure maintained during blood collection. We discourage the use of a catheter for blood drawing for TG purposes; however, when blood is drawn from a catheter that contains heparin to prevent clotting, it is recommended to flush the catheter prior to blood collection and to discard the first 5 ml to avoid the possible presence of heparin.[38]

The majority (73%) of participants of the survey used plastic blood drawing tubes,[17] which are recommended over glass tubes to avoid contact activation by the glass material.[39] It is recommended to draw blood directly into the tube instead of drawing blood into a syringe and subsequently filling up the tubes. The latter may lead to activation of coagulation as the syringe does not contain the anticoagulant and, more importantly, under- or overfilling of blood tubes leads to inaccurate results. Some closed blood collection systems based on manual aspiration of blood may be effective to reduce the burden of hemolysis.[40] Precautions should be taken to ensure the quality of the samples as certain factors can affect TG (e.g., presence of hemolysis or bilirubin).[41] We recommend reporting pre-analytical conditions, which may potentially influence test results.

The use of citrate tubes at a concentration of 0.109 M as an anticoagulant is recommended to make comparability between laboratories easier. While corn trypsin inhibitor (CTI) can be used when

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contact activation is expected, it is not recommended as a routine additive. Whenever used, we recommend adding CTI not to exceed 1.6 µM to the tube before blood collection.[42,43] The presence of CTI has a significant impact on TG parameters when using 1 pM TF or less, with only minor differences on peak and time-to-peak results observed when using 5 pM TF.[44] Patient and control population within the same study should be handled exactly under the same sampling and analytical conditions.

2.2 | Blood/plasma handling and processing

The time from blood collection to processing varies between TG users.[17] After collection, it is recommended to keep the blood at room temperature and to process it as soon as possible, preferably within 1 h.[31,45] The impact of pre-analytical variables on TG results is highly dependent on the TF concentration. Results obtained using the 1 pM TF are particularly sensitive to centrifugation.[44] To obtain PPP, we recommend centrifugation at a speed and time that results in $<10 \times 10^9$/L platelet count. This can be achieved by double centrifugation or by single high-speed/duration (e.g., a two-step centrifugation of 10 min at ≥2500 g). For PRP, a one-step centrifugation for 15 min at 150 g is recommended. Centrifugation should occur at controlled room temperature, to avoid precipitation and cold activation. Based on the stability of coagulation factors, PPP should be used within 4 h,[46] while PRP should be used within 2 h from preparation[43] or within 6 h when CTI is present.[45] Adjustment for platelet number is not recommended, unless required for specific research purposes.

2.3 | Sample storage

When testing cannot be performed soon after preparation, according to the Clinical Laboratory Standards Institute (CLSI) guidelines it is advised to freeze the plasma as soon as possible.[38] Based on the SSC-ISTH survey, the majority of laboratories performing TG store plasma at either −80°C or −20°C, but sometimes also at 4°C.[17] The best method to freeze plasma samples is snap-freezing in liquid nitrogen. When liquid nitrogen is not available, dry ice can be used or, alternatively, plasma can also be placed directly into the −80°C freezer. Based on studies that focus on coagulation tests, plasma can be stored at −80°C for a minimum of 2 years and at −20°C for a maximum of 1 month. It is not recommended to store plasma at 4°C as cold activation might occur.[45,47]

Thawing of frozen samples should be done in a water bath at 37°C. Water baths are preferred over heating blocks to ensure an evenly heat distribution. The preferred thawing duration is from 2 to not more than 5 min for a 1 ml sample and needs to be increased based on the sample volume until there is no more frozen plasma visible. Samples should be completely thawed before testing to prevent protein precipitation.[48,49] After thawing and prior to testing, samples should be gently mixed[38] and can then be left at room temperature for a maximum of 4 h prior to testing.[38,46]

3 | ANALYTICAL AND POST-ANALYTICAL FACTORS

3.1 | Reagent concentrations and sources

For clinical applications, we suggest standardized commercial reagents, which are more robust and with more stabilized composition, to be used according to the manufacturer.

When in a research setting investigators do use in-house reagents such as triggers (e.g., TF, phospholipids) or a thrombin-sensitive substrate, we recommend standardizing assay conditions and validating the in-house method. In such cases, the CLSI guidelines for coagulation tests could be used as a directive. When performing a specific TG assay in a research setting, other activators such as factor IXa (FIXa) or factor XIa (FXIa) can be used, for example, when measuring factor VIII (FVIII) levels under 1%.[50,51] For the use of an in-house TF trigger, the source and concentration should be stated to indicate whether a low or high TF concentration is being used as a low TF predominantly activates the intrinsic coagulation pathway via factor IX (FIX), while a high TF mainly activates the extrinsic pathway via factor VIIa (FVIIa). When using in-house manufactured phospholipids, it is recommended to use the standard composition of 1,2-dioleoyl-sn-glycero-3-phosphoserine, 1,2-dioleoyl-sn-glycero-3-phosphoetanolamine, and 1,2-dioleoyl-sn-glycero-3-phosphocholine in the proportion of 20/20/60 (PS-PE-PC; 20%-20%-60%) at a final concentration of 1 or 4 µM, depending on the application.[7,25,52] For the substrate, the Z-Gly-Gly-Arg-AMC is mostly used and is recommended for performing a fluorogenic-based TG assay. Any other substrate should be well characterized kinetically and checked for thrombin specificity. For specific purposes, thrombomodulin or other modulators can be used, and source and concentration should be stated.

3.2 | Plasma dilutions

All commercially available TG assays should be performed according to the manufacturers' recommendations. The addition of TG reagents to PPP and PRP results in a dilution of the original plasma sample. Hence, it is strongly recommended not to perform an additional plasma dilution step prior to analysis. Plasma dilution may have a larger effect on the anticoagulant proteins compared to the procoagulant proteins and may result in an overestimation of TG.[53] Additional dilution will also reduce the sensitivity of the TG assay toward the anticoagulant pathway.[54]

3.3 | Temperature and preheating

TG measurements should always be performed at 37°C. It is strongly advised to preheat the samples, triggers, and substrate at 37°C for at least 10 min for manual/semiautomated assays.[15,31,54] Be aware that some systems might require regular checks for accuracy of

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TABLE 1 Recommendations for a standardized TG protocol

Blood drawing
• Use a straight needle if possible • Discard the first milliliters of blood • Use either no tourniquet or only a light tourniquet during the blood collection • Take the blood directly into the blood drawing tube • Use a plastic blood tube containing sodium citrate (0.109 M) • If CTI is used, add it to the blood tube prior to collection and keep the CTI concentration below 1.6 µM.
Blood/plasma handling and processing
• Centrifugation should be done within 1 h at room temperature • Use PRP within 2 h and PPP within 4 h after blood collection • Avoid platelet adjustment in PRP, unless required to address specific issues in patients with platelet counts below 100 x 10^9/L
Sample storage
• Freeze PPP preferably on dry ice/liquid nitrogen; if not available, by putting directly into the freezer • Store PPP at −80°C (stable for at least 2 years) and at −20°C for up to 1 month
Reagent concentration and source
• We suggest the use of commercially available reagents according to the manufacturer • We suggest that if in-house reagents are used, assay characterization and procedure validation should be performed • We propose to establish reference values using the specific analyzer/reagent combination in a population of 120 healthy donors that are age-matched with the population being tested
Plasma dilutions
• Do not predilute PRP or PPP
Temperature and preheating
• Thaw samples in a warm water bath at 37°C • Ensure samples are completely thawed and are not kept too long in the water bath to avoid compromising the integrity of the sample • Carry out TG measurement at 37°C. Preheating of samples and reagents is necessary for manual/semiautomated methods
Calibration and replicates
• Use calibrated data to ensure an accurate conversion of the fluorescence into a thrombin concentration and correct for substrate consumption and inner filter effect (in the case of a fluorogenic substrate) • Measure TG in triplicate; when there is not enough, sample duplicate is accepted
Calculations and interpretation of results
• Use the embedded software for TG calculations, if not possible this should be mentioned and explained • Perform data normalization toward a control plasma measured in the same run as the samples • Interpret data with care, especially if there is a suspicion of the presence of an anticoagulant/oral contraceptive/use of other drugs affecting coagulation
Reference values
• We propose calculating age-specific reference values in a population of at least 120 healthy donors according to the CLSI guidelines • We propose comparing the reference values from the package insert with results of locally collected and age-matched 20 to 40 healthy controls to determine whether transference of the cutoff values is possible

Notes:: The method endorsed by the ISTH Guidance and Guidelines Committee Panel on writing guidance has been adopted. Accordingly, the wording “recommend” indicates a strong consensus among the co-authors; “suggest,” a moderate consensus; and “propose” the areas where there is limited knowledge. All are recommendations, except when indicated otherwise.

Abbreviations: CLSI, Clinical & Laboratory Standards Institute; CTI, corn trypsin inhibitor; h, hour(s); PPP, platelet poor plasma; PRP, platelet rich plasma; TG, thrombin generation.

temperature. For automated systems, the recommendations of the manufacturer should be followed.

3.4 | Calibration and replicates

For fluorogenic-based TG assays, free thrombin or alpha-2-macroglobulin-bound thrombin is generally used as a calibrator to convert the generated fluorescence into a molar thrombin concentration and to correct for substrate consumption and inner-filter effect. Sometimes a standard fluorophore is used for calibration. The downside of this method is the lack of substrate consumption correction. Calibration is necessary and it is strongly recommended to use the commercial reagents according to the instructions of the manufacturer. For manual/semiautomated methods, samples should be tested at least in duplicate (preferably in triplicate). Data should be reported as mean ± standard deviation. The imprecision between duplicate/triplicate should be preferably <10%. For automated commercial methods, samples and data should be measured and handled as recommended by the manufacturer.

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3.5 | Calculation and interpretation of results

For automated and semiautomated analyzers the software accompanying the device should be used for calculation. This accounts for the CAT-based assay (Thrombinoscope software), Technoclone (Technothrombin spreadsheet), ST Genesia (embedded software), Ceveron alpha (embedded software), and the Innovance ETP assay (embedded software). It is not advised to use in-house software/algorithm for calculation of TG variables, unless there is a specific research goal in which the standard algorithm related to the method is not appropriate. In this scenario, the algorithm used should be described. It is encouraged to report "raw" variables instead of percentages. When ratios are reported, for example in the presence/absence of thrombomodulin, it is also encouraged to report the "raw" data.

Interpretation of the results should be performed with care when there is suspicion of the presence of anticoagulants in the blood as the TG assay is sensitive to the presence of heparins, coumarins, direct oral anticoagulants, or any type of anticoagulant treatment.[5,55-58] Women on oral contraceptives have a more prothrombotic TG profile than women not on oral contraceptives due to acquired activated protein C resistance. Interpretation of TG results should always be done in view of potential interfering therapy.[59,60]

Normalization of results improves between-run comparability.[15,16] Normalization using a normal control plasma (in-house or commercial normal pooled plasma [NPP]), measured within the same run is advised to reduce inter-assay variability and improve standardization.[21,45] When TG is measured in patient PRP, we suggest including either a NPP or a PRP sample from a healthy donor as a control.

3.6 | Reference values

The use of reference values from published studies is discouraged due to the high variability of TG procedures. Reference values depend strongly on the methodological conditions and differ for adults and children.[19,61] We recommend establishment of reagent-, age-, and analyzer-specific reference values according to the CLSI guidelines using 120 healthy volunteers.[62] It should be noted that the CLSI guidelines on calculation of reference values concern all quantitative laboratory tests and are not necessarily applicable to TG. If the sample collection of 120 healthy donors is not feasible, transference of manufacturers' or previously established reference ranges after verification might represent an alternative approach.[62] These reference ranges may be acceptable if local measurements of 20 or more healthy subjects yield comparable results.[62] Furthermore, depending on the research question, reference ranges can be measured on a specific patient population (e.g., APS patients) for appropriate between-group comparisons.[63] In other applications of TG, such as monitoring of antithrombotic drugs and assessment of bleeding and thrombotic risk levels of TG, TG parameters are compared between patient groups.[5,64,65] Each study should report what reference ranges they applied for interpretation of results.

4 | RESEARCH AGENDA

There are many (pre)analytical steps to consider when standardizing the TG assay, all of which are summarized in Table 1. This guidance aims at harmonization between methods and laboratories to support the application of TG in patient diagnosis and management.

We plan to test these guidelines by distributing plasma samples, pooled normal plasma, and reagents to different laboratories worldwide. Samples should be analyzed according to the recommendations given in this guidance document and pooled normal plasma should be used to normalize the data. If we can demonstrate a reduced interlaboratory variation by following this guidance protocol, this will be helpful to further integrate TG assay as a diagnostic tool for patient management.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

MN, RdLK, KMJD, and BdL conceived the guidance and reviewed the literature. MN wrote the first draft of the manuscript. All the authors revised and accepted the manuscript, which was then accepted by the Scientific and Standardization Committee for Lupus Anticoagulant/Antiphospholipid Antibodies of the International Society on Thrombosis and Haemostasis (ISTH).

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