User Manual for Performing In vitro Thrombogenicity Assessment of Blood-Contacting Materials using Platelet and Coagulation Activation Molecular Biomarkers

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User Manual for Performing In Vitro Thrombogenicity Assessment of Blood-Contacting Materials using Platelet and Coagulation Activation Molecular Biomarkers

This document provides a detailed test protocol to perform in vitro thrombogenicity evaluation of biomaterials and medical devices using platelet and coagulation molecular biomarkers. It was adapted from the test method of the research paper by Patel et al.[1] that described the verification study performed at FDA.

Test Protocol

Blood Source and Preparation

Draw venous blood from healthy human adult donors per an appropriate protocol (e.g., Institutional Review Board approved protocols). Blood can be collected directly into polypropylene tubes or appropriate blood bag containing Anticoagulant Citrate Dextrose Solution A (ACDA) with a final blood to ACDA volume ratio of 85:15. To minimize the impact of blood property changes over time, start the experiments within 2 hours post blood draw and complete the tests within 4 hours. Blood from at least 3 different donors is needed to account for blood variability. It is recommended that blood from different donors be tested separately (not pooling the blood) on the test materials.

Test and control materials

There are two stages to carry out this test method, an initial validation stage to establish a specific test protocol for each individual test laboratory and a routine testing stage after the test protocol is appropriately validated. For the initial validation testing the use of at least two negative reference controls (e.g., polytetrafluoroethylene[PTFE], High-density polyethylene [HDPE]), two positive reference controls (e.g., Buna-N rubber, Latex), and a material with an intermediate thrombogenic potential (e.g., silicone or stainless steel) are necessary to validate this test method for individual test laboratories. For routine testing, a negative reference control (e.g., PTFE, HDPE) and a positive reverence control (e.g., Buna-N rubber, Latex) are still needed to be tested concurrently with the test materials using the same donor blood to account for blood variability among different blood donors. The information for all control materials that were used for the FDA validation study[1] is shown in Table 1 below. It is also recommended that a comparison article (a device with similar blood-contacting nature and a clinical use history) be tested concurrently with the test article to facilitate test result interpretation.

Table 1: *List of materials tested in the in vitro study.*

ID: Inner Diameter, OD: Outer Diameter

Test material	Description	Supplier*	Relative thrombogenic potential
Polytetrafluoroethylene (PTFE) cord	4.77 mm OD	McMaster Carr, (Princeton, NJ) Catalog # 84935K71	Low (Negative control)
High density polyethylene (HDPE) sheets	0.79 mm Thickness	United States Plastic Corporation (Lima, OH) Catalog # 46009	Low (Negative control)
Silicone (Si) tubing	1.59 mm ID x 4.76 mm OD	Qosina (North Ronkonkoma, NY) Catalog# T2010	Intermediate
316 stainless steel (SS) sheets	0.10 mm Thickness	Trinity Brand Industries (Burr Ridge, IL). Part # 2316-4	Intermediate
Glass beads	4 mm Diameter	Fisher Scientific (Pittsburgh, PA) Cat#: NC9559146	High (Positive control)
Buna-N rubber (BUNA) sheets	0.79 mm Thickness	McMaster-Carr (Catalog # 8969K72)	High (Positive control)

* Materials from other alternative suppliers can also be used to validate this test method. The mention of any commercial products and/or manufacturers does not imply endorsement by the FDA or the U.S. Department of Health and Human Services.

Experimental procedures

A summary of the major steps for the test procedures is shown in below Figure 1.

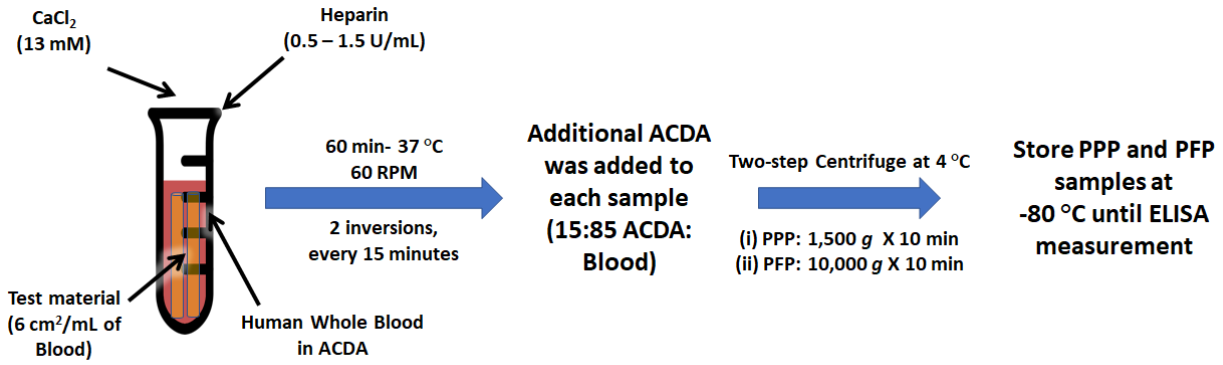


Figure 1. *Summary of the major experimental steps. For positive control: Test was run with 20 µM adenosine diphosphate in recalcified blood. For negative control: Test was run without any test material. Additional ACDA was added to the blood after the 1-hour incubation to avoid any non-test related coagulation and platelet activation. PPP: Platelet-Poor Plasma, PFP: Platelet-Free Plasma*

Detailed Procedures are Listed Step-by-Step Below.

1. Test and control material preparation:
    a. For both validation study and routine testing, prepare a negative control (a test tube without test materials) and an appropriate positive control (e.g., 20 µM Adenosine diphosphate) in the round bottom polypropylene (PP) tubes. .
    b. For validation study, prepare two different materials for each of the following reference material groups with varying thrombogenic potentials (see Table 1 above): negative reference materials, positive reference materials, and intermediate reference materials. Prepare these test samples in duplicate using a sample surface area to blood volume ratio of 6 cm^2/1 mL and put them in the round bottom PP test tubes.
    c. For a routine testing, prepare one negative reference control, one positive reverence control, the test article and comparison article (if applicable) in duplicate using a test sample surface area to blood volume ratio of 6 cm^2/1 mL and put them in the round bottom PP test tubes.
2. Before initiating the experiment, perform a complete blood count assay using a hematology analyzer to confirm that blood cell counts are in physiological range.

3. Heparinize the ACDA whole blood by adding an appropriate amount of heparin*
   *Note: Based on our validation study, a fixed heparin concentration of 1.0 or 1.25 U/ml heparin concentration can be used to achieve adequate test sensitivity for this method. However, other heparin concentration can also be used if validated by the test lab using appropriate controls (Table 1 above). Variation in blood coagulability between blood donors is one of the major challenges in developing standardized thrombogenicity tests. Alternative to using a fixed heparin concentration for all donors in the test, donor-dependent heparin concentration adjustment based on Activated Clotting Time (ACT) measurement can be used to improve test sensitivity. In the FDA validation study [1] , it was found that using a minimum heparin concentration (called ACT-Hep) that yields an ACT value of 190-240s in recalcified blood (see step 3 below) could improve test sensitivity for all the biomarkers. In this validation study, the ACT of the recalcified whole blood with different amounts of added heparin was measured using a Hemochron® Signature Elite system (Werfen, Bedford, MA) with Hemochron® JACT+ Cuvettes. Please note that, if an alternative ACT measurement system is used, the donor-dependent heparin ACT range may be different. The ACT range used for this methodology should be validated by each individual test lab using appropriate controls (Table 1 above).
4. Immediately after adding the heparin, recalcified the blood with a 2M calcium chloride solution (1:150 $CaCl_2$: blood volume ratio) to reverse the effect of ACDA anticoagulant and achieve physiological ionized Ca^{+2} concentrations of 1 to 1.4 mM.
5. Add the heparinized and recalcified blood to polypropylene test tubes containing different test and control materials (one material or control material sample per tube) to achieve a final material surface area to blood volume ratio of 6 cm^2/mL.
6. Incubate the samples for 1 hour ± 5 minutes at 37 ± 1 °C in a shaking water bath at a rotational speed of 60 rpm. To ensure uniform contact between the blood components and the test material surface, manually invert the test tubes twice every 15 minutes.
7. After 1 hour of incubation, add additional ACDA solution to the test tubes to achieve a blood to ACDA volume ratio of 85:15 to inhibit any non-test related coagulation or platelet activation.
8. Transfer the blood to new polypropylene tubes and keep on ice until centrifugation to further inhibit the coagulation processes.
9. (Optional) Perform complete blood count measurements (which include platelet and leukocyte counts) on the blood samples before and after 1-hr incubation with test materials to measure change in blood cell counts (e.g., platelets and leukocyte count) to supplement the outcome of molecular marker data.
10. Centrifuge the blood samples in two stages to collect platelet free plasma (PFP).
    a. Centrifuge the blood sample at a lower speed of 1,500 g for 10 min at 4 °C to collect supernatant called platelet poor plasma (PPP).
    b. Centrifuge PPP at a higher speed of 10,000 g for 10 min at 4 °C to collect PFP supernatant.

11. Divide PFP samples into multiple aliquots and stored at -80 °C until enzyme-linked immunosorbent assays (ELISA) of the molecular markers are performed.
12. Measuring each sample in duplicate, perform ELISAs for platelet activation markers (such as β-TG: beta-thromboglobulin, PF4: platelet activation factor-4) and coagulation activation markers (such as TAT: Thrombin-antithrombin complex) to measure the markers' levels in plasma according to ELISA kit manufacturer's instructions.

Data Collection and Statistical Analysis

1. To characterize thrombogenicity of the test samples, calculate each marker concentrations from standard curves constructed using a 4-parameter logistic regression curve fit method or per the ELISA kit manufacturer's recommended method.
2. Perform statistical analysis on marker concentrations in different test samples using a one-way analysis of variance followed by Fishers Least Significant Difference (LSD) post hoc test or other appropriate statistical test method.
    a. The results are considered statistically significant if $p < 0.05$, and marginal statistically significant if $0.05 < p < 0.1$.
3. For the current test system:
    a. A medical device or biomaterial can potentially be considered passing the test if (i) the thrombogenicity marker concentrations for the test article are not significantly higher from the negative control, the negative reference control, or a comparison article with prior histroy (If used) and (ii) the thrombogenicity marker concentrations for the test article are significantly less than the positive control and positive reference control material.
    b. A medical device or biomaterial would be considered failing the test if the thrombogenicity markers concertation are not significantly lower than the positive control or a positive reference control.
    c. For situations between the above pass and fail scenarios, the thrombogenic risks should be evaluated on a case-by-case basis and additional testing may be needed.

Test Validation Information

The followings are key information summarized from our validation study [1] that support the above test protocol**:**

Effect of Heparin Concentration on Test Sensitivity:

Variation in blood coagulability between blood donors is one of the major challenges in developing standardized thrombogenicity tests. Results (Figure 2 below) from our verification study showed that the fixed heparin concentrations of 1.25 U/ml and 1 U/ml were able to more reliably differentiate the materials with varying thrombogenic potentials than the heparin concentration of 1.5 U/ml and 0.75 U/ml. However, none of the fixed heparin concentrations were able to differentiate materials based on the three platelet activation markers for every

donor evaluated. To improve the reliability of the test system, we investigated whether ACT measurements could be used to predict a donor-dependent heparin concentration. We found that the minimum heparin concentration (called ACT-Hep) that yielding an ACT value of 190-240s allowed for the differentiation of the test materials with varying thrombogenicity potentials based on all four of the thrombogenicity biomarkers (the 3 platelet activation markers and TAT) for all for six donors (100% donors)

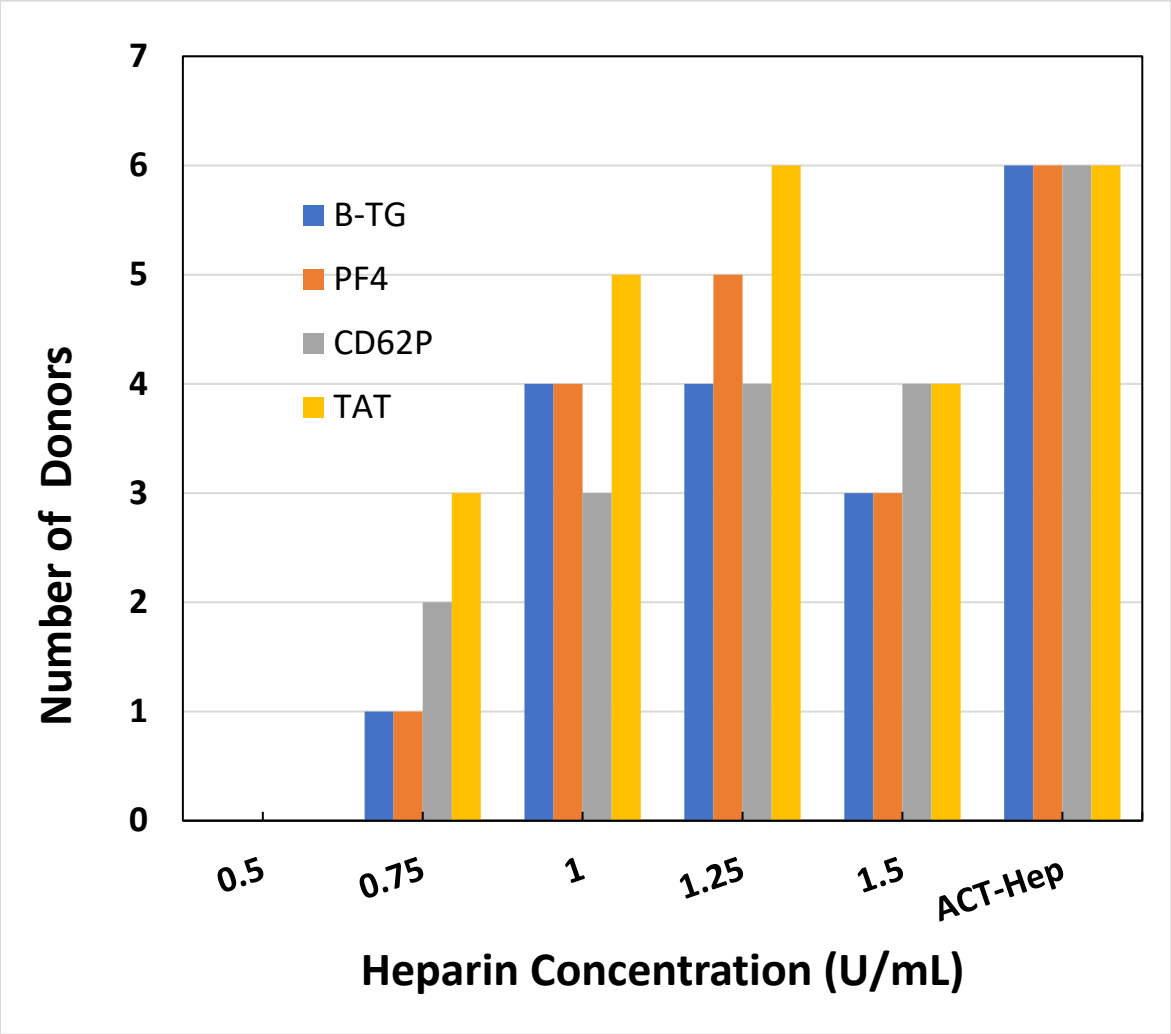
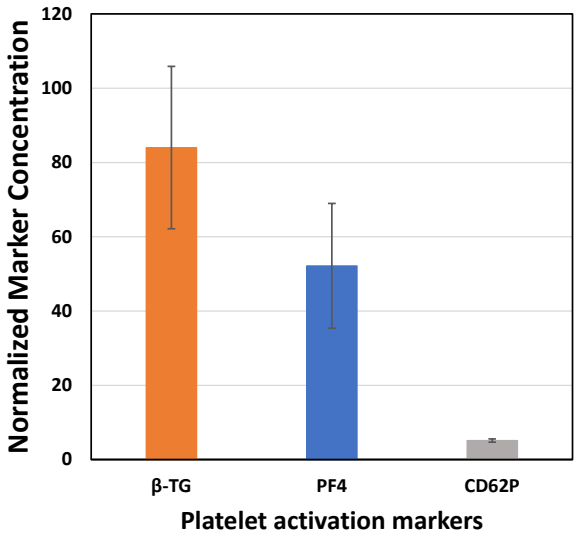


Figure 2. *Number of donors (from a total of n=6 donors) that enabled differentiation (>= 10% difference in the concentration of a marker) of the materials in different thrombogenic potential groups (Low- PTFE, HDPE; intermediate- Si, SS; high- Glass, BUNA) at different heparin concentrations. ACT-Hep is the minimum heparin concentration that achieved the targeted ACT value between 190-240s for blood from each donor.*

Comparison of Different Platelet Activation Markers

In our verification study, the test sensitivity of different platelet activation markers was studied by comparing the normalized marker concentrations of positive reference materials (Buna and Latex) to the baseline (Figure 3A) and the negative control values (Figure 3B and 3C) at fixed (1.0 and 1.25 U/mL) and donor-dependent (ACT-Hep) heparin concentrations. These results indicated that β-TG and PF4 markers may be more sensitive compared to CD62P in differentiating the positive control and positive reference materials from the negative control.

A



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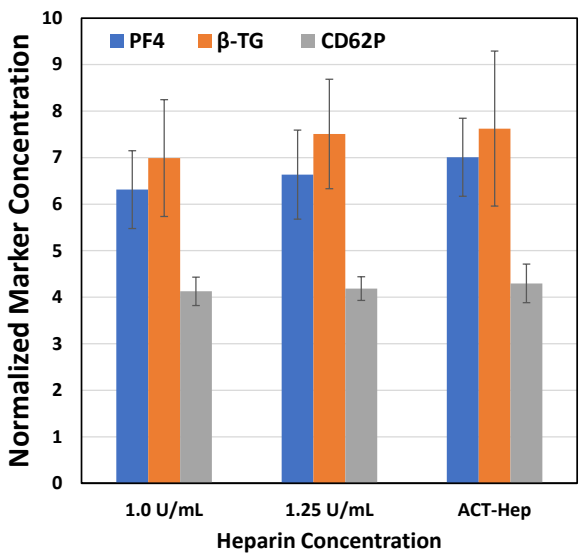

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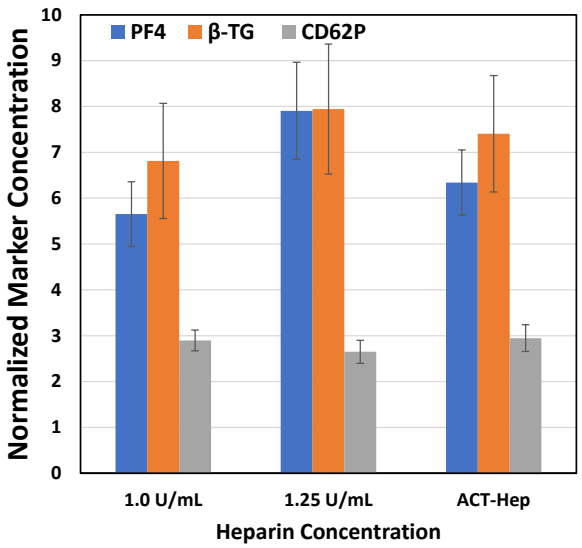

D

p Value	
Ratio of PC to Baseline	
β-TG vs CD62P	<0.05
PF4 vs CD62P	0.05<p<0.1
Ratio of Glass to NC	
β-TG vs CD62P	<0.05
PF4 vs CD62P	<0.05
Ratio of BUNA to NC	
β-TG vs CD62P	<0.05
PF4 vs CD62P	0.05<p<0.1

Figure 3. *Comparison of platelet activation marker concentration normalized to the baseline and negative controls for the (A) positive control, and thrombogenic materials (B) Glass and (C) BUNA at different test heparin concentrations (1.0 U/mL, 1.25 U/mL, and ACT-Hep). (D) Statistical significance comparison of platelet activation markers, determined by Fishers LSD post hoc test. Data are shown as mean ± SE (n=6). PC: positive control, NC: negative control.*

Reference:

1. Patel M, Parrish A, Serna III C, Jamiolkowski MA, Srinivasan K, Malinauskas RA, et al. Molecular Biomarkers for In Vitro Thrombogenicity Assessment of Medical Device Materials. Journal of Biomedical Materials Research: Part B - Applied Biomaterials. 2024; DOI: 10.1002/jbm.b.35491.