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Assessing blood clotting and coagulation factors in mice

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Abstract

The mammalian blood coagulation system was designed to restrict blood loss due to injury as well as keep the blood fluid within the blood vessels of the organism. Blood coagulation activity in inbred mouse strains varies widely among strains, suggesting that many genomic variants affect hemostasis. Some of these molecules have been discovered and characterized, however many are still unknown. Genetically modified mouse technologies are providing a plethora of new mouse models for investigating the regulation of blood coagulation. Here we provide a protocol for the tail bleeding time as a primary assessment of *in vivo* blood coagulation, as well as *in vitro* methods such as the prothrombin time, activated partial thromboplastin time, thrombin generation assay, and alternative protocols for the assessment of the activities of specific known factors involved in blood coagulation.

Keywords

Blood coagulation; hemostasis; thrombosis; bleeding; mouse models

INTRODUCTION

In vivo blood coagulation (hemostasis) depends on interactions between the vasculature and circulating plasma molecules as well as molecules found in platelets and other blood cells

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(Harris et al., 2012). Since many genes/genomic regulatory regions play a role in hemostasis, many gene targeted/genome edited mice that have been produced may exhibit blood coagulation defects leading to bleeding or excessive blood clotting (thrombosis) phenotypes. Researchers have been developing and using a diverse array of *in vivo* and *in vitro* methods for assessing hemostasis. Here, we provide protocols for assessing blood clotting that provide an introduction into assessing genetically modified mice for blood coagulation disorders.

The careful collection and isolation of the blood and plasma is one of the most important aspects for analyzing blood coagulation and its factors. In Basic Protocol 1, we detail a simple and rapid terminal blood collection method, the cardiac puncture from the surgically exposed heart. In Alternative Protocol 1, we describe another widely used terminal blood collection method from the surgically exposed inferior vena cava (IVC). In Basic Protocol 2, we outline the steps to isolate platelet poor plasma from whole blood without activating coagulation. Proper collection and handling of platelet poor plasma is essential for success in the downstream protocols. Additionally, platelet-rich-plasma (PRP) and platelet poor plasma (PPP) may be isolated from the same mouse, as described in Alternative Protocol 2. Following these protocols will enable you to minimize experimental variability introduced by improperly drawing, handling, and processing your blood samples.

In Basic Protocol 3, we detail the mouse tail bleeding time, a global *in vivo* assay designed to assess the ability of the mouse's hemostatic system to stop blood loss. While this provides valuable information regarding the hemostatic system as a whole, other complementary assays such as those listed below will be necessary to elucidate the particular mechanism responsible for the defect (Greene et al., 2010). In Basic Protocols 4 and 5, we provide protocols for *in vitro* biochemical measurements of hemostasis, such as the endpoint methods prothrombin time (PT) and activated partial thromboplastin time (APTT). These assays provide functional assessments of the extrinsic (PT) and intrinsic pathways (APTT) of blood coagulation. We have provided Alternate Protocols 3 and 4 for measuring the activity of extrinsic and intrinsic coagulation factors, respectively. Alternate Protocol 5 describes a method to measure activated protein C (APC) resistance, as APC resistance is commonly associated with the Factor V Leiden mutation.

Basic Protocol 6 describes the calibrated automated thrombogram (CAT) assay. Thrombin is a central mediator of blood clotting that converts fibrinogen to its active form called fibrin, which then activates platelets and other blood cells. The CAT assay enables thrombin generation to be continuously measured, providing the rate of thrombin generation, which can indicate a predisposition to bleeding or clotting.

Platelets are critical mediators of hemostatic blood clot formation and many methods to assess platelet function have been developed, including platelet aggregation (Getz et al., 2015; Hughes, 2018); methods of platelet analysis form an entire subgroup of blood coagulation assessment techniques and will not be addressed here.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

STRATEGIC PLANNING

The methods described in protocols 4–6 and alternative protocols 3–5, require the isolation of anticoagulated plasma from the mouse. The isolation of plasma from blood without activating blood coagulation is critical for obtaining high quality results from these assays, as any activation can deplete the coagulation factors and lead to invalid results. Both terminal and survival methods can be used for collecting mouse blood. However, obtaining blood by survival methods such as via the saphenous or tail veins yields low volumes and can expose the blood to activating factors such as the coagulation protein, tissue factor (Pawlinski et al., 2004).

As described by Rathkolb *et al.*, deciding which blood collection method to use depends on several parameters such as: the required assay volumes, allowable methods (IACUC approved) and whether the mouse must remain alive after the blood collection procedure (Rathkolb et al., 2013). Survival blood draw protocols can produce enough plasma to perform a limited number of assays. However, methods of terminal blood collection, e.g. via cardiac puncture or inferior vena cava (IVC), enable the isolation of higher plasma volumes required to perform a multitude of assays from the blood of a single mouse.

It is essential that the coagulation system remains inactive during blood sample collection and processing. For this reason, anticoagulants are added. Choosing which anticoagulant to use for your particular blood draws is essential for successful assays/experiments. There are many anticoagulants such as EDTA, heparin, acid citrate dextrose, buffered sodium citrate, and each can influence parameters such as concentrations of specific proteins in the blood (Tuck et al., 2009). Therefore, consistently using the same anticoagulant is important for your studies. For Basic Protocols 3 and 4, we use 3.2% buffered sodium citrate to a final ratio of 9 parts blood to 1 part citrate as an anticoagulant.

Contact activation is initiated by FXIIa, which occurs when blood comes in contact with surfaces like tubes and pipette tips. Contact activation is a confounding factor in tissue factor (TF)-initiated coagulation studies (Hansson et al., 2014), like the CAT assay described in Protocol 6. We use corn trypsin inhibitor (CTI) to effectively neutralize contact activation by inhibiting FXIIa, along with sodium citrate, in order to collect blood for the CAT assay (Mann et al., 2007). Note that this blood is unsuitable to use in the APTT assay, as the APTT time will be artificially prolonged because contact factors (like FXIIa), which are measured in this assay (Hansson et al., 2014).

BASIC PROTOCOL 1

BLOOD COLLECTION BY CARDIAC PUNCTURE—Non-terminal blood collections are very common methods to collect blood from mice (Rathkolb et al., 2013). However, only small blood volumes can be collected by these methods, which do not produce enough plasma to be used for multiple coagulation assays. Terminal blood collection methods such

as cardiac puncture enable blood draws of up to 1mL and the subsequent isolation of large amounts of plasma (Hughes, 2018). Previously reported methods of cardiac puncture describe puncturing through the skin toward the heart without opening up the chest cavity (Adeghe et al., 1986; Doeing et al., 2003; Donovan et al., 2005; Hoggatt et al., 2016; Schnell et al., 2002). While these procedures may be suitable for obtaining blood specimens for measurements such as serum chemistry, puncturing the heart in different areas, puncturing the heart multiple times over the course of a single blood draw, or exerting excessive negative pressure by pulling back too vigorously on the syringe plunger can activate coagulation factors. Performing the cardiac puncture incorrectly can also bring in variability and inconsistency by introducing the risks of incorrectly locating the heart with the needle, reducing the amount of blood obtained, or a completely failed blood collection (Hughes, 2018). Surgically exposing the heart to perform a right ventricular cardiac puncture, as detailed in this protocol, allows for a more accurate and consistent blood draw with minimal activation of coagulation.

Materials

Mice

Parafilm “M” laboratory film

3.2% Buffered Sodium Citrate (e.g., Medicago)

Reconstituted Buffered Sodium Citrate is stored for 2 months at 4° C

3mL syringes (e.g., Becton Dickinson (BD) Luer-Lok Tip Syringe)

26G x $\frac{3}{8}$ hypodermic needles (e.g., BD Precisionglide)

1mL syringes (e.g., BD Slip Tip Syringe)

26G x $\frac{1}{2}$ hypodermic needles (e.g., BD Precisionglide)

16.7mg/mL Sodium Pentobarbital

Laboratory Tape

Heavy absorbent wipes (e.g., Fisher 8” x 9” BloodBloc)

Gauze pads

70% Isopropanol

High Quality Surgical Scissors, must easily cut through mouse tissues (e.g. Biomedical Research Instruments)

Forceps/Tweezers

Surgical Clamp

2mL microcentrifuge tubes (preferably gradated)

1. Pipette 50 μ L of 3.2% buffered sodium citrate onto a strip of parafilm. Aspirate the drop of sodium citrate from the parafilm into the 3mL syringe with the 26G x $\frac{3}{8}$ hypodermic needle.

Aspirating the liquid with the needle and syringe coats the needle with the anticoagulant to ensure that the blood encounters anticoagulant at the earliest possible instance, optimizing anticoagulation. Prepare the number of syringes needed for the number of mice to be exsanguinated.

2. Draw the required amount of sodium pentobarbital anesthetic (dose is based on mouse weight: 50 μ g/g) into the 1mL syringe with 26G x $\frac{1}{2}$ needle.

Visually inspect syringe to ensure that there are no air bubbles. Ensure that the sodium pentobarbital completely fills the needle by pressing the syringe plunger just until sodium pentobarbital can be seen on the tip of the needle.

3. Firmly scruff the mouse with one hand, flip the mouse over so that its belly is exposed. Hold the syringe in your other hand at an approximately 30° angle to the mouse. Anesthetize mouse by intraperitoneal injection of the pentobarbital. Wait 5–10 minutes until mouse is recumbent and unresponsive to toe pinch.

Holding the mouse in this manner causes the peritoneal (belly) internal organs to settle toward the back of the mouse, this is helpful for ensuring that you strike no internal organs. Once needle is in the peritoneal cavity, refrain from pushing the needle too deeply, as you may hit an internal organ.

4. Place the mouse in dorsal recumbency, tape the paws to the heavy absorbent wipe as shown in Figure 1, and wet the fur with gauze pad saturated with 70% alcohol. Wipe off the excess alcohol with an additional gauze pad, while leaving the fur thoroughly wet.

Wetting the fur helps reduce the chance that individual hairs will contaminate your surgical site

5. Cut the skin from muscle around the rib cage using scissors and forceps. Cut at the xyphoid process and the diaphragm, then up the side of the ribs. Clamp the ribs and fold back to expose the chest cavity as shown in Video 1.

Peeling back the skin enables you to easily visualize the ribcage, the xiphoid, and makes the remainder of the surgery relatively free of the problem of contaminating hairs. Take care not to cut the liver, which sometimes sticks to the diaphragm. The clamp effectively clamps off any severed blood vessels.

6. The heart will now be exposed and should be lying to the mouse's left, with the right ventricle facing you. Carefully insert the needle into the right ventricle near the apex of the heart and toward the atria of the heart until the needle is fully

within the right ventricle. Draw blood from the right ventricle of the heart into the syringe with anticoagulant as shown in Video 1.

When inserting the needle into the right ventricle, make sure the needle is bevel side up. Take care not to move the needle/syringe around.

Whenever possible, brace your hands during surgeries so only minimal movements are possible, this keeps you steady and ensures that fatigue won't lead to you excessively moving the needle around and potentially scraping additional cardiac tissue into the syringe. From the right ventricle, 500–1000µL of blood can reliably be collected.

7. Use the syringe to quantitate the amount of blood that you have drawn by removing all air bubbles from the newly collected blood and estimating the total based on the gradations on the syringe. Remove the needle from the syringe and slowly transfer the blood into the 2mL microcentrifuge tube.

Run the blood down the side of the tube and watch carefully as you are doing so for the presence of any blood clots that may have formed during the process of your blood draw. Once the blood is transferred, you can also use the gradations on the 2mL tube to quantitate the amount of blood.

8. Add additional 3.2% citrate to bring the final amount of anticoagulant 1:9 of the total blood volume. Use the calculation:

$$\frac{\text{Total amount collected} - 500}{9}$$

You initially added 50µL of anticoagulant to the syringe in anticipation of collecting at least 500µL of blood. If a total volume of 500µL is obtained (50µL of citrate + 450µL of blood), no additional citrate needs to be added to the blood because the anticoagulant is at a 1:9 ratio to the blood. If you collected more blood, you will need to supplement with additional citrate to restore the 1:9 ratio. Keeping this ratio is very important for obtaining high quality data from the coagulation assays. When adding more citrate, add to the side of the tube, not directly into the blood, and gently invert to mix. Whole blood cannot be frozen as it causes the red blood cells to lyse. When conducting blood coagulation activity assays or RNA analyses, the necessary blood components (plasma, platelets etc.) should be isolated as quickly as possible. The half life of platelet RNA is relatively short (Angenieux et al., 2016) and activities of the coagulation factors can diminish over time. The blood is viable up to 2 hours at room temperature, after 4 hours the blood should be discarded.

ALTERNATE PROTOCOL 1

BLOOD COLLECTION BY INFERIOR VENA CAVA—Terminal blood collection by the inferior vena cava (IVC) has the same benefits as the cardiac puncture. However, since the IVC consists chiefly of the endothelial layer, with little tissue factor (TF) containing media

or adventitia, potentially less TF is released with this method. Less endogenous TF would make this the preferred blood collection method for analyses dependent upon addition of exogenous TF. The IVC surgical protocol has been well described (Day et al., 2004; Stewart et al., 2019). The protocol we describe here includes key variations such as bending the needle to make the blood draw easier. The addition of CTI as a contact activation pathway inhibitor (primarily inhibition of coagulation Factor XIIa) is suitable for studies with TF. The users of this protocol should read the cardiac puncture protocol to glean additional details on blood drawing that may be applicable to this procedure as well.

Additional Materials

1.5mL microcentrifuge tubes

3.2mg/mL Corn Trypsin Inhibitor (e.g., Hematologic Technologies Inc.)

Ketamine (100mg/kg)/xylazine (10mg/kg) (see recipe)

1. Place 50 μ L of 3.2% citrate and 7.8 μ L of CTI (50 μ g/mL final concentration of CTI) in a 1.5mL microcentrifuge tube.

Prepare the number of tubes needed for the number of mice to be exsanguinated.
2. Aspirate the citrate/CTI mixture into the 1mL syringe with the 26G x $\frac{3}{8}$ needle. Remove any excess air from the syringe and needle.
3. With the bevel side up, carefully press the needle on a heavy absorbent wipe and lift the syringe until the needle is bent to a $\sim 30^\circ$ angle as shown in Figure 2A.

Bending the needle at a $\sim 30^\circ$ angle enables you to more easily access the IVC.
4. Aspirate the anesthetic into a different 1mL syringe with the 26G x $\frac{1}{2}$ needle.
5. Anesthetize mouse by intraperitoneal injection of the ketamine (100mg/kg)/xylazine (10mg/kg). Wait 5–10 minutes until mouse is recumbent and unresponsive to toe pinch.

To anesthetize the mice, weigh the mouse and multiply the weight in grams by 4.45 to get the amount of ketamine/xylazine volume to inject. You can also use sodium pentobarbital anesthetic as in Basic Protocol 1.
6. Place the mouse in dorsal recumbency, tape the paws to the heavy absorbent wipe as shown in Figure 1, and wet the hair with gauze pad and 70% alcohol.
7. Make an abdominal midline incision using scissors and forceps from the xiphoid to the pubis. Cut the skin laterally along the ribcage and flip the skin down toward the dorsal side of the mouse.
8. Displace the intestines to the right of the abdomen (to your left side) to expose the IVC.

The IVC runs along the dorsal midline (spine) of the mouse, so the organs will have to be securely displaced so they don't shift into the surgical field while you are drawing the blood.

9. Insert the bent needle into the surgically exposed IVC at the level of the left renal artery as shown in Figure 2B. Draw blood from the IVC into the syringe with anticoagulant as shown in Figure 2C. Collect enough blood so that it brings the total volume in the syringe to at least 500 μ L.

When inserting the needle into the IVC, make sure the needle is bevel side up.

10. Similar to the cardiac puncture procedure, transfer blood to a new 1.5mL microcentrifuge tube.

As in the cardiac puncture protocol, keep the ratios of anticoagulant: blood consistent by supplementing with additional anticoagulant as necessary. If you do not collect enough blood to attain the proper anticoagulant: blood ratio, those samples cannot be used for plasma coagulation assays.

BASIC PROTOCOL 2

PLASMA ISOLATION—Proper plasma isolation is one of the most important factors for accurate and consistent coagulation assay results. Effects of poor plasma sample collection have been well documented in humans (Favaloro, 2017), and many of these effects apply to mice as well. Of particular relevance here, hemolysis and/or activation of coagulation, leading to clots in the sample can lead to false shortening or prolongation of coagulation test times (Favaloro, 2017). To prevent this from happening, proper plasma collection actually starts at the blood draw, and isolation of plasma from the blood is a simple, yet critical, procedure outlined here. We have designed this protocol for isolation of plasma for the cardiac puncture blood draw using only buffered sodium citrate as an anticoagulant.

Materials

Blood samples (from Basic Protocol 1)

1.5mL microcentrifuge tubes

0.5mL microcentrifuge tubes

Centrifuge (preferably with swinging bucket rotor for 1.5mL tubes)

1. Centrifuge the whole blood in a swinging bucket rotor for 10 minutes at 5,000 x g with the soft brake on.

The swinging bucket rotor keeps the tubes level and the soft brake on the centrifuge prevents the liquid in the tube from shifting, keeping the blood level as shown in Figure 3A.

2. Remove pseudo-plasma without drawing up red blood cells, and place in a 1.5mL microcentrifuge tube as shown in Figure 3B.
3. Centrifuge in a swinging bucket rotor again for 10 minutes at 5,000 x g.

At this point, the soft brake is not necessary. The second centrifugation pulls down any remaining blood cells (red, white, platelets).
4. Aliquot plasma into 0.5mL microcentrifuge tubes and proceed to experimental procedure or store at -80°C .

Aliquot plasma into amounts needed for experiments, for example, the PT assay requires 50 μL of plasma sample run in duplicate, so aliquot 100 μL of plasma for the assay with 10 μL additional.

ALTERNATE PROTOCOL 2

PLATELET-POOR PLASMA AND PLATELET-RICH PLASMA ISOLATION—This protocol details an isolation method to isolate both platelet-poor plasma (PPP) and platelet-rich plasma (PRP) from one mouse. It is designed for isolation of PPP and PRP from the IVC blood draw with sodium citrate and CTI used as an anticoagulant, which is needed for the CAT assay and other TF-dependent assays.

Additional Materials

Hemocytometer (e.g., Improved Neubauer) or blood analyzer (e.g., Advia 2120 from Bayer Healthcare)

1. Centrifuge whole blood from Alternative Protocol 1 for 5 minutes at 150 x g.
2. Remove the plasma and the top of the red blood cells (RBCs), which contains the PRP, and place them into a new microfuge tube.

Pulling up RBCs/buffy coat is necessary to maximize the amount of PRP obtained. Based on a final volume of 500 μL of blood, pull up 200 μL to remove all of the PRP and ~50 μL of RBCs/buffy coat. Do not discard the tube with remaining RBCs as it will be used in a later step.
3. Centrifuge for 5 minutes at 150 x g.
4. Remove the PRP, place it in a new 1.5mL microcentrifuge tube, and set aside for later.
5. Centrifuge the remaining RBCs (from step 2) for 10 minutes at 3000 x g. Remove the PPP and place in a new 1.5mL microcentrifuge tube.
6. Centrifuge the remaining RBCs again for 10 minutes at 9000 x g. Combine the PPP with the previously collected PPP (from step 5).
7. Aliquot PPP into 0.5mL microcentrifuge tubes and proceed to experimental procedure or store at -80°C .

Aliquot plasma into amounts needed for experiments, for example, the CAT assay requires 10 μ L of plasma sample run in duplicate, so aliquot 20 μ L of plasma for the assay with 10 μ L additional.

Count Platelets in PRP

8. Perform a platelet count on the collected PRP using a hemacytometer or Advia 2120.

The number of platelets in the PRP are likely to be at least 10-fold more concentrated than in whole blood. Therefore, you may have to dilute an aliquot of your PRP sample in 4% Bovine Serum Albumin (BSA) in PBS prior to counting.

9. Adjust the platelet count to $6.0 \times 10^5/\mu\text{L}$.
 - a. If the platelet count is $> 6.0 \times 10^5/\mu\text{L}$, add the amount of plasma to bring platelets to the appropriate count.
 - b. If the platelet count is $< 6.0 \times 10^5/\mu\text{L}$, centrifuge the platelets at 700 x g for 10 minutes at room temperature. Remove a known calculated amount of PPP and then add back the appropriate calculated amount of PPP to bring the platelet pellet to $6.0 \times 10^5/\mu\text{L}$. This may be problematic in that additional centrifugation of platelets may activate them prior to testing.
10. Move directly to experiment. PRP cannot be stored as the platelets will lose their functionality at -80°C .

BASIC PROTOCOL 3

IN VIVO CLOTTING TIME BY TAIL CLIP ASSAY—The mouse tail clip assay is the most commonly used bleeding model to measure bleeding time and the amount of blood loss, and it is one of only a few *in vivo* murine assays. This assay has proven useful to compare the differences between mouse models of hemostatic and thrombotic disorders, especially evaluating hemostasis in genetically deficient mice (Broze et al., 2001; Sambrano et al., 2001), as well as testing anti-hemostatic (Bi et al., 1995; Broze et al., 2001) or anti-thrombotic (Saito et al., 2016) compounds/drugs. Both total blood loss and time to cessation of blood flow are used as measures of the hemostatic system. There are two standard methods for measurement of the amount of blood collected for the tail bleed: gravimetrically and by hemoglobin concentration (Greene et al., 2010; Schiviz et al., 2014). The injury is induced mechanically with a scalpel, followed by the tail immersion into pre-warmed saline, and monitoring of the bleeding for a period of time. However, many variables can affect the consistency of this assay, like sex and age, position of tail clip, sharpness of the blade to cut the tail, anesthetic used, and body temperature and blood pressure of the mouse (Greene et al., 2010). Variability of these factors can lead to different results and make it difficult to compare findings between research laboratories. This is mainly due to the lack of a standardized tail clip assay, and as a result each research laboratory uses an adapted protocol that presumably is the same method. Thus, a clear and detailed description of the model is

necessary to eliminate some of these variables. Here, we describe our standardized experimental details of the tail clip assay. Following this procedure will enable investigators to achieve rigorous and reproducible results.

Materials

Mice

37°C water bath

Tail clip platform (rectangular plexiglass plate with custom made holes to fit 15mL tubes)

15 mL conical tubes

0.9% Saline (see recipe)

Needle-holder (e.g., Integra Miltex MeisterHand needle holder, 5.75in or 14.6cm)

4–0 Vicryl suture (e.g., Ethicon)

Rodent restrainer (e.g., Broome-Style from Plas Labs)

1mL syringes (e.g., BD Slip Tip Syringe)

25G x ½ hypodermic needles (e.g., BD Precisionglide)

Phosphate Buffered Saline (PBS)

Small animal anesthesia induction chamber (e.g., Harvard Apparatus #72–6468) and nose cone (e.g., Harvard Apparatus #73–4838 mouse anesthesia mask)

Heating Pad (e.g., Kent Scientific Far Infrared Warming Pad)

French catheter scale (e.g., Keysurgical)

Permanent non-toxic marker (e.g., Sharpie)

Stainless steel disposable scalpel (e.g., INTEGRA #21)

Silver nitrate sticks

Buprenorphine (0.05–0.1mg/kg as needed)

Red Blood Cell Lysis Buffer (see recipe)

ELISA plate (e.g., Costar Assay Plate 96-well, medium binding, Corning)

Spectrophotometer

Isoflurane vaporizer

Multichannel pipette

Centrifuge

Preparation Before Assay

1. Set up water bath and tail clip platform as shown in Figure 4A. Add 14mL of saline to a 15mL conical tube and place in a 37°C water bath for 10 minutes before the start of the experiment.

Prepare one conical tube of saline for each mouse being used in the experiment.
2. Prepare the needle holder with looped 4-0 Vicryl suture.

If Using Therapeutic Agents

3. Place mouse into rodent restrainer following recommended guidelines.
4. Intravenously inject with a 25G x ½ hypodermic needle and syringe the therapeutic agent being used into the mouse lateral tail vein 5 minutes before initiation of tail amputation.

The therapeutic agents are typically calculated per body weight. The compounds are diluted in sterile saline or PBS for a final injection volume of 0.2mL.

Tail Clip Procedure

5. Anesthetize mouse in small anesthesia induction chamber with 2% isoflurane in 30% O₂ and 70% N₂ blend at a flow rate of 1L per minute.

The anesthesia induction chamber has an attached charcoal canister filter to capture excess isoflurane gas. We found that the percentage of isoflurane is very important as it affects the mouse breathing and thus, the results. We usually use this percentage for both normal and transgenic mice and see no difference in their breathing.
6. Wait 2–3 minutes, then remove mouse from the chamber. Make sure the mouse is recumbent and unresponsive to toe pinch.
7. Weigh the anesthetized mouse, apply a nose cone (which is attached to the vaporizer), and position it horizontally on the platform and/or heating pad as shown in Figure 4B.

Anesthesia is maintained at 2% isoflurane/1L per min flow rate and the breathing rate is monitored throughout the procedure (a constant 40–60 breaths per minute is the target breath rate).
8. Insert the mouse's tail into the 3mm hole of the French catheter scale and move along the tail upward until the tail fills the 3mm diameter hole as shown in Figure 4C. Mark this location with a sharpie.

If the French catheter is hard to push forward or the tail looks constricted in the 3mm hole, the French catheter scale is not at the right

mouse tail diameter and the scale should be withdrawn and the process repeated.

9. Place the mouse's tail (marked at 3mm diameter) into the 15mL conical tube with saline for 2 minutes at 37°C as shown in Figure 4D.
10. Remove the tail from saline and place on the platform.
11. Place two fingers from your non-dominant hand on either side of the mark on the tail. Apply a fixed, downward pressure with the scalpel to cut at a 90° angle on the mark, as shown in Figure 4E, to amputate the tail.

Use a new scalpel blade for each mouse to prevent crushing of the tail, which can disrupt the vessel diameter and alter the flow of blood.

12. Place the injured tail back into the 15mL conical tube with saline for 30 minutes at 37°C as shown in Figure 4F. Observe visually the blood flowing from the amputated tail and record the time from the injury to bleeding cessation (bleeding time).

If the re-bleeding occurs, the sum of all bleeding times during the procedure is used as the total bleeding time.

13. After 30 minutes, remove tail from the saline and close the wound with looped 4-0 Vicryl tourniquet-type suture. Cauterize the end of the tail with silver nitrate stick to prevent further bleeding.
14. Observe animals during recovery and dose with 0.05–0.1mg/kg buprenorphine as needed in accordance with your approved IACUC animal procedures.

The animals usually do not attack each other's tails.

Hemoglobin Assay to Quantitatively Assess the Amount of Blood Loss

15. For the standard curve, place 8 15mL conical tubes with 14mL of the saline solution in the 37°C water bath for 10 minutes.
16. Add known amounts of wildtype mouse blood (10, 25, 50, 75, 100, 150, 175 and 200µL) to each tube of saline solution at 37°C.

In order to construct the standard curve with known amounts of whole blood, a wildtype mouse is tail bled into a tube with no saline. The appropriate quantity of blood for each point on the standard curve is immediately pipetted into the tubes and brought up to 14mL with saline solution.

17. Centrifuge the 15mL conical tubes of saline and blood (both standards and samples) at 4°C for 10 minutes at 1500 x g to pellet the blood cells.
18. Remove the supernatant and resuspend the blood cell pellet in 6mL of the Red Blood Cell Lysis Buffer. Incubate at room temperature for 10 minutes.
19. Centrifuge the 15mL conical tubes again at 4°C for 10 minutes at 1500 x g.

20. Using a multichannel pipette, add 100µL of the standards and samples supernatant to an ELISA plate.

In cases of massive bleeding (as would be observed with hemophilic mice), the samples are supersaturated and it is difficult to accurately assess the blood loss. Thus, we recommend serially diluting the samples in lysis buffer before reading the absorbance, and multiplying the results by the dilution factor.

21. Read the ELISA plate with a spectrophotometric measurement at the 575nm wavelength to detect the hemoglobin.

Typically, for a wildtype mouse the total amount of blood loss within 10 minutes is between 20–100µL (this varies between genders and body weight). We exclude from the study wildtype mice with excessive bleeding, as defined by >100µL total blood loss (sometimes this happens if the incision is not made correct; that is why we recommend to always have a wildtype mouse as a control every time a tail clip procedure is performed).

BASIC PROTOCOL 4

CLOTTING OF PLASMA WITH THE PROTHROMBIN TIME (PT)—The Prothrombin Time (PT) is one of the most widely used lab tests of coagulation. It measures the extrinsic pathway, which is initiated by TF, and changes in fibrinogen, thrombin or factors V, VII or X may alter the PT. The clotting times are measured after the addition of exogenous phospholipid and the activator of the extrinsic pathway: thromboplastin (Kasthuri et al., 2010). The protocol workflow is detailed in Figure 5.

Materials

Plasma samples from Basic Protocol 2, stored at –80°C

Thromboplastin reagent (e.g., RecombiPlasTin 2G from Instrumentation Laboratory)

37°C water bath

37°C mini dry bath with 0.5mL tube block (e.g., USA Scientific)

1000µL Combitip (e.g., Eppendorf Combitips Advanced)

Cuvettes (These are specific for the automated coagulation analyzer)

Automated coagulation analyzer (e.g., Sigma Amelung KC4 ; Diagnostica Stago SStart Hemostasis Analyzer)

NOTE: This protocol and analysis is based on the Sigma Amelung KC4 Micro-Coagulation Analyzer and steps or amount of sample/reagent may change depending on the coagulation analyzer used.

1. Turn on machine to warm up to 37°C.

2. Take the Thromboplastin reagent out of the 4° refrigerator, aliquot the appropriate amount of reagent for testing the desired number of plasma samples, and place in warming slot in machine to warm reagent to 37°C.

100µL of the Thromboplastin reagent is needed for each sample and each mouse sample is assayed in duplicate. Allow the reagent to warm for at least 5 minutes.

3. Thaw frozen plasma samples in a 37°C water bath for no more than 1 minute.

Once samples are thawed, place them in a 37°C mini dry bath to keep warm until use.

4. Select the preprogramed PT assay in the machine and enter the sample ID (if applicable).
5. Place the cuvettes into the holding position of the machine.
6. Add 50µL of the plasma sample into the cuvette, gently swirl 4–5 times, and place cuvette into the test position.

The sample cuvette contains a metal ball that is magnetically held in place by the machine. Visually inspect each cuvette to ensure that it contains a metal ball prior to use. The plasma samples are assayed in duplicate, so 50µL of the same sample will be added to two different cuvettes. Gently swirling of the cuvette makes sure that the sample is completely distributed at the bottom of the cuvette. For the Sigma Amelung KC4 machine, the test position is where the magnet is that holds the metal ball in the cuvette.

7. Start the incubation time on the machine and incubate the plasma sample for 1 minute.
8. After the incubation is complete, initiate the start measurement on the machine and use the automated pipette with 1000µL Combitip to add 100µL of the Thromboplastin reagent to the cuvette.

Make sure to invert to mix the Thromboplastin reagent before use. Adding the thromboplastin reagent in the appropriate region of the cuvette (just to the right of the metal ball) is essential for reproducible results. The time should automatically start once the reagent has been added by the automated pipette. The time will stop automatically when the machine detects that the metal ball has been released from the magnet due to clotting in the cuvette, which traps the metal ball.

9. Record the elapsed times to clot formation.

ALTERNATE PROTOCOL 3

PT FACTOR ACTIVITIES—The activity of specific factors, in this case thrombin, TF, FV, FVII, and FX, can be determined by creating a standard activity curve (using wildtype plasma) and diluting the plasma samples with factor deficient plasma. Then the PT assay is

performed normally and the clotting time of the samples can be plotted against the curve and the activity determined (Everett et al., 2014). The protocol workflow is detailed in Figure 5.

Additional Materials

Imidazole Buffer (see recipe)

Factor deficient plasma (e.g., Instrumentation Laboratory; George King Bio-Medical)

NOTE: To measure thrombin activity, thrombin (FII) deficient plasma is only available from Instrumentation Laboratory.

1. Follow steps 1–3 of Basic Protocol 4.
2. A standard activity curve is made with wildtype mice. The first dilution is a 1:10 dilution of plasma to imidazole buffer. The remaining dilutions are made serially, up to eight times.

The 1:10 dilution is 100 μ L of plasma and 900 μ L of imidazole buffer. This represents 100% factor activity. To make the serial dilutions, 500 μ L of the 1:10 dilution is added to 500 μ L of imidazole buffer. That is now a 1:20 dilution that represents 50% factor activity. Then 500 μ L of the 1:20 dilution is added to 500 μ L of imidazole buffer to make the next dilution, and so on.

3. The experimental samples are diluted 1:10 with imidazole buffer.
4. Select the preprogramed PT assay in the machine and enter the sample ID (if applicable).
5. Place the cuvettes into the holding position of the machine.
6. Add 50 μ L of the standard curve dilution or diluted plasma sample into the cuvette.
7. Add 50 μ L of the Factor deficient plasma into the cuvette, gently swirl 4–5 times, and place cuvette into the test position.

The plasma samples are run in duplicate so 50 μ L of the same sample will be added to two different cuvettes. Gently swirling the cuvette makes sure that the sample is completely distributed. For the Sigma Amelung KC4 machine, the test position is where the magnet is located that holds the metal ball in the cuvette.

8. Start the incubation time on the machine and incubate the plasma sample for 1 minute.
9. Once the incubation is complete, initiate the start measurement on the machine and use the automated pipette with 1000 μ L Combitip to add 100 μ L of the Thromboplastin reagent to the cuvette.

Make sure you mix the Thromboplastin reagent before use by inverting the tube. Adding the thromboplastin reagent in the appropriate region of

the cuvette (just to the right of the metal ball) is essential for reproducible results. The time should automatically start once the reagent has been added by the automated pipette. The time will stop automatically when the machine detects that the metal ball has been released from the magnet due to clotting in the cuvette, which traps the metal ball.

10. Record the times. Plot the standard curve and the average times for the experimental samples against the curve to determine factor activity.

BASIC PROTOCOL 5

CLOTTING OF PLASMA WITH THE ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)—The Activated Partial Thromboplastin Time (APTT) is another one of the most widely used lab tests of coagulation. It measures the intrinsic pathway, or contact pathway, and changes in contact factors or factors VIII, IX, XI, or XII may alter the APTT. The clotting times are measured after the addition of exogenous phospholipid and the activator of the intrinsic pathway: kaolin, micronized silica, etc. (Kasthuri et al., 2010). The protocol workflow is detailed in Figure 5.

Materials

Plasma samples from Basic Protocol 2, stored at -80°C

APTT Reagent or Contact activator (e.g., APTT-SP from Instrumentation Laboratory)

0.025mol/L CaCl_2 (e.g., APTT-SP from Instrumentation Laboratory)

37°C water bath

37°C mini dry bath with 0.5mL tube block (e.g., USA Scientific)

1000 μL Combitip (e.g., Eppendorf Combitips Advanced)

Cuvettes (These are specific for the automated coagulation analyzer)

Automated coagulation analyzer (e.g., Sigma Amelung KC4 ; Diagnostica Stago STart Hemostasis Analyzer)

NOTE: This protocol and analysis is based on the Sigma Amelung KC4 machine and steps or amount of sample/reagent may change depending on the coagulation analyzer used.

1. Follow steps 1–6 of Basic Protocol 4, except aliquot the APTT reagent and CaCl_2 to prewarm before use in step 2.
 50 μL of both the APTT reagent and CaCl_2 is needed for each sample and each sample is run in duplicate. Allow reagents to warm for at least 5 minutes.
2. Start the incubation time on the machine and incubate the plasma sample for 2 minutes.

3. Halfway through the incubation, add 50µL of the APTT reagent to the cuvette, and let incubate for 1 minute.

Make sure you mix the APTT reagent before use by inverting. If CaCl₂ is added at this step instead of the APTT reagent, the sample will not clot.

4. After the incubation is complete, initiate the start measurement on the machine and use the automated pipette to add 50µL of the CaCl₂ to the cuvette.

Make sure to you mix the CaCl₂ before use by inverting. The time should automatically start once the reagent has been added by the automated pipette. The time will stop automatically when the metal ball is released from the magnet due to the sample clotting.

5. Record the elapsed times to clot formation.

ALTERNATE PROTOCOL 4

APTT FACTOR ACTIVITIES—The activity of specific factors, in this case FVIII, FIX, FXI and FXII, can be determined by creating a standard activity curve (using wildtype plasma) and diluting the plasma samples with factor deficient plasma. Then the APTT assay is performed normally and the clotting time of the samples can be plotted against the curve and the activity determined (Everett et al., 2014). The protocol workflow is detailed in Figure 5.

Additional Materials

Imidazole Buffer (see recipe)

Factor deficient plasma (e.g., Instrumentation Laboratory; George King Bio-Medical)

1. Follow steps 1–7 of Alternate Protocol 3, except aliquot the APTT reagent and CaCl₂ to prewarm before use in step 2.

50µL of both the APTT reagent and CaCl₂ is needed for each sample and each sample is run in duplicate. The standard curve dilution made for Alternate Protocol 1 can be used for this as well, so a new curve does not have to be made.

2. Start the incubation time on the machine and incubate the plasma sample for 2 minutes.

3. Halfway through the incubation, add 50µL of the APTT reagent to the cuvette, and let incubate for 1 minute.

Make sure to invert to mix the APTT reagent before use. If CaCl₂ is added at this step instead of the APTT reagent, the sample will not clot.

4. After the incubation is complete, initiate the start measurement on the machine and use the automated pipette to add 50µL of the CaCl₂ to the cuvette.

Make sure you mix the CaCl_2 before use by inverting. The time should automatically start once the reagent has been added by the automated pipette. The time will stop automatically when the metal ball is released from the magnet from the sample clotting.

- Record the times. Plot the standard curve and the average times for the experimental samples against the curve to determine factor activity.

ALTERNATE PROTOCOL 5

ACTIVATED PROTEIN C (APC) RESISTANCE—The APTT test can be used to test resistance to activated protein C (APC). In normal plasma samples, adding APC to the sample will cause a prolongation of the APTT assay. However, alterations in certain factors can diminish this result, for example, the Factor V Leiden mutation (Harris et al., 2012; Yang et al., 1998). The protocol workflow is detailed in Figure 5.

Additional Materials

Activated Protein C (e.g., Chromogenix Coatest APC Resistance)

- Follow steps 1–3 of Basic Protocol 5, except APC/ CaCl_2 also needs to be aliquoted out to prewarm before use in step 2.

CaCl_2 and APC/ CaCl_2 are run for each sample. 100 μL of the APTT reagent and 50 μL of CaCl_2 and APC/ CaCl_2 are needed for each sample and each sample is run in duplicate. Allow reagents to warm for at least 5 minutes.

- After the incubation is complete, initiate the start measurement on the machine and use the automated pipette to add 50 μL of the CaCl_2 or APC/ CaCl_2 to the cuvette.

Make sure you mix the reagents before use by inverting. The time should automatically start once the reagent has been added by the automated pipette. The time will stop automatically when the metal ball is released from the magnet from the sample clotting.

- Record the times. Calculate the average times per sample. Use the average times to calculate the APC ratio, which is determined by:

$$\text{APC Ratio} = \frac{\text{Clot time APC/CaCl}_2}{\text{Clot time CaCl}_2}$$

A ratio close to 1 indicates APC resistance.

BASIC PROTOCOL 6

CALIBRATED AUTOMATED THROMBOGRAM (CAT) TO MEASURE THROMBIN GENERATION—Thrombin is a pivotal enzyme in hemostasis and thrombosis, since there are no hemostatic mechanisms that bypass thrombin (Hemker et al., 1993). It enables the production of more thrombin through feedback activation of the coagulation cascade and the

activation of platelets. However, it also initiates anti-coagulant biochemical reactions that gradually cease its production. Assays measuring thrombin generation have existed for many decades (Kintigh et al., 2018). These assays have documented decreased thrombin generation in several coagulation factor deficiencies and in patients using anticoagulant therapy, as well as increased thrombin generation in some congenital and acquired thrombophilias. Although there are many tests to evaluate individual components of the coagulation system, there is no test that accurately evaluates the thrombotic risk of an individual. The Calibrated Automated Thrombogram (CAT) Assay was developed to measure disorders of hemostasis and thrombosis.

Assays for evaluation of the coagulation system in mice are not as widely available as those for humans and are typically limited to evaluation coagulation factor levels, clotting times and systemic measures of clotting activation, such as d-dimer and thrombin-antithrombin complex (Emeis et al., 2007; Furie et al., 2005; Tchaikovski et al., 2007). The CAT assay can be used to measure thrombin generation in platelet-poor or platelet-rich plasma collected from mice. It provides a global assessment of prothrombotic and anti-thrombotic factors present in plasma and platelets working concurrently to generate or inhibit the generation of thrombin.

Materials

PPP or PRP samples from Alternative Protocol 2

15 mL conical tubes

ddH₂O

FluCa Kit containing Fluca-Buffer and Fluca-Substrate (e.g., Diagnostica Stago)

37°C water bath

CAT calibrator (e.g., Diagnostica Stago)

HEPES-buffered saline (see recipe)

Tissue factor at 0.6 pM in HEPES-buffered saline containing 0.1% BSA

Phospholipids: 20 mol% phosphatidylcholine, 50 mol% phosphatidylethanolamine and 30 mol% phosphatidylserine (e.g., Avanti Polar Lipids) at 6 µM in HEPES-buffered saline

Small glass flask

Small glass beaker

Fluoroskan Ascent™ FL Microplate Fluorometer (CAT Analyzer)

1. Turn on the Calibrated Automated Thrombogram (CAT) Analyzer and allow warm up to 37°C for 20 minutes.

If using PRP, allow the platelets to sit and rest during this time.

2. Warm 15 mL of ddH₂O and Fluca-Buffer to 37°C in a water bath.

You will need 1122µL for half a plate and is the minimal amount needed to prime the pump on the CAT Analyzer.

Preparation of the CAT Analyzer and Sample Plate

3. Enter your username and password on the CAT Analyzer. Adjust the read length to 120 minutes and the read interval to 40 seconds.
4. Set up a plate template on the CAT Analyzer. You will need a CAT calibrator (in duplicate) for each PPP or PRP sample.
5. Set up the test plate by adding the following into each well:
 - a. 10µL PPP or PRP sample
 - b. 30µL HEPES-buffered saline if testing PRP; or 30µL HEPES-buffered saline with 24 µM phospholipids if testing PPP
 - c. 10µL Tissue Factor
6. Click “plate out” on CAT Analyzer. Put plate in plate drawer.
7. Follow the on-screen instructions. There is a 10-minute incubation period. Set a timer for 9 minutes once the incubation period begins.
8. When the timer sounds, add 28µL Fluca-Substrate to the conical tube with 1122µL pre-warmed Fluca-Buffer (1:40 substrate to buffer).
9. Follow the on-screen instructions:
 - a. Fill small glass flask with the warmed ddH₂O. Place the aspirating tube into the water and the dispenser tip above the small glass beaker.
 - b. Press next, the water will be pumped through the lines and dispensed into the beaker.
 - c. Remove the aspirating tube from the water.
 - d. Press next, the water will be emptied from the tubing into the beaker.
 - e. Place the aspirating tube into the warmed Fluca-substrate. Make sure it is at the bottom of the tube. Place the dispensing tip against the inside of the tube just above the Fluca-substrate. The Fluca-substrate will be circulating through the tubing.
 - f. Remove the “M” pin and put the dispenser tip in the hole. Make sure the aspirating tube is still at the bottom of the Fluca-substrate tube.
 - g. Press next, Fluca-substrate will be dispensed into the plate.
 - h. Remove the dispenser tip and put it in the flask containing the water. Let the aspirating tube hang free. Close the cover and let the assay run.

Terminate Assay

10. At the end of the assay run, replace the pin into the “M” hole.
11. Clean the dispenser with 10mL of ddH₂O. On the CAT Analyzer, go to “Instrument”, then “Prime Instrument”, change the volume to “10,000”. Place aspirating tube into a conical tube containing at least 10mL ddH₂O. Put the dispenser tip above the beaker.
12. Press “Start”. Empty the tube once the priming is over.
13. Press “Instrument”, then “Empty Instrument”.

Collect and Analyze Data

14. To analyze the data, Press “export to .xls” and the data will be downloaded to an excel file. A histogram can be visualized by double clicking on the graph, and averages of the duplicate samples will be seen. Averages of the duplicate parameters can then be used in a graphing/statistical program for further analysis. Detailed information on the software is provided by the manufacturer.

REAGENTS AND SOLUTIONS

HEPES-buffered Saline

20 mM HEPES

140 mM NaCl

pH 7.4

Store up to 1 year at 4°C

Imidazole Buffer

0.05M Imidazole

0.1M NaCl

Store up to 1 year at 4°C

Ketamine (100mg/kg)/xylazine (10mg/kg)

2mL 50mg/mL Ketamine (Vetalar, Ketaset, Ketalar)

0.8mL 20mg/mL Xylazine (Rompun)

Fill with d²H₂O to 10mL

Red Blood Cell Lysis Buffer

0.15 M NHCl

10 mM KHCO₃

1 mM EDTA

Store up to 1 year at 4°C

0.9% Saline

9g NaCl

Fill with d²H₂O to 1000ml

Store up to 1 year at 4°C

16.7mg/mL Sodium Pentobarbital

1mL 390mg/mL Sodium Pentobarbital

22.4mL diluent (40% propylene glycol, 10% ethanol, 50% water)

Store up to 1 year at room temperature

COMMENTARY

Background Information

Tail Clip Assay—Numerous procedures have been developed to assess various aspects of the hemostatic system. However, there are many variables to consider when performing blood coagulation models. The hemostasis community has been striving for a standardized tail clip assay protocol that is both simple to perform and rigorous (Greene et al., 2010). This assay has been developed from the human template bleeding time, which is a test that is commonly used to assess to risk of bleeding. Despite attempts at standardization of the human template bleeding time, it is still not as accurate as taking a careful patient history for predicting bleeding risk in patients undergoing surgical procedures. The tail-bleeding model was first developed in rats for the purpose of studying its practicality in assessing the effects of drugs on platelet function and primary hemostasis (Dejana et al., 1979; Stella et al., 1975). Consequently, two different techniques were established in order to measure these two variables: (a) a template model, where a small, standard incision on the dorsal part of the tail is made using a “template” device (Dejana et al., 1979; Stella et al., 1975) or (b) a “free-hand” or transection model, in which the tail artery and two veins are all transected, resulting in amputation of the tail tip (Dejana et al., 1979). Over the years, the tail clip assay has been adapted to mice and subjected to several modifications.

The tail bleeding assay detailed here is conceptually similar to the human template bleeding time in that the amount of bleeding from an induced injury and time to cessation of bleeding is quantitated. However, the mouse tail bleeding assay has not been completely standardized, and thus “mouse tail bleeding assay” is presently a blanket term for all assays involving the measurement of bleeding from an induced injury to the tail of a mouse (Greene et al., 2010; Saito et al., 2016). The non-standardization of this assay causes varying results between labs and makes communication between the labs difficult on agreeing what exactly has been assayed when a mouse tail bleeding assay has been performed. It is our sincere intent that

the protocol described in Basic Protocol 3 will help standardize this assay to allow for better experimental outcomes (leading to a reduction in the variance of the mouse tail bleeding assay and therefore reduced numbers of experimental mice necessary to assess hemostasis via this assay) and enhancing the inter-laboratory reproducibility of this important assay.

In terms of the measurement of the hemoglobin in Basic Protocol 3, we have also used human hemoglobin (Sigma Aldrich item #H7379) rather than mouse blood from a wildtype mouse to construct a standard curve for measuring hemoglobin. We have constructed a standard curve of hemoglobin diluted in phosphate buffered saline (PBS) (2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, 0.156mg/mL, 0.078mg/mL, and PBS alone). We assayed the undiluted samples and these were read in a spectrophotometer at 416 nm wavelength. Recently, Saito *et al.* described a potentially more sensitive method to determine hemoglobin concentration by immersing the cut tail directly into 2.5mL of a solution containing Drabkin's reagent (Sigma Aldrich item #D5941)(Saito et al., 2016).

Plasma Coagulation Assays—The PT, APTT, and thrombin time/generation assays are used ubiquitously in human clinical settings to evaluate the activity of coagulation and the risk of bleeding in humans (Kasthuri et al., 2010). Using adaptations of these assays to analyze coagulation in mice enables us to analyze the same factors and tests to better provide human clinical correlations of our mouse studies. The Sigma Amelung KC4 coagulation assay protocols are standardized for humans and call for a 50µL of the plasma sample to be used. 25µL may also be sufficient for this and other machines, as these machines were designed for human use, which would cut down on the amount of sample need. In order to provide a method of determining day-to-day variability in your assay conditions, it is useful to develop a large plasma pool from equal numbers of male and female control mice. Aliquots of this pooled sample can be run each time you perform the assay. Divergent results of this sample over different days can provide useful information regarding your assay conditions.

Both the PT and APTT assays do not provide much information about global coagulation due to the high concentration of activator needed to overcome the added citrate (Kasthuri et al., 2010), but it can give you information about what factors are altered. As we have described here, measuring activity levels of proteins such as FV can provide additional information regarding how changes in those proteins actually alter coagulation (Emeis et al., 2007; Sommeijer et al., 2005). For example, as qualitative defect in FV activity due to a polymorphism or mutation in the FV gene/protein would be easily detected in the activity assay. Having the ability to measure activity levels of these proteins is especially important given the lack of suitable antibodies available for measuring coagulation factor antigen levels in mice.

Critical Parameters/Troubleshooting

Blood Draws—Critical parameters should be considered when performing the cardiac puncture. The first is the positioning of the mouse's heart. The heart should lie toward the mouse's left side after opening the thoracic cavity. If it is not in this position, take your gloved finger and gently flip it to the left and/or slightly adjust the mouse's recumbent

position to slant toward the left. The heart should then stay in this advantageous position. The mouse may involuntarily move and flip the ribcage back on to the right ventricle while you are collecting blood. If you have the clamp appropriately secured with the forceps wedged between the clamps handle, this should not occur. If it does, do not panic and cease blood drawing while flipping the ribcage back toward the mouse's head.

Similarly, several parameters should be considered when performing the IVC procedure. Blood could leak out of penetration site if the needle is not sufficiently inserted into the IVC. When drawing blood from the IVC, be careful to continuously monitor the blood flow going into the needle through the wall of the IVC (which is very transparent). Collecting the blood too quickly will cause the IVC to collapse and begin to be drawn into the needle. If this occurs, cease pulling back on the plunger of the syringe until blood again collects in the IVC, as evidenced by the red color you will see through the IVC. In cases where more blood is needed but no more blood is pooling by the needle tip, postural venous return (carefully lifting up the posterior end of the mouse) may help to bring additional blood to the IVC. For this reason, these blood collection surgeries may be performed on a tilt table that can be level at the beginning of the procedure but easily tilted by the operator during the latter part of the procedure so that the mouse's tail is above its head. This ensures the maximum postural venous return.

Prolonged blood draw times and insufficient mixing of the blood with the anticoagulant during the blood draw can lead to insufficiently anticoagulated blood and thus, unusable samples. Make sure to draw the blood as efficiently and carefully as possible to prevent this problem (Day et al., 2004). As with any surgical procedure, there is a learning curve, so you may have to perform each of the blood collection techniques many times to truly master them. However, this is time well spent and your newfound expertise will likely become evident in your experimental results.

Plasma Isolation—Samples should be excluded from further analysis if there is evidence of poor blood draw, leading to partial activation of the blood coagulation system or macro or microthrombi that could result in consumption of the plasma hemostatic factors. Some of the hallmarks of bad blood drawing, such as hemolysis, have been excellently described (Rathkolb et al., 2013). Here we would like to reiterate the importance of clean blood draws. Hemolysis is usually observed when a blood sample has been poorly drawn, as is shown by the samples on the middle and right sides of Figure 6. An additional indicator of a poor blood draw can be seen after separating the plasma from the blood cells by centrifugation. Normally, the white blood cell buffy coat should be smooth all the way across the tube, as in the sample on the left in Figure 6. If there is bumpiness or unevenness in the white cell buffy coat, as seen in the sample on the right in Figure 6, this indicates clotting occurred in this sample.

Whichever blood collection method you choose for collecting the blood, it is best to consistently use that method. Differences in several blood values have been identified in blood collected from various anatomical sites, so using blood obtained under standardized conditions from the same anatomical site will enable you to produce the highest quality data (Hoggatt et al., 2016). In addition, it is of the utmost importance to clearly communicate

your blood drawing method and type of anticoagulant in your scientific report so others may be able to accurately interpret and reproduce your experiments if necessary.

Tail Clip Assay—Since gender can affect many hemostatic parameters, including the bleeding time (Greene et al., 2010), using equal numbers of males to females for experimental groups will reveal any sex difference but allow for the average between the groups to be determined for the tail clip assays. There are likewise profound differences in hemostasis due to mouse strain background (Westrick et al., 2007), so strain background should be carefully controlled. Clearly stating which mouse strain and where the strain came from will inform other research and potentially reduce skewed results (Schiviz et al., 2014). We strongly advocate the use of littermates for these experiments. Although the age of the animal is important (ideally between 8–12 weeks old), we have found that using mice within a certain body weight (25–30g) and thus, comparable tail dimensions, helps to limit the variability of the assay.

Further, the experimental outcomes of the tail clip assay are highly dependent on the surgical procedure, type of anesthesia, temperature, mouse age, body weight and strain (Greene et al., 2010; Liu et al., 2012). These aspects appear to be the most important factors to control. For example, respiratory function and heart rate can have an impact on the amount of blood released from the tail of the mice. That is why an anesthetic should be used that has minimal effect on respiratory function (Anesthetics such as pentobarbital can affect breathing and heart rates of the mice). Monitoring the heart rate could allow for correction of the amount of blood collected from the tail bleed, so more accurate, consistent measurements can be made.

Anesthesia affects the body temperature, leading to hypothermia. To maintain the animal's body temperature at 37°C, the anesthetized mouse is placed on a heating platform. The platform could be a transparent rectangular Plexiglas board that can fit on top of the water bath calibrated to 37°C as shown in Figure 4A, or a far infrared warming pad (Kent Scientific). If neither of these options are available, the mouse can be placed on a Styrofoam box and a heat lamp or a heating pad can be used as previously described (Liu et al., 2012).

Plasma Coagulation Assays—Sodium citrate is added to the blood to remove calcium ions that are essential for coagulation (Mann et al., 2007). The PT and APTT assays require the addition of calcium, so if the percentage of calcium is not exact, greater variability between samples may be seen, which could skew results for the assays. Improper thawing of the plasma samples can lead to the formation of insoluble fibrin polymers in your plasma samples before you even begin your experiments. Carefully visually inspect your samples before adding them to the cuvettes for the presence of fibrin strands or other visible material in your plasma samples, which are indicative of coagulation activation. Sometimes there may even be an insoluble fibrin gel in your sample that cannot be broken up with a pipet because the activated fibrin strands have high tensile strength. These samples should be excluded from further analysis.

All samples should be assayed in duplicate. Make sure the PT and APTT reagents are used before the expiration date, using the reagents after the expiration date will skew the assay results as the reagents will not function properly.

Statistical Analyses

For the PT, APTT, factor activity, and bleeding time/hemoglobin amounts, the average value for each mouse is used (if samples were run in duplicate) and mice are grouped based on strain, genotype, treatment group, etc. Then a Mann-Whitney nonparametric test statistical analysis is run between each group to determine if there is a significant difference between them. With more than two experimental groups, it is common to adjust the p -values to q -values using the False Discovery Rate, and significance is determined at $q < 0.05$. However, significance determined based off a $p < 0.05$ is acceptable (Althouse, 2016).

Tail Clip Assay—To construct the standard curve, plot the absorbance against the known amount of blood added to each standard tube. We use the plate reader software to draw the best-fit curve through the points of the graph or create a standard linear curve. The sample concentrations are determined by using its absorbance value to interpolate the blood loss using the curve function of the standard curve. If samples have been diluted, we multiply the concentration obtained by standard curve fitting by the dilution factor.

Understanding Results

Understanding Results for Tail Clip Assay—In some variations of the method, the tail is cut at 1 to 5 mm from the tip (Maroney et al., 2012; Sambrano et al., 2001; Tranholm et al., 2003), while in others the tail is amputated at 1 to 4 mm tail diameter (Holmberg et al., 2009; Ivanciu et al., 2015). The measurable parameters are: the bleeding time, measured as cessation of blood flow due to hemostatic plug formation, and total blood loss, measured as the hemoglobin content of blood. Many authors report either one or the other of these values but not both. Since changes in one parameter can occur independently of the other, we highly recommend obtaining (and reporting) both in order to more accurately assess bleeding.

Understanding Results for Plasma Coagulation Assays—Significant differences in the PT suggest that the mouse carries a genomic variant affecting either fibrinogen, thrombin or factors V, VII or X of the extrinsic coagulation pathway. Likewise, with the APTT assay, significant differences suggest that the mouse carries a genomic variant affecting a limited number of coagulation factors including: fibrinogen, thrombin or factors V, VIII, X, XI or XII. Depending on the results of the PT and APTT assays, information regarding potential defects in coagulation can be obtained. Subsequent analyses of individual coagulation factors can then determine more specifically where the effect is acting.

Understanding Results for CAT Assay—The individual parameters of the thrombogram can be evaluated to assess abnormalities. The Lag Time is the time between initiation of the assay and the appearance of the first traces of thrombin. It will vary depending on the activator used, its concentration and the amount of plasma in the assay. The Endogenous Thrombin Potential (ETP) is measured by taking the integral (dx/dt) of the

relative change in fluorescence and is the total thrombin generated in the assay. It is proportional to the area under the curve. Peak Thrombin is the maximal amount of thrombin being generated at any point during the reaction. The Time to Peak Thrombin is the time from initiation of the reaction to the time of maximal thrombin generation. The velocity index is the rate of thrombin generation. These parameters are calculated using software that compares fluorescence intensity in the sample to that of a calibrator added to a parallel sample of platelet-poor or platelet-rich plasma. The calibrator consists of thrombin trapped within α 2macroglobulin, which blocks the interaction of thrombin with other macromolecules in plasma, but leaves it accessible to cleave small substrates (Hemker et al., 2002). As the calibrator consumes the substrate, fluorescence increases and the calibration constant is measured through a range of fluorescence levels providing a sample-specific constant for each level of fluorescence.

Time Considerations

Time Considerations for Blood Collections—With experience, approximately 6, 6–12 week old mice can be easily exsanguinated per hour. If the mice are considerably older, it will take additional time. This is because the proper anesthetic dosing becomes more unpredictable with the increased adiposity of the mice, so mice may have to be supplemented with additional anesthetic. Additional pericardial and peritoneal visceral fat deposits will make the cardiac and IVC blood draws more difficult. In addition, older mice have decreased venous blood volume, presumably due to their increased adiposity.

Time Considerations for Plasma Isolation—Once blood samples have been obtained, move directly to plasma isolation as some of the coagulation factors, like coagulation FV are labile (Linskens et al., 2018). It is best to have all microcentrifuge tubes pre-labeled before beginning the blood draw/plasma isolation to ensure that you can move as quickly as possible. The samples should be aliquoted immediately after they are centrifuged and stored at -80°C . If the samples are stored at -20°C , the assays should be performed within a month of the date the samples were obtained. It is useful to keep track of the time it takes to isolate, aliquot and store the plasma samples in your laboratory notebook.

Time Considerations for Tail Clip Assay—The tail clip assay usually takes less than 30 minutes per mouse. With practice, more than one mouse can be undergoing the procedure at any given time.

Time Considerations for PT and APTT Assays—A single sample to clot in the PT assay could take anywhere from 5–10 seconds. The plasma samples are diluted in the PT Factor Activity assay, which extends the time to clot anywhere from 25–50 seconds. A single sample to clot in the APTT assay could take anywhere from 25–50 seconds. The plasma samples are diluted in the PT Factor Activity assay, which extends the time to clot anywhere from 50–80 seconds. However, the time it takes the samples to clot may vary depending on the mouse model used. With the Sigma Amelung KC4 machine, only two samples can be run at a time in duplicate. The total amount of time to complete the assay is dependent on how many samples are being used for the experiment/analysis. With the

inclusion of the 30 seconds required to thaw the plasma samples, with practice, up to 30 samples can be analyzed within 1 hour.

Time Considerations for CAT Assay—It takes about an hour per 96 well plate to perform the CAT assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

Blood coagulation is a complex process involving a complex network of molecules. Many methods have been developed for assessing the murine blood clotting system *in vivo* and *in vitro*. However, the use of many different nuanced protocols makes it difficult to interpret the results of these studies in the greater context of hemostasis. Therefore, we provide basic *in vivo* and *in vitro* protocols for several of the most common methods to assess blood clotting. Proper collection and processing of blood samples is critical for assessing coagulation activity *in vitro*. Thus, we have included protocols for obtaining high quality blood samples critical for downstream biochemical assays, such as prothrombin time (PT), activated partial thromboplastin time (APTT), and the calibrated automated thrombogram (CAT) assay.

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Figure 1:
An anesthetized mouse in dorsal recumbency with the paws taped to a heavy absorbent wipe.

Figure 2:

A) A 1mL syringe with the 26G x $\frac{3}{8}$ needle bent at $\sim 30^\circ$ angle. **B)** The abdominal incision with the intestines to the right of the abdomen (to your left side) to expose the inferior vena cava (IVC). The white arrow is indicating the IVC at the level of the left renal artery. **C)** The bent needle inserted into the IVC to draw blood.

Figure 3:

A) The phase separation between plasma and the red and white blood cells after centrifugation in a swinging bucket rotor to keep the liquid level. **B)** The pseudo-plasma (without drawing up red blood cells) transferred to a 1.5mL microcentrifuge tube.

Figure 4:

The tail clip assay. **A)** The water bath and tail clip platform with a transparent rectangular Plexiglas board. **B)** The mouse with a nose cone and positioned horizontally on the tail clip platform. **C)** The mouse's tail in the 3mm hole of the French catheter scale. **D)** The mouse's tail (marked at 3mm diameter) placed in the 15mL conical tube with saline for 2 minutes at 37°C. **E)** The cutting of the tail with two fingers from your non-dominant hand on either side of the mark on the tail and the scalpel at a 90° angle to the tail. **F)** The injured tail placed in the 15mL conical tube with saline for 30 minutes at 37°C.

Figure 5:

The workflow of the PT and APTT coagulation assays. Black arrows are steps that overlap between assays. Blue arrows represent PT assay steps. Orange arrows represent factor activity steps for the PT assay. Green arrows represent APTT assay steps. Yellow arrows represent factor activity steps for the APTT assay. Red arrows represent APC resistance steps for the APTT assay.

Figure 6:

Plasma separation from blood draws. The middle and right tube show hemolysis from a blood sample that has been poorly drawn. The left tube shows a normal plasma separation from the blood cells with a smooth buffy coat; and the right tube shows bumpiness/unevenness in the white cell buffy coat, which indicates clotting occurred in this sample.