

Published in final edited form as:

Methods Mol Biol. 2012 ; 788: 101–107. doi:10.1007/978-1-61779-307-3\_8.

## Clot retraction

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### Abstract

The study of clot retraction *in vitro* has been adopted as a simple and reproducible approach to assess platelet function. Plasma clots should retract away from the sides of a glass tube within a few hours allowing the rapid characterization of outside-in signalling through platelet integrin  $\alpha_{IIb}\beta_3$  within individuals or in response to test compounds. In this chapter we describe the role of platelets in fibrin clot retraction and provide a detailed description of the methods used to assess this process.

### Keywords

Clot retraction; platelets; integrin  $\alpha_{IIb}\beta_3$ ; outside-in signaling

## 1. Introduction

The conversion of fibrinogen to fibrin at sites of injury result in the formation of threads of fibrin which form a clot trapping platelets and red blood cells to form a plug at the site of injury, and in the arterial circulation consolidating the developing platelet thrombus. Clot retraction is a process driven by outside-in signaling by platelet integrin  $\alpha_{IIb}\beta_3$  that results in the contraction of the fibrin mesh. The contraction of the fibrin clot, results in the blood clot becoming smaller and excess fluid is extruded. This draws the edges of damaged tissue together and forms a mechanically stable clot.

The study of the retraction of plasma clots formed *in vitro* has been adopted as a simple and reproducible approach to characterise outside-in signalling through platelet integrin  $\alpha_{IIb}\beta_3$ .

### Clot formation, stabilization and retraction

Exposure of sub-endothelial collagen upon vessel injury activates platelets (1-3) and also initiates coagulation resulting in the production of thrombin, a potent platelet agonist (4). Platelet activation results in spreading, the secretion of granules containing pro-thrombotic factors, and the affinity of the fibrinogen receptor, integrin  $\alpha_{IIb}\beta_3$  for its ligand fibrinogen is increased (5). These ‘inside out’ signals result in platelet aggregation via bivalent binding to fibrinogen. As a consequence of fibrinogen binding, the cytoplasmic tail of  $\beta_3$  integrin subunit becomes phosphorylated triggering an ‘outside-in’ signal that provide a second wave

of activatory signaling that is necessary for irreversible platelet thrombus formation. Such 'outside-in' signaling through integrin  $\alpha_{IIb}\beta_3$  plays a key role in clot retraction (6).

The role of platelets in fibrin clot formation is long established. At sites of injury the activation of the tissue factor- (extrinsic) and contact- (intrinsic) dependent coagulation pathways leads to the activation of factor X, and through subsequent processing of prothrombin to thrombin, lead to the conversion of plasma fibrinogen to fibrin (Figure 1). Activated platelets provide phospholipids released in the form of microparticles that act along with calcium as cofactors for the actions of factor X. The exposure of amino phospholipids such as phosphatidylserine on the surface of activated platelets provides a surface for the assembly of the prothrombinase complex, and thereby thrombin may be produced in the vicinity of a platelet thrombus as it forms, ensuring the incorporation fibrin for thrombus stabilization. Through activation of protease activated receptors on the platelet surface, thrombin also acts as a powerful platelet agonist.

Platelets generate force to contract the fibrin matrix and draw the edges of the wound together. The retraction is driven by the interaction between the fibrin outside the cells and the actin - myosin cytoskeleton of the platelets, which is mediated by integrin  $\alpha_{IIb}\beta_3$ . Myosin binding occurs upon phosphorylation of the  $\beta_3$  subunit of the integrin (7) and the ANK domain-containing Bcl-3 co-localises with the cytoskeleton, which have been shown, along with Src-family kinases to play key regulatory roles in this process (8, 9, 10). Phospholipase  $C\gamma_2$  (PLC $\gamma_2$ ) is also implicated, and following fibrinogen - integrin  $\alpha_{IIb}\beta_3$  binding, becomes tyrosine phosphorylated by a Src family kinase-dependent mechanism (10). Evidence suggests that tyrosine kinases regulate the attachment of the cytoskeleton to integrin  $\alpha_{IIb}\beta_3$  and are essential for the transition of cellular contractile forces to the fibrin polymers (11). The process of retraction is energy demanding and the metabolite sensing kinase adenosine monophosphate (AMP)-activated protein kinase  $\alpha_2$  (AMPK  $\alpha_2$ ) affects the phosphorylation of the Src-family kinase Fyn, which in turn affects integrin  $\alpha_{IIb}\beta_3$  signalling and thus clot retraction (12).

### Clot retraction as an assay of integrin $\alpha_{IIb}\beta_3$ outside-in signalling

The ability of platelets to drive clot retraction has become a surrogate measure of outside in signalling following engagement of the integrin  $\alpha_{IIb}\beta_3$  that may be readily measure with little required equipment. In this chapter we describe a simple *in vitro* technique that can be used assess platelet function in human or mouse blood.

## 2. Materials

1. Modified Tyrodes-Hepes buffer (134 mM NaCl, 0.34 mM  $\text{Na}_2\text{HPO}_4$ , 2.9 mM KCL, 12mM  $\text{NaHCO}_3$ , 20 mM HEPES, 5 mM glucose, 1 mM  $\text{MgCl}_2$ , pH 7.3)
2. 4% (w/v) sodium citrate (*see* <sup>Note 1</sup>)

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<sup>1</sup> Sodium citrate is used as an anticoagulant as this reduces the availability of ionized calcium and magnesium without reducing the pH, which would reduce platelet function.

3. Thrombin (Sigma, Poole, UK), make a stock of 20 units ml<sup>-1</sup> in Tyrodes-Hepes buffer.
4. Syringes, sealed glass pipettes (*see*<sup>Note 2</sup>), reusable tack adhesive (such as Blu-Tack), Glass tubes (10mm diameter tubes with a total capacity of 3ml for mouse clot retraction assays, and 10mm or 12 mm diameter tubes for assays with human blood) and test tube rack
5. Camera and microbalance

### 3. Method

In this section we describe the measurement of clot retraction *in vitro* using platelet rich plasma (PRP), and note considerations for performing and designing experiments.

#### 3.1 Preparation of human blood

1. Human Blood is drawn into a syringe containing 4% (w/v) sodium citrate and PRP obtained by centrifugation at 100g for 15 - 20 min at room temperature in the presence of prostacyclin (0.1µg ml<sup>-1</sup>) (*see*<sup>Note 3</sup>).
2. Remove the PRP into a clean tube and keep back some red blood cells (RBC) (*see*<sup>Note 4</sup>).

#### 3.2 Preparation of mouse blood

1. Mouse blood (1ml) is drawn into a syringe containing 100µl 4% (w/v) sodium citrate as an anticoagulant. PRP is prepared from whole blood by centrifugation at 200g for 8 min at room temperature in the presence of prostacyclin (0.1µg ml<sup>-1</sup>).

#### 3.3 Clot retraction assay

1. Rest the platelets in a warm water bath (30°C) for 30 min.
2. Count platelet numbers and adjust if necessary using platelet poor plasma (PPP) to ensure consistency (*see*<sup>Note 5</sup>).
3. Fill glass tubes with 745 µl of warm (30°C) Tyrodes-Hepes buffer and 5 µl RBC (used to colour the clot (*see*<sup>Note 4</sup>)). Place all the tubes in the rack on a plain piece of paper or clean bench where it can undisturbed for several hours.
4. Add 200 µl PRP (this can be reduced to 50µl for mouse platelets) to each tube

<sup>2</sup>Glass pipettes can easily be sealed by holding the tip over a Bunsen flame for a few seconds.

<sup>3</sup>Do not add ACD during platelet preparation, since this will lower pH and chelate ions preventing clot retraction from occurring.

<sup>4</sup>Red blood cells are used to colour the clot for easy visualization. Use the RBCs that have been pelleted in the preparation of PRP as these are more concentrated than whole blood and will provide more defined colour.

<sup>5</sup>When comparing the effect of a compound on an individual each donor provides a full set of data. However when comparing different individuals the platelet number is likely to be variable. To keep platelet levels standardised samples can be diluted with platelet poor plasma. With human work this will be more easily available as blood volumes can be larger, however, with mouse work when comparing test and control animals this may involve taking blood from another animal (test animal for test samples and control for control samples) in order to prepare some PPP. For this type of work it is worth preparing samples from several animals at once to allow pairing up similar platelet concentrations and use of PPP can be shared. PPP may be prepared by centrifugation of PRP (1400g for 10 minutes at room temperature) to remove platelets, and can be stored at -20°C for future use).

5. At this point any test compounds that may affect clot retraction or platelet function should be added and incubated if required (*see* <sup>Note 6</sup>).
6. Add 50  $\mu\text{l}$  thrombin 20 unit  $\text{ml}^{-1}$  (final concentration of 1 unit  $\text{ml}^{-1}$ ) and mix by flicking the tube quickly.
7. Finally and immediately after adding the thrombin add a sealed glass pipette to the centre of the tube and secure with sticky tac (*see* <sup>Note 7</sup>).

#### Summary of assay setup—

Tyrodes-Hepes buffer	745 $\mu\text{l}$
PRP	200 $\mu\text{l}$
Thrombin (20 units /ml stock)	50 $\mu\text{l}$
RBC	5 $\mu\text{l}$

Amounts can be reduced for the study of mouse platelets or where the volumes of blood samples are limited, using narrow glass tubes (10 mm diameter or less).

Tyrodes-Hepes buffer	175 $\mu\text{l}$
PRP	50 $\mu\text{l}$
Thrombin (20 units /ml stock)	20 $\mu\text{l}$
RBC	5 $\mu\text{l}$

**Assessment of results—**Images of the experiment enable the kinetics of clot retraction to be observed, and photographs should be taken at time 0 and then every 30 minutes (or more frequently if desired). Images may also be analysed to provide numerical data, such as apparent thrombus area, using image analysis software.

Results from clot retraction are assessed numerically by the weight of the clot or weight/volume of extruded serum. Typically an experiment is ended after 120 min (although platelet concentration, temperature, and type of experiment will effect this). A point to end the assay should be chosen before all samples reach completion since differences may be less apparent after several hours since all samples may eventually completely contract (some treatments may result in delay in contraction, rather than inhibition of contraction). This will require optimization for particular experimental outcomes. (*see* <sup>Notes 8 & 9</sup>)

An example of clot retraction using this procedure is shown in Figure 2, where the Src family kinase inhibitor PP2 was used to reduce inhibit clot retraction.

<sup>6</sup>When testing compounds that may influence clot retraction it is important to incorporate a vehicle control.

<sup>7</sup>Developing clots may collapse if disturbed. The sticky tac is used to ensure that the glass pipette does not move.

<sup>8</sup>Setting up experiments in duplicate will allow clots to be weighed to be taken at a point where differences are pronounced while keeping another set to examine if the same end point is eventually reached.

<sup>9</sup>Temperature can affect the speed of clot retraction. If the laboratory temperature is variable, assays should be conducted within an incubator at 20°C.

**Alternative methods**—One limitation of the standard method is that it may prove difficult to differentiate between coagulation and platelet defects. Plasma free clot retraction in the presence of purified fibrinogen can be used to investigate the function of integrin  $\alpha_{IIb}\beta_3$ , without the complication of the effects of the coagulation cascade (10, 13, 14).

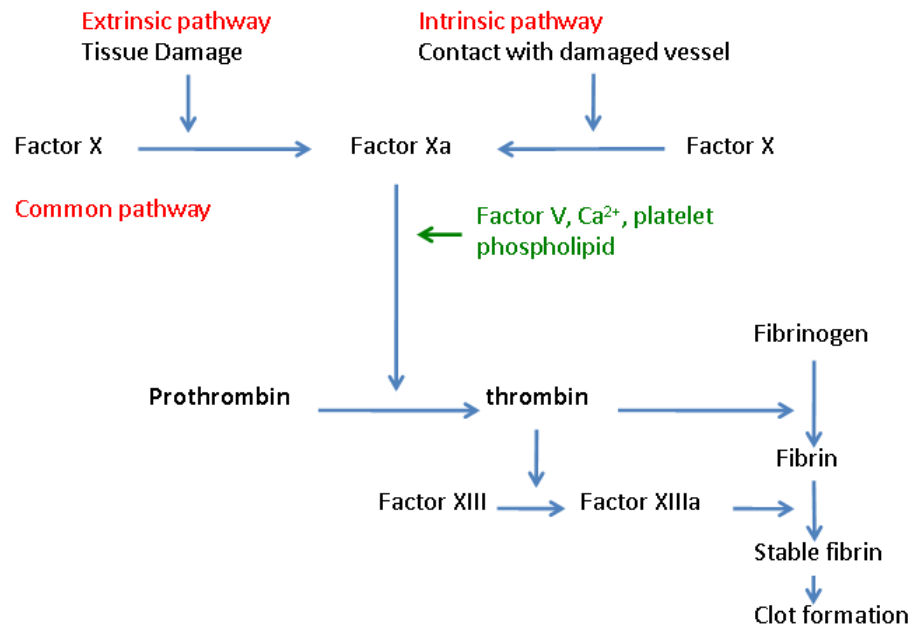
## Abbreviations

<b>PRP</b>	platelet rich plasma
<b>RBC</b>	red blood cell
<b>PP2</b>	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine
<b>PLC<math>\gamma</math>2</b>	phospholipase C $\gamma$ 2
<b>AMPK <math>\alpha</math>2</b>	adenosine monophosphate (AMP)-activated protein kinase $\alpha$ 2

## References

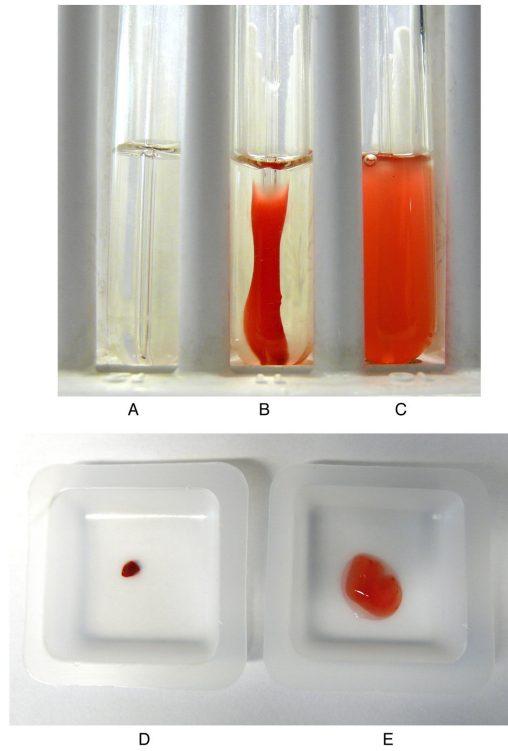
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**Figure 1. The regulation of fibrin clot formation**

Diagram of the final stages of the coagulation cascade where the intrinsic and extrinsic pathway meet and combine in the final common pathway, that leads to the conversion of fibrinogen to fibrin.



**Figure 2. Clot retraction of human PRP**

Photograph was taken after two hours of (A) no thrombin, (B) 1unit ml<sup>-1</sup> thrombin (and DMSO solvent control 0.1%), (C) with 1 unit ml<sup>-1</sup> thrombin PP2 (25μM) Src family kinase inhibitor (dissolved in DMSO). Clots were weighed after 3 hours (D) thrombin control and (E) thrombin and PP2 Src inhibitor.