PART 10 Platelet

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TOPICS COVERED

- ✓ Platelet Count
- ✓ Platelet Function Testing
- ✓ Platelet Function Testing by Flow Cytometry

Platelet Count: Blood is mixed with a diluent that causes hemolysis of red cells. A hemocytometer is filled with the diluting fluid, and the platelets are counted under the microscope, preferably by using phase-contrast, if available.

Materials/equipment:

- ✓ Flat-bottom, thin counting chamber (phase-contrast hemocytometer with Neubauer ruling)
- ✓ Phase-contrast microscope equipped with long-working-distance phase condenser, if available; otherwise an ordinary light microscope
- ✓ 20 µl pipette
- ✓ 2 ml graduated pipette
- ✓ 12 x 75 mm tube
- ✓ Mechanical mixer

Reagent: Diluting fluid: 1% ammonium oxalate in distilled water. Store in the refrigerator and always filter just before using.

Specimen: If the blood sample is from a finger prick, the puncture must be clean and the blood free flowing. Wipe away the first drop of blood. If the blood sample is from venous blood, it must be collected into a dry plastic (or siliconized glass) syringe with a short needle not smaller than 21 gauge. The needle must be removed before the blood is delivered into a plastic container with EDTA. The blood and anticoagulant must be mixed gently, to avoid frothing, without any delay.

Method: Pipette 0.38 ml of diluting fluid into a test tube. Fill the 20 µl pipette to the mark and wipe off the outside of the pipette. Expel the contents of the pipette into the diluting fluid, and wash out the pipette by drawing up the blood and expelling it into the tube a few times. Mix for at least 10 minutes by hand or, preferably, by mechanical mixer. Fill the hemocytometer, as described below. Cover the chamber with a petri dish for 10 to 20 minutes to allow the platelets to settle. Leave a piece of wet cotton or filter paper in the dish to prevent evaporation. Using a microscope, count the platelets in the large 1 mm squares (= 0.1 µl). Count the platelets in as many squares as necessary to reach a count of at least 100. The platelets appear round or oval, and their internal granular structure and purple sheen allow them to be distinguished from debris. Ghosts of the red cells that have been lysed by the ammonium oxalate are seen in the background. If phase contrast is not available, an ordinary light microscope can be used, provided the condenser is racked down to provide a low intensity of light. Calculate the number of platelets per liter of blood according to the formula below.

The hemocytometer: The hemocytometer counting chamber, with Neubauer or improved Neubauer ruling, is constructed so that the distance between the underside of the cover glass and the surface of the chamber is 0.1 mm. The surface of the chamber contains two specially ruled areas with dimensions as

shown in Figure 21. The central 1 mm^2 has double or triple boundary lines. In the central areas, there are 25 squares in the improved Neubauer and 16 squares in the Neubauer ruling. Each square has an area of 0.04 mm^2 (0.2 x 0.2 mm). These squares are, in turn, divided into smaller squares, each 0.0025 mm^2 (0.05 x 0.05 mm). The outer quadrants of the ruled area are each 1 mm^2 and are divided into 16 squares.

Calculations:

The formula for calculating the cell count is:

Count (cells/l) = N x D/A x 10 x 10^6

Where N = total number of cells counted

D = dilution

A = total area counted (in mm^2)

10 = factor to calculate volume in µl from area (in mm^2) and depth of chamber (0.1 mm)

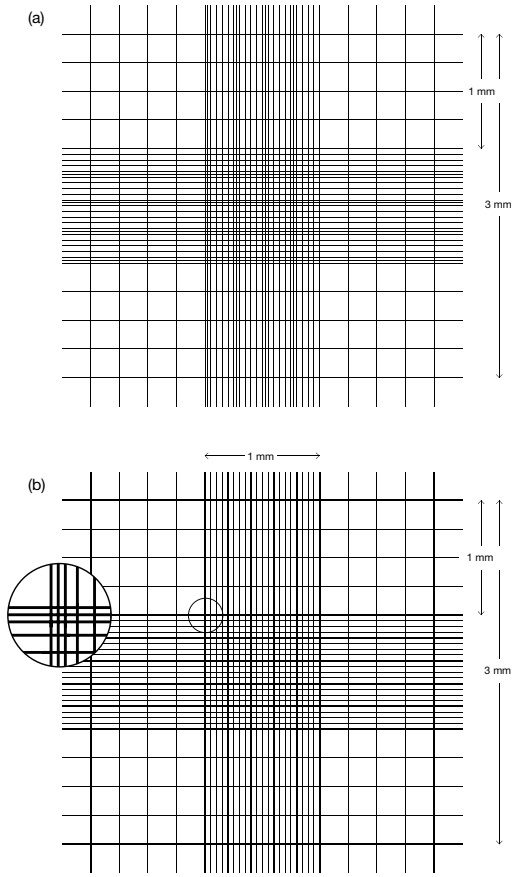
10^6 = factor to convert count/µl to count/l

Sources of error in cell counting: When capillary blood is used, a free-flowing drop must be obtained. When anti-coagulated blood is used, the specimen must be carefully mixed by inverting the tube of blood at least 20 times before a sample is taken. Do not shake the tube, because shaking introduces foam, which makes accurate pipetting impossible. Tilt the well-mixed tube to an angle of 45° or slightly more, and pipette from the lip of the tube, following the same procedures as for capillary blood. The blood-sampling pipettes must be clean and dry. The pipette must be filled quickly, and the blood must be drawn accurately by using a pipette suction device attached to the pipette, filling up to the desired line. If the line is overshot slightly, the excess blood may be expelled by touching the lip of the pipette on a piece of filter paper or soft tissue. If the line is overshot, a fresh pipette must be used. No air bubbles should be present in the blood column. The outside of the pipette must be wiped free of blood (being careful not to pull blood from the tip) before it is introduced into the diluting fluid. After the contents of the pipette have been discharged into the diluent, diluting fluid must then be drawn into the pipette with steady suction several times, to ensure that all the blood is discharged into the fluid. The tube containing the diluted blood must be shaken gently for at least two minutes by hand or, preferably, in a mechanical shaker. After the tube has been shaken, the chamber is immediately filled by means of a Pasteur pipette or capillary tube. The chamber is filled by capillary action, with the flow of fluid from the pipette or capillary regulated so that it fills quickly and smoothly. It must be filled completely, but fluid must not spill over into the moats. Allow the cells to settle in the counting area for 10 to 20 minutes, then proceed with the counting. The hemocytometer chamber and glass cover must be clean and dry before they are used. Important errors can be introduced by fingerprints or an oily film. A sufficient number of cells must be counted to reduce error due to chance distribution of cells. In practice, at least 100 cells should be counted. As a further check on correct distribution of cells in the chamber, the number of cells counted in each area (i.e. in the large squares) should not differ by more than 10%.

Controls: Two dilutions must be made, and the mean of the two counts taken; the two counts should agree within 10%.

Sources of error in platelet counting: Blood obtained by a venipuncture is preferable to capillary blood, because platelets adhere to the wound and successive dilutions from a finger prick are not always reproducible. The general errors of pipetting and hemocytometry are described above. In addition, special attention must be paid to ensuring that the counting chamber is scrupulously clean, since dirt and debris

may be counted as platelets. Wash the chamber with soapy water, then rinse with distilled water, allow to drain dry, and wipe with lint-free tissue. Be sure that the cover slip is clean before using it. The presence of platelet clumps precludes reliable counts. If the sample contains clumps, a fresh sample must be collected. The ammonium oxalate diluent should be kept refrigerated and must be discarded if there is evidence of bacterial contamination. The specimen must be counted within three hours of collection.



10.2 Platelet Function Testing

10.3 Clot Retraction

10.4 Measurement of Platelet Aggregation

Figure 21. Hemocytometer counting chamber (a) Neubauer and (b) Improved Neubauer

Platelet Function Testing: The primary role of platelets is to support hemostasis by formation of platelet plug at the sites of vascular injury. When the blood vessel is injured and the subendothelial microfibrils and collagen fibers are exposed, platelets bind to the vessel wall, a process called platelet adhesion. At sites of high shear, platelets bind to vessels indirectly with the help of high molecular weight VWF multimers. VWF binds to exposed collagen through its A3 domain, and the platelets bind to the collagen bound VWF through its GPIbα receptor. Platelets can also adhere directly to collagen in the sub endothelium with the glycoprotein VI (GPVI) and integrin α2β1 receptors. Once platelets adhere to the vessel wall, they undergo a series of changes: Through the reorganization of platelet cytoskeleton, they change from small disc shaped structures to spiculated spheres with the development of filopodia. Membrane flip flop translocates anionic procoagulant phospholipids, mainly phosphatidyl serine, to the outer surface of platelets, which forms a good platform for thrombin generation. Platelets release the contents of alpha (e.g. fibrinogen, FV, VWF, and growth factors) and dense granules (e.g. ADP, ATP, calcium, and serotonin). This causes further platelet activation. Formation of thromboxane A_2 (TxA_2) occurs from arachidonic acid via phospholipase A2, cyclooxygenase-1 (COX-1), and TXA_2 synthase. Platelet agonists (ADP, TxA_2, and thrombin) bind to specific membrane receptors and initiate platelet aggregation by activation of the integrin receptor, α2β3 (GPIIb-IIIa) that binds to fibrinogen and/or VWF to form the platelet plug. Abnormalities in any of these pathways causing platelet adhesion, activation, degranulation, and aggregation can cause

platelet dysfunction. Platelet disorders include both quantitative (thrombocytopenia) or qualitative defects and can be inherited or acquired. Bleeding symptoms are primarily mucocutaneous, like ecchymosis, gum bleeding, easy bruising, menorrhagia, post-partum hemorrhage, and gastrointestinal bleeding (malena, hemestemesis, or hematochezia). Symptoms can be mild or severe depending on the abnormality. A list of inherited platelet function disorders (IPFDs) with associated salient clinical and laboratory features are shown in Table 33. The true prevalence of platelet disorders is not known. It is thought that platelet function disorders are more common than previously appreciated due to underdiagnosis. In areas of the world where consanguinity is common, autosomal recessive disorders like Glanzmann thrombasthenia and Bernard Soulier syndrome have a higher prevalence. While diagnosis of Glanzmann thrombasthenia and Bernard Soulier syndrome are relatively easy due to typical aggregometry pattern, the diagnosis of most other IPFDs is cumbersome and requires complex assays. In the investigation of patients suspected of having an IPFD, it is important to collect a detailed clinical history, including personal and family history. The use of a validated BAT like the ISTH BAT is strongly encouraged. History should also include drug history, recent food intake which can possibly interfere with platelet function, and the presence of other features (e.g. eczema, recurrent infections, familial cases of myelodysplasia, AML) and potential syndromic features (e.g. hearing loss, heart defects, face or bone dysmorphism, albinism) which can help in diagnosis. Preliminary laboratory investigations should include full blood count including platelet count, examination of a blood film to look for platelet morphology and other blood cell features (see below), determination of the skin bleeding time (described in Section 11), screening tests for secondary hemostatic tests (PT and APTT), and fibrinogen assay to exclude other coagulation disorders. It is recommended to perform screening tests for VWD (i.e. VWF activity, VWF antigen) either concurrently or prior to more extensive work-up for platelet function disorders. VWD, type 2B VWD, and platelet type VWD can also present with macrothrombocytopenia and should also be considered in patients with low platelet counts. Another simple test that can give an indication that to the presence of a platelet function defect is the clot retraction method, described below.

Clot retraction: The retraction of the clot in clotted whole blood can give an indication of platelet number and function. When the clot retracts, serum is expressed, and the degree of clot retraction can be measured.

Method: Collect 1ml of blood into a glass test tube (75 mm x 10 mm) and place at 37°C. Examine the tube visually until a firm clot is present. Leave undisturbed at 37°C for another hour. Measure the distance from the base of the tube to the meniscus. Carefully remove the clot with a thin wooden stick (e.g. a cocktail stick), leaving the serum that has been expressed from the clot in the tube. Measure the distance from the base of the tube to the meniscus of the serum. Divide the serum distance by the total distance and multiply by 100 to give a percentage.

Interpretation: Normally, more than 40% serum is expressed. A decreased expression is present in some platelet defects, notably Glanzmann's thrombasthenia. It can also be abnormal in severe thromobocytopenia, Wiskott Aldrich syndrome, and Stormorken syndrome.

Notes: The tubes and wooden stick must be absolutely clean to prevent the clot from adhering to the tube. The clot must be removed carefully and gently to avoid squeezing and therefore more serum being expressed.

Platelet count, morphology, and examination of peripheral blood smear: Initial testing for a suspected platelet function disorder should include a complete blood count along with evaluation of peripheral blood smear. This helps to detect abnormalities of platelet number, size and morphology which can provide important clues for further laboratory testing. Platelet count can be measured by manual microscopic method, automated counting in hematology analyzers, and flow cytometry based immunological labelling. Estimation of the platelet count on the peripheral smear should be performed to confirm platelet count, especially in cases of macrothrombocytopenia or platelet anisocytosis. In a well prepared and stained smear, the average number of platelets are counted in 10 oil immersion fields (100x). This value should be multiplied by 15,000 to determine the platelet count/µL. It is important to check the smear to

evaluate platelet clumps, platelet size, morphology and granularity. Pseudo-thrombocytopenia due to EDTA-dependent platelet clumping and satellitism (platelets rosetting neutrophils) should be excluded. It is important to remember that platelet clumps can also be seen in platelet type or type 2B VWD and should not be mistaken for EDTA-induced clumping. Platelet type VWD can also show large platelets in the smear. An abnormal platelet size is often a feature of inherited platelet disorders (with or without platelet dysfunction) as shown below:

- Small platelets: Wiskott-Aldrich syndrome, X-linked thrombocytopenia, ADAP deficiency
- Large/giant platelets: Bernard Soulier syndrome (giant), MYH9-related disorders (giant), Grey platelet syndrome and α-granule deficiency syndromes, platelet type VWD, ITGA2B/ITGB3-related thrombocytopenia (GT variant), Filaminopathy, SLFN14-related thrombocytopenia, GATA1-defects, Velocardiofacial syndrome
- Normal platelets: All the remaining disorders

The mean platelet volume (MPV) is a readily available parameter on impedance-based platelet counting hematology analyzers and gives an estimate about the platelet size. Laboratories should establish their own reference ranges as MPV can vary between instruments. Small platelets can be easily missed by light microscopy and a reduced MPV might be a first clue for possible Wiskott Aldrich syndrome. Various factors can affect the measurement of MPV and interfere with impedance-based platelet counting, like abnormalities of red cells and platelets. The presence of microcytic and fragmented red cells can overestimate platelet counts while presence of large and/or giant platelets can be missed causing underestimation of platelet counts. In these scenarios, platelet counts and MPV will not be reliable. It is very useful to concomitantly look at platelet histogram which can identify platelet size abnormalities, and possible interferences/contamination. In macrothrombocytopenia, the optical or fluorescent platelet counts give truer platelet counts. Large pale platelets with bluish grey cytoplasm with absent or markedly reduced azurophilic granules can be seen in Gray platelet syndrome due to deficiency of α-granules. Paris Trousseau syndrome shows large to giant platelets, some of them showing clumped/fused α-granules forming one large, clumped granule. Platelet anisocytosis (i.e variation in platelet size) and anisogranularity (i.e variation of platelet granularity) with presence of pale staining empty looking platelets is a classical feature of Acquired platelet dysfunction with eosinophilia (APDE). APDE is a transient bleeding diathesis associated with eosinophilia, commonly presenting in children from South-East Asia, and reversed upon treatment of eosinophilia. Morphologic abnormalities can also be seen in other blood cells. Presence of Dohle-like inclusion bodies in neutrophils in MYH9-related disorders, giant peroxidase positive cytoplasmic granules in neutrophils and/or other leucocytes in Chediak Higashi syndrome, Howell Jolly bodies in Stormoken syndrome and dyserythropoiesis in GATA-1 mutations.

Measurement of platelet aggregation by light transmission aggregometry: Light transmission aggregometry (LTA) is the gold standard for platelet function testing. It was first independently described by O'Brien and Born in the 1960s. The principle is based on the changes in the optical density of platelet rich plasma (PRP) as platelets get activated and form aggregates. PRP is turbid due to the suspension of platelets compared to PPP. Once agonists are added to PRP, platelets form aggregates and settle down, thereby clearing the plasma and allowing more light to pass through. The change in transmitted light is measured over time by an aggregometer and represented graphically. By comparing the pattern of aggregation response to each agonist, the type of platelet function disorder can be suspected and diagnosed. A number of excellent reviews and guidelines for platelet function testing and diagnosis of IPFDs have been published.

Precautions prior to studying platelet aggregation (pre-analytical variables in platelet aggregation): Blood samples for LTA should be collected after a short period of rest. Subjects should refrain from smoking for at least 30 minutes before and from caffeine at least 2 hours before testing. A record of all drugs, including native/herbal medications taken during the last 10 days should be documented. For a list of drugs that can interfere with platelet function, see the British Society for Haematology's guidelines on platelet function testing referenced below. Drugs known to reversibly inhibit platelet function (e.g. NSAIDs) should be stopped at least 3 days before sampling. Drugs known to irreversibly inhibit platelet function (e.g. Aspirin,

thienopyridines) should be stopped at least 10 days before sampling, unless their effect is being specifically investigated. Patients should ideally be fasting, preferably after overnight fasting. If not fasting, avoid sampling after fatty meals. Many other "normal" dietary constituents, including alcohol, onions, garlic, pepper, and ginger, may also inhibit platelet aggregation. This should be kept in mind when evaluating results. Blood should be drawn with minimal, or no venostasis using a needle of at least 21 gauge into a 109 mM or 129 mM sodium citrate, buffered anticoagulant. The first 3-4 ml of blood drawn should be discarded or used for tests other than LTA (e.g. PT/APTT). When sample collection is difficult, under-filled tubes may only be used to exclude severe platelet function defects, such as Glanzmann thrombasthenia or Bernard-Soulier syndrome.

Preparation of platelet-rich and platelet-poor plasma: Blood samples should be allowed to 'rest' at room temperature for at least 15 minutes before centrifugation. Refrigeration of platelets can cause activation and hence samples should always be maintained at ambient temperature. PRP should be prepared by centrifuging blood samples at 200g for 10 minutes at ambient temperature (~21°C) without using a brake. For samples with very large platelets, like Bernard-Soulier syndrome, PRP should be prepared by blood sedimentation for 30 minutes. The PRP is carefully removed, avoiding contamination with red cells or the buffy coat (aggregation will be diminished if either are present), into a capped polypropylene tube kept upright at 20–25°C. PPP should be prepared by centrifuging whole blood, or the tubes of blood from which PRP was removed, at ambient temperature at 1500g for 15 minutes. Grossly hemolyzed samples should be rejected. If the sample is lipemic, indicate this in the final report. It is necessary to check the platelet count of the PRP. The results of LTA studies could be inaccurate when the platelet count in the PRP sample is lower than 150 x 10^9/l. Samples with low platelet counts should be interpreted with caution but may be tested to exclude severe platelet function disorders, like Glanzmann thrombasthenia, Bernard Soulier syndrome, type 2B VWD, and platelet VWD. The platelet count of PRP samples should NOT be adjusted to a standardized value with autologous PPP. Platelet count in PRP in samples from subjects with normal platelet count (200–600 x 10^9/l) do not affect the results of LTA studies. Adjusting the platelet count in PRP using autologous PPP within this range may inhibit platelet responsiveness. This is probably because the PPP may contain substances released from platelets during the additional trauma from the higher speed centrifugation used in PPP preparation. Uncertainty remains over what is the best practice to follow when the platelet count in PRP exceeds 600 x 10^9/l. For exceedingly high PRP counts (>1000 x 10^9/l), it may be advantageous to adjust the platelet count to a more suitable level with the patient's PPP.

Aggregating agents or agonists: There are two types of agonists, weak and strong agonists. Weak agonists (e.g. ADP and epinephrine) at critical concentrations show an initial primary wave of aggregation due to direct agonist induced effect, followed by a secondary wave of aggregation caused by the release of platelet granules and TXA_2 synthesis. Strong agonists (e.g. thrombin, collagen, TXA_2) on the other hand show only a single curve without distinction between primary and secondary aggregation as these agonists directly induce platelet aggregation, TXA_2 synthesis, and granule release. The different phases of platelet aggregation are shown in Figure 22, which is evident only with weak agonists.

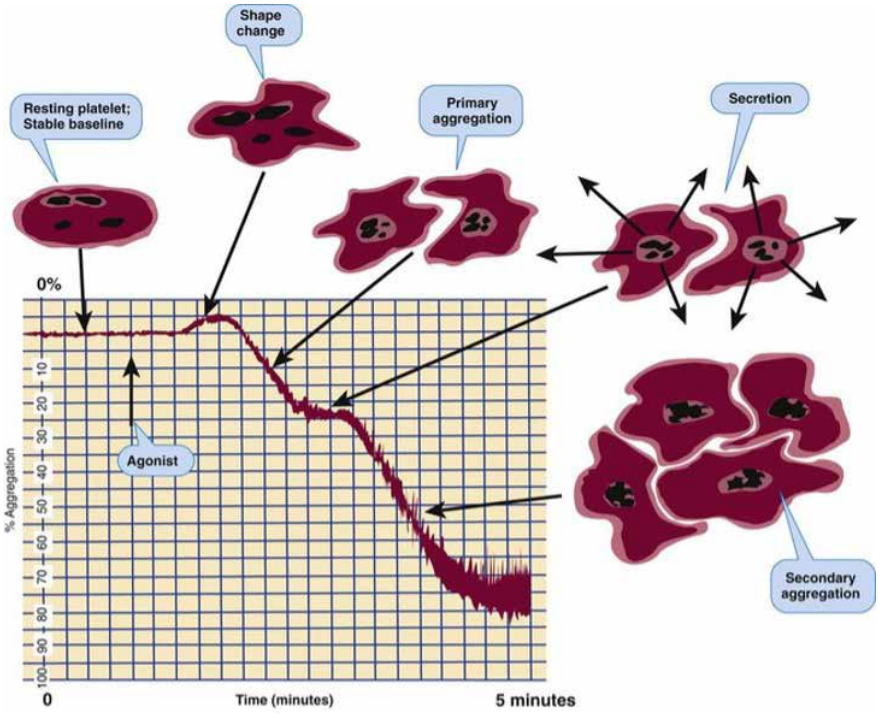


Figure 22. Image from Rodak's Hematology: Clinical principles and applications, 5th edition

Table 33. Summary of recommended agonists along with concentrations that can be used as an initial panel and extended panel (concentrations as per ISTH guidelines)

Agonist	Initial panel and concentration	Extended panel and concentration
ADP*	2 µM	5 µM, 10 µM
Epinephrine*	5 µM	10 µM
Collagen (Horm or type 1 fibrillary)	2 µg/ml	
Arachidonic acid	1mM	
Ristocetin (low dose)	0.5-0.7 mg/ml	
Ristocetin (high dose)	1.2-1.5 mg/ml	
Thrombin receptor activating peptide (TRAP)*, PAR1	-	10 µM
Thromboxane A2 mimetic U46619	-	1 µM

*Higher concentrations should be used if abnormal results are seen with the initial concentration.

Adenosine-5-diphosphate (ADP): A stock solution of 1mM/l of the disodium salt is prepared in OBS and stored in small amounts at -40°C. This is stable for at least three months. Once thawed, the solution should be used within three hours or discarded. For use, further dilutions are prepared in OBS. The pattern of response to ADP depends on its final concentration. At 2 µmol/l, clearly defined primary and secondary waves can be seen: the first represents the direct agonist-induced effect and the latter is due to release of endogenous ADP and generation of TXA_2, which itself aggregates platelets. Below 2 µmol/l, progressively fewer normal subjects show a secondary response, and the primary wave usually reverses as the ADP is enzymatically degraded. Above 3 µmol/l, the primary phase is usually so intense that the distinction between it and the secondary phase is masked. ADP induces a change in shape of the platelets from a disc to a spiky sphere. This initially causes a slight increase in the optical density of the platelet suspension, which can be seen only if primary aggregation is impaired.

Adrenaline (epinephrine): A stock solution of 1mM/l of the bitartrate salt is prepared in OBS. It should be stored and used as for ADP. With adrenaline, the concentrations used and the patterns of response are similar to those of ADP. However, in the absence of a secondary wave, the primary wave does not reverse, nor is it ever so intense that the secondary wave is masked.

Collagen: A very stable suspension of equine tendon collagen fibrils (1 mg/ml), available from Hormon-Chemie, Munich, Germany, is widely used. A number of other materials are equally suitable. It is stored at 4°C and must be well mixed immediately prior to dilution in the buffer packaged with it. It should be used at final concentration of 0.5–2.0 µg/ml in PRP, and diluted suspensions are stable for one week at 4°C. With collagen, no primary wave occurs. The response is usually defined by the duration of the lag phase prior to the onset of aggregation and by the intensity of the latter. A slight increase in the optical density caused by the shape change precedes aggregation. Collagen from a number of different sources is in use. Both the type of collagen and the species from which the preparation is prepared (e.g. equine or bovine) can have an important effect on the results obtained. Indeed, more than a hundred-fold range of concentrations are required, depending on the source material. It is therefore important to select a suitable source and to establish a local reference range for this material, which should be re-evaluated if the source is changed. For a review, see Jennings et al. (2008).

Ristocetin: At a final ristocetin concentration of 1 mg/ml in PRP, distinct primary and secondary waves are usually discernable, but above this the direct effect is so intense that the two phases merge. The primary wave is a measure of the amount of VWF present in the plasma, whereas the second wave is due to release of endogenous substances.

Arachidonic acid: Sodium arachidonate (99% purity) is dissolved in OBS to a concentration of 10mM/l. Small aliquots are placed in darkened glass vials that are flushed with nitrogen to prevent oxidation, then tightly capped and stored frozen below -20°C. Aggregation is generally monophasic and preceded by a short lag phase.

Reagents: Some examples of reagent concentrations and dilutions are shown below

Note: These concentrations are appropriate if one part is added to nine parts of PRP. Dilutions can be made in distilled water or saline, or as per manufacturer's instructions.

✓ ADP (Stock solution 1000 µM)

Make a 1 in 10 dilution = 100 µM (i.e. 0.1 ml 10,000 µM solution + 0.9 ml OBS). From this, make appropriate working strengths: 20 µM (i.e. 0.2 ml 100 µM + 0.8 ml OBS), PRP final concentration of 2–50 µM (i.e. 0.5 ml 100 µM + 0.5 ml OBS), PRP final concentration of 5 µM. In cases where hyperaggregability is being tested, working strengths of lower concentration may be needed (e.g. 10 µM, 5 µM).

- ✓ *Adrenaline (epinephrine):* Dilute as for ADP to make a working strength of 50 µM. This will give a final PRP concentration of 5 µM.
- ✓ *Collagen:* Mix well and dilute in OBS/other diluents: 1 in 500 (i.e. 0.1 ml stock + 4.9 ml OBS) = 20 µg/ml, PRP final concentration of 2 ug/ml
- ✓ *Ristocetin:* This is used at up to two different concentrations depending on the results obtained. Normal dose ristocetin at either 15 mg/ml or 12.5 mg/ml, and low ristocetin at either 7.5 mg/ml or 5 mg/ml. This will give a final PRP concentration of 1.5 mg/ml or 1.25 mg/ml, and low dose ristocetin of 0.75 mg/ml or 0.5 mg/ml respectively depending on the concentration used.

Method/procedure: LTA studies should be completed within a maximum of 4 hours after blood sampling. Because of platelet refractoriness to aggregation after centrifugation, PRP samples should be allowed to sit at ambient temperature for at least 15 minutes before testing. PRP should be stored in full, tightly stoppered tubes until tested. Before starting, bring the reagents/agonists to room temperature. Prepare fresh dilutions of reagents if required. Turn on the aggregometer and the computer/recorder and wait till

the temperature of the aggregometer reaches 37°C. Set the stirring speed to 1000 rpm unless otherwise specified by the manufacturer of aggregometer. Pipette 450 µl of PRP to glass cuvette with a magnetic stir bar. The number of cuvettes used depends on the number of agonists to be tested. Pre-warm the PRP cuvettes in the incubation wells surrounding the heater block for 2-5 minutes. Pipette 500 µl of PPP to another glass cuvette without stir bar and place in the corresponding blank reference well. PRP and autologous PPP should be used to set 0% and 100% light transmission in the aggregometer. Allow to run for 1 minute before adding the agonist. Pipette 50 µl of agonist to bottom of the PRP cuvette without producing bubbles. The volume of agonist added for LTA should be consistent, and never more than 10% of the total sample volume. A know control sample must be run parallel with the patient. Platelet aggregation should be monitored for a minimum of 3 minutes after adding an agonist, and up to 10 minutes for agonists that do not reach maximal aggregation by 3–5 minutes. Repeat the procedure for each agonist.

Notes: If an abnormal response is seen with initial panel of agonists, additional extended panel (Table 35) can be added on along with ATP release by lumiaggregometry (see below). Higher concentrations of agonists (e.g. ADP, epinephrine) should be used if abnormal response is seen at the initial concentration. If no aggregation response is seen with arachidonic acid, add on the agonist, TXA_2 receptor analogue (TRA), U46619. In TXA_2 synthesis defects (COX/TXA_2 synthase deficiency) or Aspirin effect, normal response is seen with TRA. In cases of TXA_2 receptor defect, abnormal responses are seen with both arachidonic acid and TRA. Isolated abnormal response with Epinephrine can be seen in a proportion of healthy subjects. However, this may be the only LTA feature of Quebec platelet syndrome. When studying platelet aggregation as part of a hyperaggregability assessment, ADP and adrenaline are used at lower concentrations to obtain a dose response curve. Concentrations used are: 2 µM, 1 µM, 0.5 µM, and 0.1 µM final concentration in PRP. A spontaneous aggregation is also performed before the rest of the agonists are tested. If the platelets hyperaggregate with 0.5 mg/ml ristocetin, this indicates possible type 2B VWD or platelet type VWD. Check for spontaneous aggregation by monitoring PRP under the same stirring conditions on the aggregometer without adding any agonist to stimulate aggregation. Perform mixing studies to differentiate between the two subtypes. This can be done by two methods. Basic procedure: Mix equal volumes of patient plasma with normal platelets (i.e. 225 µl of patient PPP + 225 µl of control PRP). This mixture is then assayed with ristocetin at 0.5 mg/ml. If agglutination is present, it suggests type 2B VWD. An absent agglutination may be due to platelet type VWD. It is important to note that an absent response may also be due to the additional dilution of PRP.

Alternate procedure: Wash the patient and control platelets three times with PBS-EDTA in 15 ml centrifuge tubes (low centrifugation speed to soft pellet the platelets). Carefully resuspend patient's platelets in normal plasma and normal platelets in patient's plasma. Adjust platelet count to 400 x 10^6 pelleted platelets. (Note that PBS-EDTA is prepared by adding 9 mM di-sodium EDTA to PBS and adjusting the pH to 7.0. PBS used contains 135 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, with a pH of 7.4.) Retest with ristocetin at 0.5 mg/ml. Reactions should fit one of the patterns below.

Table 34. Type 2B VWD and platelet type VWD interpretation

Tube 1	Washed patient platelets + Control PPP	No agglutination	Agglutination
Tube 2	Control washed platelets + patient PPP	Agglutination	No agglutination
	INTERPRETATION	Type 2 B VWD	Platelet type VWD

The 2021 VWD guidelines favor the use of targeted genetic testing for the diagnosis of type 2B and platelet type VWD. However, this may not be feasible for many laboratories due to cost constraints. RIPA mixing study is still retained as one of tests in the diagnostic algorithm according to ISTH.

Interpretation and reporting of results: The platelet aggregation tracing should be evaluated based on presence of shape change, length of lag phase (some agonists like collagen may have a longer lag phase), maximal aggregation (most convenient and commonly used method for reporting results), slope of aggregation (indicates rate of aggregation), visual examination of the aggregation tracings (i.e. disaggregation,

presence of secondary wave—particularly distinct for epinephrine and low doses of ADP). Disaggregation with ADP is particularly striking with inherited P2Y12 defects. PRP prepared in the same way from a healthy normal donor should be processed and run simultaneously with the patient as a check on the reagents. This is especially important if abnormal patient results are obtained, as some agonists are labile, particularly once diluted to working concentrations. Clinical laboratories must establish appropriate reference intervals for each agonist's concentration. Results from healthy normal subjects tested alongside the patient can be used to derive reference ranges. Great caution is required when interpreting platelet aggregation patterns. A number of technical factors may influence the results. Bear in mind that there are a number of important differences between aggregation determined by nephelometry and that occurring in the body. Nevertheless, useful diagnostic information can be obtained, and some examples of aggregation patterns are shown in Table 35.

Table 35. Platelet aggregation results in various disorders

Disorder	ADP	Collagen	Ristocetin 1.25 mg/ ml	Ristocetin 0.5 mg/ ml	Arachadonic Acid	Adrenaline
VWD type 1 and 2A	N	N	A/R**	A	N	N
VWD type 2B	N	N	N	H	N	N
Bernard-Soulier syndrome	N	N	A	A	N	N
Glanzmann's thrombasthenia	A	A	N	A	A	A
Storage pool disease	P/N	R/N	N	A	R/N	P/N
Cyclo-oxygenase defect*	R/N	R	N	A	R/A	R/N

N = Normal; A = Absent; R = Reduced; H = Heightened response; P = Primary wave only
*Or Aspirin effect
**Can be normal in mild VWD type 1

Further investigation of platelet function: If an abnormal aggregation pattern is observed in an individual, it is advisable to repeat the assessment on at least one further occasion to check for consistency of the abnormality. In the presence of abnormal aggregation, further investigation may be useful. These include the measurement of platelet nucleotide content and their release during platelet aggregation. Quantitation of membrane glycoproteins can be performed for the unequivocal diagnosis of Bernard-Soulier syndrome and Glanzmann's thrombasthenia (see below).

ATP release by lumiaggregometry: Lumiaggregometry is the most widely used method to assess platelet dense granule release function. It has the advantage that LTA and ATP release from dense granules can be measured simultaneously. It is based on the principle of luciferin-luciferase reaction. When platelets are activated, dense granules release their stored ATP and ADP. The released ATP reacts with luciferin in the presence of luciferase to give luminescence which can be quantified, relative to an ATP standard. Defective ATP secretion can be seen in both dense granule deficiency and primary secretion defects. Distinction between the two entities requires additional testing, like measurement of total platelet ATP-ADP content by bioluminescent assays, serotonin release assays with radiolabeled ^{14}C-5-HT, mepacrine assay by flow cytometry (see below) or assessment of dense granules by whole mount transmission electron microscopy.

Table 36. Technical factors that influence platelet function

Anticoagulant	1/10th volume of trisodium citrate.
Time	Start tests 30 minutes after preparation of PRP. Complete studies within four hours of blood collection.
Centrifugation	Should be sufficient to remove red cells and white cells, but not large platelets. Should be done at room temperature, not at 4°C. Large platelets can be separated by sedimentation.
Platelet count	Low counts <100 x 10^9/l cause slow, weak responses. High counts >1000 x 10^9/l may show reduced response.
pH	<pH 7.7 inhibits aggregation. >pH 8.0 enhances aggregation.
Mixing speed	<800 rpm shows reduced aggregation. >1200 rpm breaks up platelet clumps.
Hematocrit	>55% shows progressively less aggregation, especially second-phase inhibition due to increased citrate concentration.
Temperature	<35°C shows decreased aggregation with regular doses of all agonists, but increased response to low doses of ADP.
Lipemia	Increased chylomicrons cause reduced aggregation.
Dirty cuvette	May cause apparent spontaneous aggregation.
No stir bar	No response on addition of aggregating agent.
Air bubbles	Rapid, large oscillations of pen prior to aggregation. Also caused by low platelet count.

Differences between in vivo and in vitro conditions for platelet aggregation: With in vitro blood tests, blood is anticoagulated, RBC and WBC are removed, vascular components are not involved, coagulation is not involved, platelet population is selected, products of platelet activation and release are retained, reagents used are unphysiological in composition and dose, platelets are unstable out of the body, and drugs may show more or less marked effects than in vivo.

Platelet Function Testing by Flow Cytometry: Flow cytometry can provide useful information for the diagnosis and classification of platelet function disorders. It can be used for analysis of glycoprotein receptors, platelet function testing after agonist stimulation, measurement of platelet procoagulant function (e.g. annexin V binding) and assessment of alpha and dense granule content. The major advantages of flow cytometry are the requirement of small sample volumes, it can be performed on whole blood, and it is not limited by thrombocytopenia. This becomes particularly useful in pediatric and thrombocytopenic subjects.

Platelet surface glycoprotein analysis by flow cytometry: Glanzmann thrombasthenia, characterized by abnormal or absent αIIbβ3 can be detected with fluorescently labelled GPIIb (CD41) or CDIIIa (CD61) monoclonal antibodies, and Bernard Soulier syndrome, characterized by abnormal or absent GP1b/V/IX can be diagnosed using antibodies directed against GPIb (CD42b) or GPIX (CD42a) antibodies. Additionally, other less common IPFDs affecting the receptors α2β1 (CD49/CD29), GPIV (CD36) or GPVI can also be diagnosed using respective antibodies. Analysis of surface molecules can be done even up to 24 hours from collection. Flow cytometric platelet activation test (PACT)**.** PACT allows for simultaneous measurement of a broad range of different activation markers of platelet function thereby allowing for a more comprehensive analysis of various pathways involved in platelet function. Platelets can be identified using their characteristic forward side scatter (FSC) and side scatter (SSC) properties and preferably by the addition of a specific glycoprotein receptor (e.g. CD41). Some of the commonly used activation markers are: PAC-1 (monoclonal antibody that specifically binds to the activated conformation form of αIIbβ3), CD62P or P-selectin (one of the contents of α-granules, can be used as a marker of α-granule release), and CD63 (can be used as a marker of δ-granule release). Testing can be done using a combination of agonists to target different

pathways, such as ADP, thrombin receptor activating peptide (TRAP), and convulxin. Expression of activation markers on the platelets is measured (using MFI and/or %) at baseline (before adding agonist) and after agonist stimulation. A reduced expression after agonist stimulation suggests a defect/abnormality in the specific pathway. It is important to run a control sample simultaneously similar to LTA.

Ristocetin-induced VWF binding by flow cytometry: The function of the GP1b/V/IX receptor and VWF can also be assessed by flow cytometry by measuring VWF expression on platelets after the addition of normal and low doses of ristocetin. Monoclonal antibody against anti VWF can be used. Mixing studies with patient plasma and control plasma and vice versa at low doses of ristocetin can differentiate between type 2B and platelet type VWD.

Mepacrine uptake and release assay by flow cytometry: The mepacrine uptake and release assay can also be used to assess δ-granule defects. Mepacrine is a fluorescent acridine derivative that binds to platelet adenosine nucleotides with high affinity. After incubation of platelets with mepacrine at 37°C for 30 minutes, it is selectively taken up by dense granules which can be measured by increase in fluorescence by flow cytometry. The release function can also be assessed by measuring mepacrine fluorescence after agonist stimulation (e.g. convulxin or TRAP). Fluorescence is measured at baseline (only platelets), after addition of mepacrine (platelets + mepacrine) and after addition of agonist (platelets + mepacrine + agonist). Uptake and release ratios can be calculated. Decreased uptake and release are seen in dense granule deficiency, while a normal uptake with reduced release is seen in secretion defects.

Platelet procoagulant activity: Upon simultaneous stimulation of platelets with convulxin and thrombin, a subset of platelets expresses anionic procoagulant phospholipid, phosphatidyl serine on the surface of activated platelets. This can be assessed using fluorescently labelled annexin V or lactadherin which binds to phosphatidyl serine. The translocation of procoagulant phospholipids facilitates the assembly of coagulation factors for thrombin generation on the surface of platelets. Flow cytometry allows the detection of impaired (Scott Syndrome) or increased (Stormoken syndrome) procoagulant activity and its associated syndromes.

Table 37a. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of glycoprotein receptors					
Glanzmann thromb-asthenia	ITGA2B, ITGB3	Normal	Absent response to all agonists except agglutination to ristocetin	Flow cytometry identifies GPIIb/ GPIIIa deficiency	Autosomal recessive, severe bleeding
ITGA2B/ ITGB3-related thrombocytopenia	ITGA2B, ITGB3	Macrothrombocytopenia with anisocytosis	Absent/impaired response to all agonists except agglutination to ristocetin	Reduced GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal dominant, mild to moderate bleeding
Bernard Soulier syndrome	GP1BA, GP1BB, GP9	Macrothrombocytopaenia	Absent agglutination response to ristocetin	Flow cytometry identifies GP1b-IX-V deficiency	Autosomal recessive, moderate/severe bleeding
DiGeorge/ Velocardiofacial syndrome/ 22q11.2 deletion syndrome	Del22q11.2	Normal/mildly reduced platelet counts with large platelets.	Normal/variable aggregation (reduced response to Ristocetin in ~30% of cases)	Reduced GP1b/ IX/V expression by flow cytometry	Cleft palate, cardiac defects, abnormal facies, developmental disabilities, immunodeficiency, mild to significant bleeding
Platelet type VWD	Gain of function mutation in GP1BA	Macrothrombocytopenia, platelet clumps	Increased agglutination with low concentration of ristocetin	Reduced high molecular weight VWF multimers (reduced VWF activity)	Autosomal dominant, mild to moderate bleeding
Defects of collagen receptors	GP6 ND	Normal	Isolated reduced aggregation response to collagen	GPVI or α2β1 deficiency by flow cytometry	Autosomal recessive, mild bleeding

Table 37b. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Abnormalities of G-protein-coupled receptors					
TXA_2 (TP) receptor defect	TBXA2R	Normal	Abnormal aggregation with AA and TXA_2 analog (U46619)		Autosomal recessive, mild bleeding diathesis
ADP receptor (P2Y12) defect	P2Y12	Normal	Markedly impaired aggregation response to ADP (only primary wave); reduced response with other agonists can also be seen	Decreased expression of activation markers on agonist stimulation by ADP	Autosomal recessive, mild bleeding after trauma/surgery
Defects of platelet granules (isolated/syndromic)					
Alpha granule defects					
Gray platelet syndrome	NBEAL2	Macrothrombocytopenia; large platelets with bluish grey cytoplasm and absent azurophilic granules	Variable, mild abnormalities; can be normal	Absent/ reduced α granules by EM Decreased/ absent P-selectin expression by flow cytometry/ELISA	Autosomal recessive, mild bleeding, progressive myelofibrosis with splenomegaly
Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome	VPS33B, VIPAS39	Large grey platelets	Variable, mild abnormalities; can be normal	Absent/ reduced α granules by EM P-selectin is reduced in some and normal in others	Flexion contractures, hypotonia, cholestatic jaundice, renal tubular acidosis, failure to thrive, ichthyosis, infection, mild bleeding diathesis
Paris-Trousseau/ Jacobsen syndrome	Del11q23 including FL1	Large platelets, few with fused α-granules	variable	Giant fused α granules in EM, dysmegakaryopoi-esis	Mild bleeding diathesis, developmental delay, cardiac defects, craniofacial anomalies

Table 37c. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Dense granule defects					
Isolated dense granule deficiency/δ-storage pool deficien cy		Normal	Variable. May be decreased to several agonists: ADP, collagen and epinephrine, or normal	Decreased ATP release by lumiaggregometry Reduced platelet ADP and increased ATP/ADP ratio Reduced/absent dense granules by whole mount TEM Reduced CD63 and reduced mepacrine uptake and release by flow cytometry	
Hermansky-Pudlak syndrome	HPS1, AP3B1 (HPS2), HPS3, HPS4, HPS5, HPS6, DNTBP1 (HPS7), BLOC1S3 (HPS8), and BLOC1S6 (HPS9)	Normal	Variable		Oculocutaneous albinism, immunodeficiency, mild bleeding diathesis, pulmonary fibrosis, granulomatous colitis, and neutropenia.
SLFN14-related thrombocytopenia	SLFN14	Macrothrombocytopenia	Variable		
Chediak-Higashi syndrome	LYST	Giant peroxidase positive cytoplasmic granules in leucocytes; lymphohistiocytosis	Variable		Variable oculocutaneous albinism, recurrent life threatening infections, mild bleeding diathesis
Combined α-granule/δ-granule disorders	ND	Pale grey platelets by light microscopy	Variable	Absent/reduced α and δ- granules on TEM, and other features as seen in alpha and dense granule disorders	Mild to moderate bleeding diathesis
Granule secretion defect or primary secretion defect	ND	Normal	Variable	Decreased ATP release by lumiaggregometry, normal mepacrine uptake with reduced mepacrine release by flow cytometry Reduced CD63 expression by flow cytometry; normal alpha and dense granules by EM	Mild to moderate bleeding diathesis.

Table 37d. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of transcription factors					
Familial platelet disorders with associated myeloid malignancy (FPD/ AML)	RUNX1	Thrombocytopenia	Abnormal aggregation to multiple agonists	δ-granule secretion defect	Predisposition to develop MDS and AML
FLI1-related δ granule defect	FLI1	Thrombocytopenia	Reduced aggregation to collagen and TRAP	δ-granule secretion defect	
GATA1- defect	GATA1	Macrothrombocytopenia	Reduced aggregation to collagen and ristocetin	Reduced α-granule content and release	
GFI1B-related defect	GFI1B	Macrothrombocytope-nia, dyserythropoiesis	Normal	Reduced α-granule release	Autosomal dominant
Signal transduction protein defects					
Cytosolic phospholipase A2 defect/ Cyclo-oxygenase deficiency/ TXA_2 synthase deficiency	PLA2G4A/ ND/TBXAS1	Normal	Absent response with AA and normal response with TXA_2		
CalDAG-GEFI defect	RASGRP2	Normal	Markedly reduced/absent response to ADP, Epinephrine, reduced/normal response to Collagen, TRAP and ristocetin	Normal GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal recessive, severe bleeding
Leukocyte adhesion deficiency-III	FERMT3	Leukocytosis	GT like pattern	Normal GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal recessive, severe bacterial infection, poor wound healing, severe bleeding

Table 37e. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of cytoskeletal proteins					
Wiskott Aldrich syndrome (WAS) /X-linked thrombocytopenia	WAS	Microthrombocytopenia		Decreased T-cell subsets, natural killer cell function, decreased α- and δ-granules by TEM	WAS: X-linked inheritance; Eczema, immune deficiency, malignancies and autoimmunity.
MYH9 related disease (formerly known as Sebastian, May-Hegglin, Fechtner, and Epstein syndromes)	MYH9	Macrothrombocytopenia, Dohle like inclusion in neutrophils	Normal		Autosomal dominant; variable association with sensorineural hearing loss, cataracts, and nephritis
ADAP defect	FYB	Microthrombocytopenia		Increased expression of P-selectin and PAC-1 but impaired expression upon activation	
Defects of membrane phospholipids					
Scott syndrome	TMEM16F	Normal	Normal	Reduced Annexin binding by flow cytometry	Autosomal recessive
Stormorken syndrome	STIM1, ORAI1	Anemia Howell Jolly bodies	Normal	Enhanced Annexin V binding and defective PAC-1 expression	Faical dysmorphism, ichtyhosis, myopathy
Enhanced platelet fibrinolytic activity					
Quebec platelet disorder	PLAU (duplication)	Variable thrombocytopenia	Abnormal response with Epinephrine	Excess platelet urokinase plasminogen activator causing proteolysis of α-granule proteins and fibrinolysis	Autosomal dominant, delayed onset bleeding after surgery/trauma, not response to antifibrinolytics but responsive to antifibrinolytics

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