NCL Method ITA-2.2

Analysis of Platelet Aggregation by Light Transmission Aggregometry

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This document describes a procedure for analysis of platelet aggregation (1-6). Platelets are small (~2 μm) anuclear cells obtained by fragmentation of megakaryocytes. Platelets, also known as thrombocytes, play a key role in hemostasis. Abnormal platelet counts and function may lead to either bleeding or thrombosis. Assessing nanoparticle effects on human platelets *in vitro* allows for quick screening of their potential anticoagulant or thrombogenic properties mediated by direct effects on platelets.

2. Principles

Platelet-rich plasma (PRP) is obtained from fresh human whole blood and incubated with either a control or test sample. Platelet Poor Plasma (PPP) is used as a background control. The instrument records change in the light transmission through PRP which occurs when platelets aggregate resulting in a decrease in the sample turbidity. In addition, the instrument allows measuring ATP release indicative of platelet activation.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

- 3.1.1 ChronoLum Reagent (Chrono-Log, 395)
- 3.1.2 ATP (Chrono-Log, 384)
- 3.1.3 Freshly drawn human whole blood anticoagulated with sodium citrate
- 3.1.4 Collagen (Chrono-Log, 385)
- 3.1.5. Epinephrine (Chrono-Log, 393); this reagent is optional
- 3.1.6 Thrombin (Chrono-Log, 386); this reagent is optional
- 3.1.7 ADP (Chrono-Log, 384); this reagent is optional

3.1.8 Arachidonic acid (Chrono-log, 390); this reagent is optional

3.1.9 Ristocetin (Chrono-Log, 396); this reagent is optional

3.1.10 Saline (Hospira, 0409-6138-03)

3.2 Materials

3.2.1 Pipettes covering range 0.05 to 10 mL

3.2.2 Polypropylene tubes, 15 mL

3.2.3 Kim wipes

3.2.4 Silicon-coated stir bars (Chrono-Log, 311)

3.2.5 Glass reaction tubes (Chrono-Log, 312)

3.3 Equipment

3.3.1 Centrifuge capable of operating at 200 x g and 2,500 x g

3.3.2 4 channel model 700 Whole Blood/Optical Lumi-aggregometer (Chrono-Log)

4. Preparation of Plasma, Test Samples and Controls

4.1 Test sample preparation

This assay requires 0.4 mL of nanoparticle solution, at 11X the desired highest test concentration. The nanoparticles should be dissolved/resuspended in saline, or other medium, which does not interfere with platelet aggregation.

The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere (7) and are summarized in Box 1 below.

Box 1. Example Calculation of Nanoparticle concentration for In Vitro Test

In this example, we assume the mouse dose is known to be 123 mg/kg.

$$human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ vitro\ concentration_{human\ maxtrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ \times 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 22 mg/mL will be prepared and diluted 10-fold (2.2 mg/mL), followed by two 1:5 serial dilutions (0.44 and 0.088 mg/mL). When 50 µL of each of these samples is added to the test tube and mixed with 0.45 mL of plasma and 50 µL Chrono-Lum reagent, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Two 50 µL replicates are tested per each sample concentration.

4.2 Plasma preparation

You will need two types of plasma to perform this experiment: platelet rich plasma (PRP), and platelet poor plasma (PPP). Plasma from individual donors can be analyzed separately or pooled together. Pooled plasma is prepared by mixing plasma from at least 2 individual donors. For initial screening experiments we use pooled plasma. Analysis of plasma from individual donors may be needed for mechanistic follow up experiments. Blood is drawn into vacutainer tubes containing sodium citrate as anticoagulant. Estimate the volume of PRP and PPP needed for this

experiment based on the number of test samples. Keep in mind that each 10mL of whole blood produce ~2mL of PRP and ~5mL of PPP. Based on the volume of each type of plasma, divide the vacutainer tubes containing whole blood into two groups. Use one group to make PRP and use the second group to make PPP. Follow the guidance below for centrifugation time and speed used to prepare each type of plasma.

PRP – centrifuge whole blood at 200 x g for 8 minutes, collect plasma and transfer to a fresh tube

PPP – centrifuge whole blood at 2,500 x g for 10 min, collect plasma and transfer to a fresh tube

Important: A) During the blood collection procedure, the first 10 mL of blood should be discarded; this is necessary to avoid platelet stimulation caused by venipuncture. B) PRP must be prepared as soon as possible and no longer than 1 h after blood collection. PRP must be kept at room temperature and should be used within 4 hours. C) Exposure of either blood or PRP to cold temperature (< 20ºC) should be avoided, as it will induce platelet aggregation; likewise, exposure to heat (> 37 °C) will activate platelets and affect the quality of test results.

4.3 Preparation of Controls

4.3.1 Negative Control (saline)

Sterile saline is used as a negative control. Store commercial stock at room temperature. After opening, store the bottle at nominal temperature of 4 °C.

4.3.2 Vehicle Control (relevant to the given nanoparticle)

When nanoparticles are not formulated in saline, the vehicle sample should be tested to estimate the effect of excipients on the platelets. This control is specific to each given nanoparticle sample. Vehicle control should match formulation buffer of the test nanomaterial by both the composition and concentration. Dilute this sample the same way you dilute the test nanomaterials. If the vehicle is saline this control can be skipped.

4.3.3 Positive Control

Several reagents listed below can be used as positive controls. The default positive control for this assay is collagen.

ADP - each vial contains 2.5 mg of lyophilized adenosine diphosphate. Reconstitute according to the manufacturer's instructions, use freshly prepared.

Collagen - each vial contains 1 mg of native collagen fibrils (type I) from equine tendons suspended in isotonic glucose solution of pH 2.7. After opening store at 4 °C and use the content of the vial within 1 week.

Thrombin - each vial contains a minimum of 10 units of lyophilized thrombin from human plasma. Reconstitute according to the manufacturer's instructions, use freshly prepared.

Arachidonic Acid - each vial contains a minimum of 10 mg of arachidonic acid. Included is a vial containing 100 mg bovine albumin, fraction V powder, 96% to 99% purity. Reconstitute, prepare and store the reagent according to the manufacturer's instruction.

Ristocetin - each vial contains 62.5 mg of stabilized freeze dried ristocetin. Reconstitute and prepare the reagent according to the manufacturer's instruction.

Epinephrine - lyophilized preparation of 1-epinephrine bitartarate with stabilizers. Reconstitute, prepare and store the reagent according to the manufacturer's instruction.

4.4 Preparation of Chrono-Lum reagent

Chrono-Lum reagent is used for measurement of ATP release. Each commercially supplied vial contains 0.2 mg luciferin, 22,000 units d-luciferase plus magnesium sulphate, human serum albumin, stabilizers and buffer. Kit includes 4 vials of CHRONO- LUME plus a vial of lyophilized adenosine 5' triphosphate for use as an ATP standard. Keep commercial stock frozen at -20 °C. After thawing use fresh or re-freeze and use within 30 days.

5. Experimental Procedure

Training to operate the 4-channel model 700 Whole Blood/Optical Lumi-aggregometer (Chrono-Log) is needed prior to performing this assay. The instructions provided below may be insufficient to a user unfamiliar with this instrument.

5.1 Instrument Setup

5.1.1 Before starting calibration set the instrument Gain to 0.005, temperature to 37°C and RPM to 1200
5.1.2 Run ATP standard: hit select until @ Gain setting and Set to 0.005
5.1.3 Insert test tubes into the corresponding slot on the instrument warming section. Add stir bar to 4 tubes (tip: count out tubes needed for experiment and place stir bar in each tube)
5.1.4 Wipe tubes off with Kim Wipes (tip: wipe off as many tubes as can fit in the warming chamber located next to the incubation chambers)
5.1.5. Transfer tubes from warming section into incubation chamber and insert the tubes into PRP slots in each chamber on Chrono-Log instrument
5.1.6. Add 450 µL of PRP to these 4 tubes
5.1.7. Warm tubes for 5 minutes (In the AggroLink software you will need to label samples as ATP standard. There is an automatic scroll down ATP standard operation/ click on this setting to make all labels read the same. Some software manipulation and editing are needed before continuing with PRP set up.)
5.1.8. Hit Run New in software
5.1.9. Add 50 µL of Chrono-LUME reagent to these 4 tubes
5.1.10. Hit OK in software
5.1.11. Warm tubes for 2 minutes
5.1.12. Add 5 µL of ATP standard (when a sample volume to be pipetted is 10 µL or less gently wipe the pipette tip with a kim wipe but avoid touching the bottom of the tip.)
5.1.13. Close the chamber
5.1.14. Hit Set (on the instrument) until spikes between 20 & 70% appear on the graph
Important: A) PRP is accepted if the luminescent line spikes up between 30% – 80% of whole gain (which is equivalent to 20% - 70% visualized on graph) and then drops back down; B) Do not exceed this gain range, if this happens repeat this step
5.1.15. Repeat for all channels
5.1.16. Hit the stop button
5.1.17. Save the ATP standard by hitting the save button in the AggroLink software
5.1.18. Change instrument select until LCD screen has everything displayed
5.1.19. Hit the RunNext button in the AggroLink Software

5.2 Optical Baseline Setup

5.2.1. Add stir bar to 4 tubes
5.2.2. Wipe tubes off with Kim Wipes
5.2.3. Place 4 tubes in PRP slot in each chamber on Chrono-LOG instrument
5.2.4. Add 450 µL PRP to those tubes with stir bars
5.2.5. Add 450 µL of PPP into a 5th tube (no stir bar)
5.2.6. Make sure that the metallic switch on the front of the Chrono-LOG instrument is switched to 1 for both machines. This links both instruments to analyze the PPP in the first chamber to the rest of the chambers eliminating the need to add PPP to each chamber.
5.2.7. Place PPP tube in the PPP slot on Chrono-LOG instrument in first chamber
5.2.8. Warm tubes for 5 minutes (This step is not necessary if tubes are pre-warmed with PRP in the warming tubes located near the chambers)
5.2.9. Add 50 µL of Chrono-LUME reagent to tubes in step 4
5.2.10. Warm tubes for 2 minutes (In the AggroLink software you will need to label samples. There is an automatic scroll down window, choose a preset sample or create one
5.2.11. Press (hold) baseline button on the front of the Chrono-LOG instrument until the illumenescent line reaches 0%.
5.2.12. Hit stop button in software
5.2.13. Save in AggroLink software
5.2.14. Hit Run next on software when ready to run samples

Note: there is no need to place new tubes for sample run. PC, NC, or some nanoparticle samples can be added to existing tubes and run against the PPP tube in the PPP slot throughout the experiment. However, when nanoparticles are turbid or aggregate in PPP to create some turbidity which will interfere with light transmission, a PPP plus given nanoparticle at a given concentration should be used as baseline.

Important: *when nanoparticle optical properties are expected to interfere with this assay and PPP+NP is used for optical baseline in each test sample, one need to place a switch on the side of the instrument to position 2,3 and 4 for chambers 2,3,4 respectively. This is important for the instrument to evaluate PRP sample treated with the test nanoparticle to a corresponding PPP+NP sample. If the switch stays in the position described in step 5.2.6 all test nanoparticle samples will be analyzed against plain PPP.*

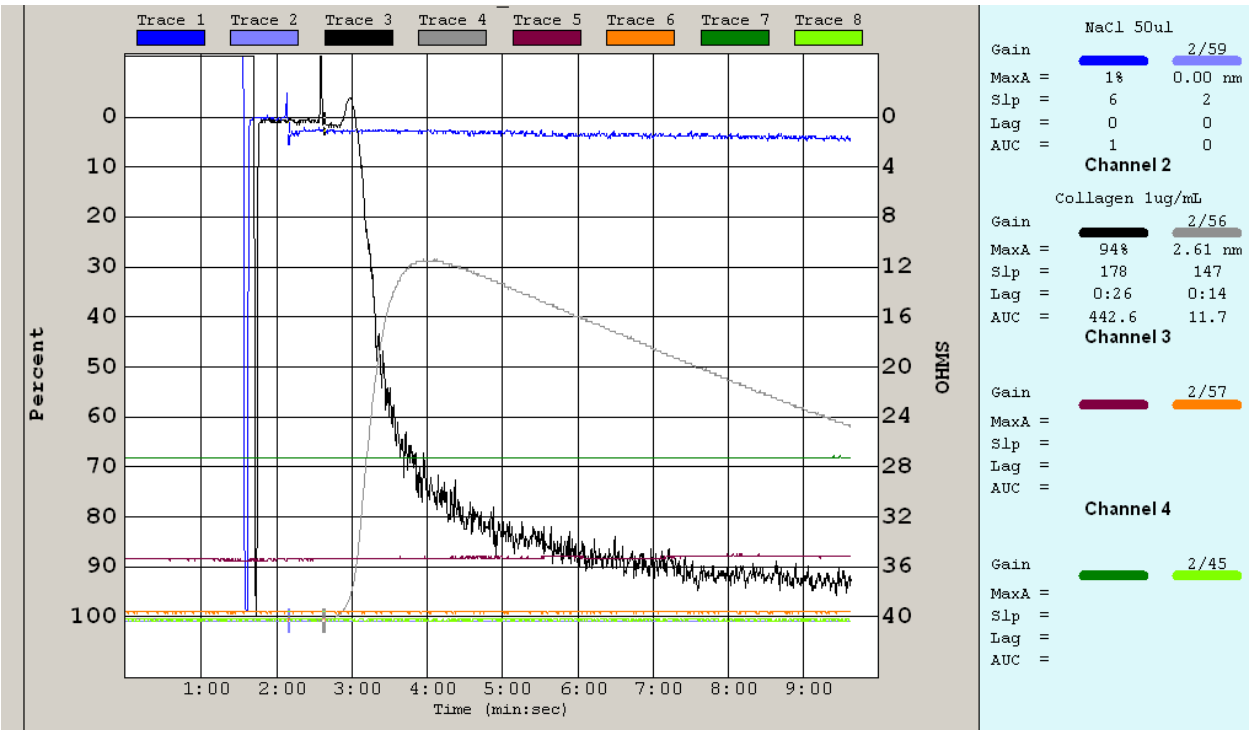
5.3. Analyzing Test-Samples

5.3.1. Add stir bars to 4 tubes
5.3.2. Wipe tubes off with Kim Wipes
5.3.4. Add 450 µL of PRP to these 4 tubes (PPP tube should still be in instrument)
5.3.5. Place tubes in PRP slots in each chamber on Chrono-Log instrument
5.3.6. Warm tubes for 5 minutes (This step is not necessary if tubes are pre-warmed with PRP in the warming tubes located near the chambers)
5.3.7. Add 50 µL of Chrono-LUME reagent to these 4 tubes
5.3.8. Warm tubes for 2 minutes (in this waiting time please set software to sample setting and edit each sample)
5.3.9. Hit ok in software
5.3.10. Add 50 µL of test-sample
5.3.11. Press Trace 1,
5.3.12. Repeat for Trace 3, Trace 5 and Trace 7 respectively.
5.3.13. Close chamber
5.3.14. Run for 6 minutes (tip: during this waiting time place PRP in tubes to pre-warm for next run)

Note: sometimes you may need to set the baseline for each sample run

6. Data Analysis and Calculations

The instrument software calculates area under the curve (AUC) for each test sample. Please see the image below for example of collagen-induced platelet aggregation. The top curve (black color) refers to the platelet aggregation and the corresponding AUC value for this curve is 442.6. The bottom curve (grey) refers to ATP release and is indicative of platelet activation; the corresponding AUC value is 11.7.



AUC values from replicate samples are analyzed to calculate mean value (Mean), standard deviation (SD) and % CV ((SD/Mean) *100%) to assess precision.

One may compare AUC or calculate percent platelet aggregation according to the following formula**:** % Platelet Aggregation = (AUC test sample/ AUC positive control) *100%
It is assumed that positive control platelet aggregation in this case is 100%.

AUC values for ATP release are analyzed to confirm platelet activation. In most traditional cases platelet aggregation data correlates with platelet activation data. In rare cases a discrepancy between aggregation and activation may occur in the samples used to study nanoparticle effect on PC-induced aggregation. Such data is suggestive of a drug or a nanoparticle effect on intracellular pathways leading to platelet aggregation. If this is the case one should consider follow up studies using ADP, epinephrine, thrombin and other reagents listed as optional in section 3.1.

7. Acceptance Criteria

7.1 %CV for each control and test sample should be within 25%.

7.2 If both replicates of positive control or negative control fail to meet acceptance criterion described in 7.1, the run should be repeated.

8. References

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8. Chrono-Log User manual

9. Abbreviations

API	active pharmaceutical ingredient
CV	coefficient of variation

PRP	platelet rich plasma
PPP	platelet poor plasma
SD	standard deviation