



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 measures ATP release, which can be indicative of platelet activation.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. ChronoLum Reagent (Chrono-Log, 395)
2. ATP (Chrono-Log, 384)
3. Freshly drawn human whole blood anticoagulated with sodium citrate
4. Collagen (Chrono-Log, 385)
5. Epinephrine (Chrono-Log, 393); this reagent is optional
6. Thrombin (Chrono-Log, 386); this reagent is optional
7. ADP (Chrono-Log, 384); this reagent is optional
8. Arachidonic acid (Chrono-log, 390); this reagent is optional
9. Ristocetin (Chrono-Log, 396); this reagent is optional
10. Saline (Hospira, 0409-6138-03)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Polypropylene tubes, 15 mL
3. Kimwipes
4. Silicon-coated stir bars (Chrono-Log, 311)
5. Glass reaction tubes (Chrono-Log, 312)

3.3 Equipment

1. Centrifuge, 200xg and 2,500xg
2. Four channel model 700 Whole Blood/Optical Lumi-aggregometer (Chrono-Log)

4. Preparation of Plasma, Test Samples and Controls

4.1 Test Samples

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

In vitro test concentrations are based on the calculated 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 have been reviewed elsewhere [7] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 22 mg/mL is prepared. This sample is then 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 sample dilutions 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 this assay

are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Two 50 µL replicates are tested per each sample concentration.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

In this example, we are assuming a known efficacious mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

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

The blood volume of a human is approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

4.2 Plasma preparation

Two types of plasma will be needed for 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 two individual donors. Pooled plasma is typically used for initial screening experiments. Analysis of plasma from individual donors may be needed for mechanistic follow up experiments.

Blood is drawn into vacutainer tubes containing sodium citrate as an anticoagulant. Estimate the volume of PRP and PPP needed for this experiment based on the number of test samples. Keep in mind that each 10 mL of whole blood produces approximately 2 mL of PRP and 5 mL 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 the second group to make PPP. Follow the guidance below for centrifugation times and speeds for preparation of each type of plasma.

PRP: Centrifuge whole blood at 200xg for 8 minutes, collect plasma and transfer to a fresh tube.

PPP: Centrifuge whole blood at 2,500xg for 10 minutes, 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 hr 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 temperatures (< 20°C) 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 Controls

4.3.1 Negative Control (Saline)

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

4.3.2 Vehicle Control (specific per given nanoparticle)

When nanoparticles are not formulated in saline or PBS, the vehicle should also be tested to estimate the effect of excipients on the platelet aggregation. This control is specific to each given nanoparticle sample. Vehicle control should match formulation buffer of the test nanomaterial by both composition and concentration. This sample should be diluted to the same concentration as the test nanomaterials. If the vehicle is saline or PBS, this control can be skipped.

4.3.3 Positive Control

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

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

ADP: Each vial contains 2.5 mg of lyophilized adenosine diphosphate.

Reconstitute according to the manufacturer's instructions. Use freshly prepared.

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 instructions.

Ristocetin: Each vial contains 62.5 mg of stabilized freeze dried ristocetin. Reconstitute and prepare the reagent according to the manufacturer's instructions.

Epinephrine: Lyophilized preparation of 1-epinephrine bitartrate with stabilizers. Reconstitute, prepare and store the reagent according to the manufacturer's instructions.

4.4 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. The kit includes 4 vials of CHRONO-LUME plus a vial of lyophilized adenosine 5' triphosphate for use as an ATP standard. Keep the 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

1. Before starting calibration, set the instrument Gain to 0.005, temperature to 37°C, and RPM to 1200.
2. Run ATP standard: Hit select until @ Gain setting and Set to 0.005.
3. Insert test tubes into the corresponding slot on the instrument warming section. Add stir bar to four tubes. (Tip: count out tubes needed for experiment and place stir bar in each tube.)

4. Wipe tubes off with Kimwipes. (Tip: wipe off as many tubes as can fit in the warming chamber located next to the incubation chambers.)
5. Transfer tubes from warming section into incubation chamber and insert the tubes into PRP slots in each chamber on Chrono-Log instrument.
6. Add 450 μL of PRP to these four tubes.
7. Warm tubes for five 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 is needed before continuing with PRP set up.).
8. Hit Run New in software.
9. Add 50 μL of Chrono-LUME reagent to these four tubes.
10. Hit OK in software.
11. Warm tubes for 2 minutes.
12. Add 5 μL of ATP standard. When a sample volume to be pipetted is ≤ 10 μL gently wipe the pipette tip with a Kimwipe but avoid touching the bottom of the tip.
13. Close the chamber.
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.
15. Repeat for all channels.
16. Hit the Stop button.
17. Save the ATP standard by hitting the save button in the AggroLink software.
18. Change instrument select until LCD screen has everything displayed.
19. Hit the Run Next button in the AggroLink Software.

5.2 Optical Baseline Setup

1. Add stir bar to four tubes.
2. Wipe tubes off with Kimwipes.
3. Place four tubes in PRP slot in each chamber on Chrono-LOG instrument.
4. Add 450 μL PRP to those tubes with stir bars.

5. Add 450 μL of PPP into a fifth tube (no stir bar).
6. Make sure 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.
7. Place PPP tube in the PPP slot on Chrono-LOG instrument in first chamber.
8. Warm tubes for five minutes. (This step is not necessary if tubes are pre-warmed with PRP in the warming tubes located near the chambers.)
9. Add 50 μL of Chrono-LUME reagent to tubes in step 4.
10. Warm tubes for two 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
11. Press (hold) baseline button on the front of the Chrono-LOG instrument until the illuminescient line reaches 0%.
12. Hit Stop button in software.
13. Save in AggroLink software.
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 interferes with light transmission, a PPP plus 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, place switch on the side of the instrument to position 2, 3 and 4 for chambers 2, 3, and 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 1 all test nanoparticle samples will be analyzed against plain PPP.

5.3 Analyzing Test-Samples

1. Add stir bars to four tubes.
2. Wipe tubes off with Kimwipes.
3. Add 450 μ L of PRP to these four tubes (PPP tube should still be in instrument).
4. Place tubes in PRP slots in each chamber on Chrono-Log instrument.
5. 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.)
6. Add 50 μ L of Chrono-LUME reagent to these four tubes.
7. Warm tubes for two minutes. During this time set software to sample setting and edit each sample.
8. Hit OK in software.
9. Add 50 μ L of test sample.
10. Press Trace 1.
11. Repeat for Trace 3, Trace 5 and Trace 7, respectively.
12. Close chamber.
13. Run for six minutes (Tip: during this time place PRP in tubes to pre-warm for next run.)

Note: 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 Figure 1 for an example of collagen-induced platelet aggregation. The top curve (black) 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.

1. AUC values from replicate samples are analyzed to calculate mean value (Mean), standard deviation (SD), and %CV $[(SD/Mean)*100\%]$ to assess precision.
2. One may compare AUC or calculate percent platelet aggregation according to the following formula:

$$\% \text{ Platelet Aggregation} = (\text{AUC test sample} / \text{AUC positive control}) * 100\%$$

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 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 nanoparticle effect on intracellular pathways leading to platelet aggregation. If this case, one should consider follow up studies using ADP, epinephrine, thrombin or other reagents listed as optional in section 3.1.

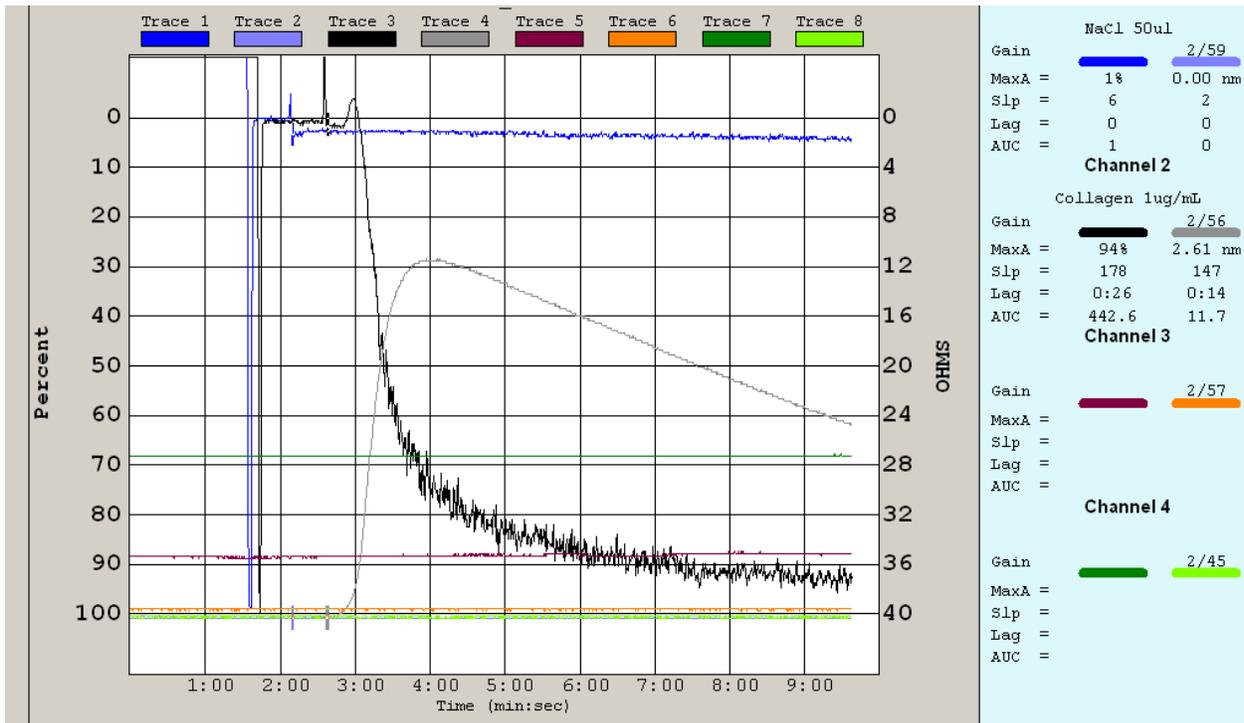


Figure 1. Example of collagen-induced platelet aggregation.

7. Acceptance Criteria

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

CV	coefficient of variation
PBS	phosphate buffered saline
PRP	platelet rich plasma
PPP	platelet poor plasma
SD	standard deviation