NCL Method ITA-2.1

Analysis of Platelet Aggregation by Cell Counting

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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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and

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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. Following incubation, PRP is analyzed using the Z2 particle count and size analyzer to determine the number of active platelets. Percent aggregation is calculated by comparing the number of single (unaggregated) platelets in the test sample to the number of single (unaggregated) platelets in the control baseline sample.

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 Calibration standard 5 μm (Beckman Coulter, 6602794)
- 3.1.2 Isoton II diluent (Beckman Coulter, 8320312)
- 3.1.3 Coulter Clenz solution (Beckman Coulter, 8546931)
- 3.1.4 Freshly drawn human whole blood anticoagulated with sodium citrate
- 3.1.5 Collagen (Helena Laboratories, 5368)
- 3.1.6 RPMI-1640 Cell Culture Media (GE Life Sciences, Hyclone, SH3060501)
- 3.1.7 PBS, (GE Life Sciences, SH30256.01)

3.2 Materials

3.2.1 Pipettes covering the range 0.05 to 10 mL

3.2.2 Polypropylene tubes, 15 mL

3.2.3 Plastic beakers

3.2.4 Blood cell counter vials with snap caps (VWR, 14310-684)

3.2.5 50 µm aperture tube (Beckman Coulter, 8320517)

3.3 Equipment

3.3.1 Water bath set at 37ºC

3.3.2 Centrifuge capable of operating at 200xg, 2,500xg and 18,000xg

3.3.3 Z2 particle count and size analyzer (Beckman Coulter)

4. Preparation of Plasma, Test Samples and Controls

4.1 Test Sample Preparation

This assay requires 0.4 mL of nanoparticle solution, at 5X the highest test concentration. The nanoparticles should be dissolved/resuspended in RPMI, 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.

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 10 mg/mL will be prepared and diluted 10-fold (1 mg/mL), followed by two 1:5 serial dilutions (0.2 and 0.04 mg/mL). When 25 µL of each of these samples is added to the test tube and mixed with 0.1 mL of

plasma, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Three 25 µL replicates are tested per each sample concentration.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

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

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

Blood volume constitutes 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 blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

4.2 Plasma Preparation

You will need three types of plasma to perform this experiment: platelet-rich plasma (PRP), platelet-poor plasma (PPP) and platelet-free plasma (PFP). 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 PFP needed for this experiment based on the number of test samples. Keep in mind that each 10 mL of whole blood produces ~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 use the second group to make PPP, which is needed to produce PFP. Follow the guidance below for centrifugation time and speed used to prepare 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 min, collect plasma and transfer to a fresh tube.

PFP – centrifuge PPP at 18,000xg for 5 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 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 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 (PBS)

Sterile Ca^{2+}/Mg^{2+}-free PBS is used as a negative control. Store at room temperature for up to 6 months.

4.3.2 Vehicle Control (relevant to each given nanoparticle)

When nanoparticles are not formulated in saline or PBS, the vehicle sample should 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 the composition and concentration. Dilute this sample the same way you dilute the test nanomaterials. If the vehicle is PBS this control can be skipped.

4.3.3 Positive Control (collagen)

Collagen is provided as a solution with a final concentration 100 µg/mL contained in the sealed glass vials. Keep it at a nominal temperature of 4°C. After opening, the content of the vial should be used within 4 weeks.

5. Experimental Procedure

5.1 Prepare the Z2 instrument as described in the owner's manual [8]. Pre-warm all racks and tubes to 37ºC.

5.2 Prepare PRP, PPP and PFP as described in step 4.2, then proceed to next step.

5.3 *Part A (Nanoparticle Ability to Induce Platelet Aggregation)*

In a microcentrifuge tube, combine: 1) 100 µL PRP with 25 µL test material; 2) 100 µL PRP with 25 µL of positive control (collagen); 3) 100 µL PRP with 25 µL of negative control (PBS) and 4) 100 µL PRP with 25 µL of vehicle control (a buffer or media used to formulate nanoparticle; if nanoparticle is formulated in PBS, this sample can be skipped). Prepare three replicates for each sample.

Part B (Nanoparticle Ability to Interfere with Collagen-Induced Platelet Aggregation)

In a separate set of tubes combine: 1) 100 µL of PRP with 50 µL of negative control (PBS); 2) 100 µL of PRP with 25 µL of positive control (collagen) and 25 µL of RPMI; 3) 100 µL of PRP with 25 µL of positive control (collagen) and 25 µL of test nanomaterial; 4) 100 µL PRP with 25 µL of vehicle control (a buffer or media used to formulate nanoparticle; if nanoparticle is formulated in PBS, this sample can be skipped) and 25 µL of RPMI. Prepare three replicates for each test combination.

Note: The final concentration of nanoparticles in this case is slightly (1.2 times) lower than that tested in part A. If there is a reason to expect that this difference will affect the test results, adjust the concentration of the stock nanoparticles accordingly.

Part C (Assessment of Nanoparticle Interference with the Assay)

Prepare 1 control tube, by combining 100 µL of platelet free plasma (PFP) and 25 µL of nanoparticle solution. *If nanoparticles aggregate to a micron size particulates, they either create artificially high number of single platelet count (if the aggregates resemble platelet size and pass the aperture) or will not pass*

through the aperture and prevent accurate counting of single platelets, resulting in false-negative or false-positive result, respectively.

5.4 Briefly vortex all samples to mix ingredients and incubate for 15 min at a nominal temperature of 37ºC.

5.5 Add 10 mL of Isoton II diluent into a blood cell counter vial. Prepare two (2) vials for each sample replicate. Each replicate will be diluted into two Isoton II containing vials and a platelet account will be obtained using Z2 counter. The mean response will then be calculated for each replicate.

5.6 Add 20 µL of PRP treated with positive control, negative control, vehicle control (if applicable) or test-nanomaterial prepared in step 5.3 to the Isoton II containing vials from step 5.5. Cover vials and gently invert them to mix diluted samples. Proceed with platelet count determination using the Z2 counter immediately.

Notes:

1) *Dilutions of tested samples and controls should be performed ex tempore. Counts should be performed within two (2) hours after removing from the incubator. When planning the experiment, consider that analysis of 1 nanoparticle sample with all controls takes approximately 1 hr. If the nanoparticle interferes with the assay and results in clogging of the aperture, additional time will be needed to clean the instrument and continue counts. This is not a high throughput screening assay. Plan your time accordingly.*
2) *Perform platelet count of the blank PRP used for the experiment in the beginning and the end of the run to confirm that quality of plasma is not affected by storage and handling. Normal platelet count in human plasma should be between 125-690 x 10^9/L (8). Average platelet count in our experiments is between 300-450 x 10^9/L.*

6. Calculations and Data Interpretation

The following parameters should be calculated for each control and test sample:

6.1 Percent Coefficient of Variation

$$\%CV = \frac{standard\ deviation}{mean} * 100\%$$

6.2 Platelet Count

$$\frac{5 * Instrument\ count\ value}{100} = \#platelets\ x\ 10^9/L$$

6.3 Percent Platelet Aggregation

$$\%Platelet\ Aggregation = \frac{Platelet\ Count_{negative\ control} - Platelet\ Count_{test\ sample}}{Platelet\ Count_{negative\ control}} * 100\%$$

6.4 Positive Sample

Assay threshold is 20%. Percent platelet aggregation values above 20% are considered positive, i.e. test material induces platelet aggregation.

6.5 Inhibition of collagen-induced platelet aggregation

There is no formal guidance on what degree of inhibition is considered significant. Apply scientific judgement to interpret results from the part B of the study. Statistically significant inhibition does not necessarily mean it is physiologically relevant. If an inhibition is observed one should consider a relevant follow-up *in vivo* study to verify *in vitro* findings.

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.

7.3 Within the acceptable run, if two of three replicates of unknown sample fail to meet acceptance criterion described in 7.1, this unknown sample should be re-analyzed.

8. References

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9. Abbreviations

CV	coefficient of variation
PBS	phosphate buffered saline
PRP	platelet rich plasma
PPP	platelet poor plasma
PFP	platelet free plasma
RPMI	Roswell Park Memorial Institute
SD	standard deviation