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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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; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. Calibration standard 5 µm (Beckman Coulter, 6602794)
2. Isoton II diluent (Beckman Coulter, 8320312)
3. Coulter Clenz solution (Beckman Coulter, 8546931)
4. Freshly drawn human whole blood anticoagulated with sodium citrate
5. Collagen (Helena Laboratories, 5368)
6. RPMI-1640 Cell Culture Media (GE Life Sciences, Hyclone, SH3060501)
7. PBS, (GE Life Sciences, SH30256.01)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Polypropylene tubes, 15 mL
3. Plastic beakers

4. Blood cell counter vials with snap caps (VWR, 14310-684)
5. 50 µm aperture tube (Beckman Coulter, 8320517)

3.3 Equipment

1. Water bath, 37ºC
2. Centrifuge, 200x*g*, 2,500x*g*, and 18,000x*g*
3. Z2 particle count and size analyzer (Beckman Coulter)

4. Preparation of Plasma, Test Samples and Controls

4.1 Test Samples

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.

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 10 mg/mL is prepared. This sample is then 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 sample dilutions is added to the test tube and mixed with 0.1 mL of, the final nanoparticle concentrations tested in this 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 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:

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

4.2 Plasma Preparation

Three types of plasma will be needed for 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 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 PFP 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, which is needed to produce PFP. Follow the guidance below for centrifugation times and speeds for preparation of each type of plasma.

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

PPP: Centrifuge whole blood at 2,500x*g* for 10 minutes, collect plasma and transfer to a fresh tube.

PFP: Centrifuge PPP at 18,000 x g 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 temperatures (< 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. Controls

4.3.1 Negative Control (PBS)

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

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 PBS or saline, 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 sealed glass vials. Maintain at a nominal temperature of 4°C. After opening, the contents of the vial should be used within 4 weeks.

5. Experimental Procedure

1. Prepare the Z2 instrument as described in the owner's manual [8]. Pre-warm all racks and tubes to 37ºC.
2. Prepare PRP, PPP and PFP as described in step 4.2, then proceed to next step.

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 (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 (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 lower (1.2 times) than that tested in part A. If this difference is expected to affect the test results, adjust the concentration of the stock nanoparticles accordingly.

   Part C – Assessment of Nanoparticle Interference with the Assay

   Prepare one control tube, by combining 100 µL of PFP and 25 µL of nanoparticle solution. If nanoparticles aggregate to micron size particulates, they either create artificially high number of single platelets (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.
4. Briefly vortex all samples to mix ingredients, and incubate for 15 min at a nominal temperature of 37ºC.
5. Add 10 mL of Isoton II diluent into a blood cell counter vial. Prepare two vials for each sample replicate. Each replicate will be diluted into two Isoton II containing vials and a platelet count will be obtained using Z2 counter. The mean response will then be calculated for each replicate.
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.

Note: Dilutions of tested samples and controls should be performed ex tempore. Counts should be performed within two hours after removing from the incubator. When planning the experiment take into account that analysis of one nanoparticle sample with all controls takes approximately one hour. If the nanoparticle interferes with the assay and clogs the aperture, additional time will be needed to clean the instrument and continue counts. This is not a high throughput screening assay.

Note: 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]. The average platelet count in our experience has been 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:

$$\frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$$

6.2 Platelet Count:

$$\frac{5 \text{ x Instrument Count Value}}{100} = \text{Number of platelets x } 10^9/\text{L}$$

6.3 Percent Platelet Aggregation:

$$\frac{(\text{Platelet Count}_{\text{negative control}} - \text{Platelet Count}_{\text{test sample}})}{\text{Platelet Count}_{\text{negative control}}} \times 100\%$$

6.4 Positive Sample

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 part B of the study. Statistically significant inhibition does not necessarily mean it will be physiologically relevant. If an inhibition is observed one should consider relevant follow up in vivo studies to verify in vitro findings.

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.
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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7. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013;172(2):456-66

8. Beckman Coulter Z series User manual # 991 4591-D, section A8.4.2.

9. Abbreviations

CV	coefficient of variation
PBS	phosphate buffered saline
PRP	platelet rich plasma
PPP	platelet poor plasma
PFP	platelet free plasma