



## **NCL Method ITA-17**

### **In Vitro Induction of Leukocyte Procoagulant Activity by Nanoparticles**

**Nanotechnology Characterization Laboratory**  
Frederick National Laboratory for Cancer Research  
Leidos Biomedical Research, Inc.  
Frederick, MD 21702  
(301) 846-6939  
[ncl@mail.nih.gov](mailto:ncl@mail.nih.gov)<http://www.ncl.cancer.gov>

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.

Method written by:

Timothy M. Potter, B.S.

Barry W. Neun, B.S.

Jamie Rodriguez, B.S.

Anna N. Ilinskaya, Ph.D.

Marina Dobrovolskaia, Ph.D.

## 1. Introduction

Leukocyte procoagulant activity (PCA) is accepted as an important component in the onset of disseminated intravascular coagulation (DIC). DIC is common in acute promyelocytic leukemia (APL) and other forms of cancer [1-5]. DIC in cancer patients is often observed after initiation of therapy with cytotoxic oncology drugs that act by altering DNA replication (e.g., doxorubicin, daunorubicin, and vincristin) [3, 6]. Cytotoxic oncology drugs acting by other mechanisms, (e.g., methotrexate and paclitaxel) do not induce DIC [7-8]. DIC is also a common complication in sepsis [9-12]. Cytotoxic drugs (doxorubicin, vincristin, and daunorubicin) and endotoxin have previously been shown to induce leukocyte PCA in vitro and DIC in vivo [13-21]. In vitro, doxorubicin-induced leukocyte PCA has previously been linked to DIC in vivo [3].

## 2. Principles

This document describes a protocol for assessing the ability of a nanoparticle formulation to induce leukocyte procoagulant activity. This protocol includes two cell models: normal leukocytes, represented by PBMC isolated from healthy blood donor volunteers, and acute promyelocytic leukemia cells, represented by the HL-60 cell line. Briefly, cells are treated with nanoparticles, and then undergo a wash step to remove excess particles. Isolated cells are then used to initiate plasma coagulation, which is measured using coagulometer (following NCL protocol ITA-12) for analysis of prothrombin time.

## 3. Reagents, Materials, Cell Lines, 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. Human blood from at least 3 donors, anti-coagulated with Li-heparin for PBMC isolation and anti-coagulated with Na-citrate for plasma coagulation test
2. HL-60 promyelocytic cells (ATCC, CCL-240)
3. Ficoll-Paque Plus (GE Life Sciences, 17-1440-02)
4. Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
5. Ultrapure LPS (InvivoGen, tlr1-pek1ps)

6. Doxorubicin hydrochloride (Bedford Labs, NIH DVR pharmacy)
7. Fetal bovine serum (FBS) (GE Life Sciences, Hyclone , SH30070.03)
8. RPMI-1640 (Invitrogen, 11835-055)
9. Hanks balanced salt solution (HBSS) (Invitrogen, 24020-117)
10. Pen/Strep solution (Invitrogen, 15140-148)
11.  $\beta$ -mercaptoethanol (Sigma, M7522)
12. L-glutamine (Hyclone, SH30034.01)
13. Trypan Blue solution (Invitrogen, 15250-061)
14. Neoplastine CI (Diagnostica Stago, 00666)
15. CoagControl N+ABN (Diagnostica Stago, 00676)
16. Calcium ionophore (Sigma, C7522)

### 3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. 6-well plates
3. Polypropylene tubes, 50 and 15 mL
4. Finntip, 5 mL (ThermoScientific, 9404180)
5. 4-well cuvettes (Diagnostica Stago, 38876)
6. Polystyrene tubes, 5 mL (Falcon, 352058)
7. Scintillation vials, 20 mL (Research Products International, FS74511-20)

### 3.3 Equipment

1. Centrifuge
2. Refrigerator, 2-8°C
3. Freezer, -20°C
4. Cell culture incubator with 5% CO<sub>2</sub> and 95% humidity.
5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemocytometer
9. Coagulometer STart4 (Diagnostica Stago) or comparable

#### 4. Reagent and Control Preparation

##### 4.1 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature or overnight at 2-8°C, and allow to equilibrate to room temperature. Incubate 30 minutes in a 56°C water bath, mixing every 5 minutes. Fifty (50) mL single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.

##### 4.2 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents: heat inactivated FBS (10% for PBMC and 20% for HL-60), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Store at 2-8°C, protected from light, no longer than one month. Before use, warm in a 37°C water bath.

##### 4.3 Lipopolysaccharide, 1 mg/mL (LPS, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS to the vial and vortex to mix. Store daily use aliquots at a nominal temperature of -20°C.

##### 4.4 Doxorubicin, Stock

Doxorubicin (adriamycin) is provided as a solution at 2 mg/mL and stored at 2-8°C.

##### 4.5 Positive Controls

###### Positive Control for PBMC

Dilute stock LPS solution in cell culture medium to a final concentration of 1 µg/mL. Store at room temperature. Discard unused portion after experiment. Alternatively, calcium ionophore at a final concentration of 500 ng/mL can be used.

###### Positive Control for HL-60 Cells

Dilute doxorubicin stock solution in cell culture medium to a final concentration of 50 µg/mL. Store at room temperature. Discard unused portion after experiment. Alternatively, calcium ionophore at a final concentration of 50 ng/mL can be used.

##### 4.6 Negative Control

Use PBS as the negative control. Process it the same way as the study samples.

##### 4.7 Buffer A

Prepare Buffer A by dissolving NaCl to a final concentration of 150 mM and CaCl<sub>2</sub> to a final concentration of 6.6 mM in 20 mM HEPES, pH 7.4.

##### 4.8 Coagulation Controls (Coag N+ABN)

Reconstitute lyophilized control plasmas with 1 mL of distilled water. Allow the solutions to stand at room temperature 30 min prior to use. Mix thoroughly before use. Keep unused portion refrigerated and use within 48 hr after reconstitution. These plasma samples are used as instrument controls.

#### 4.9 Plasma for Coagulation Test

Use freshly collected whole blood anti coagulated with Na-citrate (blue top vacutainers) within 1 hr after collection. Spin the blood 10 min, 2500xg at 20-22°C; collect plasma and pool. Pooled plasma is stable for 8 hr at RT. Do not refrigerate or freeze. Analyze two duplicates of test-plasma in each coagulation assay, run one duplicate before nanoparticle-treated plasma samples and the second duplicate at the end of each run.

#### 4.10 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

#### 4.11 Neoplastin Reagent

This reagent is supplied as a lyophilized powder along with reconstitution buffer. Reconstitute according to the manufacturer's instructions and use fresh or refrigerate and use within the time specified by the manufacturer.

### **5. Preparation of Study Samples**

This assay requires 3.0 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 10X the highest final test concentration. If using only PBMC or HL-60 model, only 1.5 mL nanoparticle is required. 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 [22] 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 20 mg/mL is prepared. This sample is then diluted 10 fold (2 mg/mL), followed by two 1:5 serial dilutions (0.4 and 0.08 mg/mL). When 400 µL of each of these sample dilutions are combined in a culture plate well with 3.6 mL of cells, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Each nanoparticle concentration is plated in duplicate.

**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:

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

## 6. Cell Preparation

### 6.1 Isolation of human lymphocytes

1. Place freshly drawn blood anticoagulated with Li-heparin (green top vacutainers) into 15 or 50 mL conical centrifuge tubes. Add an equal volume of room-temperature PBS and mix well.
2. Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing the Ficoll-Paque at the bottom of the blood sample tube. Alternatively, blood/PBS mixture may be slowly layered over Ficoll-

Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. To maintain Ficoll-blood interface it is helpful to hold tube at 45° angle.

3. Centrifuge 30 minutes at 900xg, 18-20°C, without brake.
4. Using a sterile pipet, remove the upper layer containing plasma and platelets, and discard.
5. Using a fresh sterile pipet, transfer mononuclear cell layer into a fresh centrifuge tube.
6. Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20 °C. The HBSS volume should be approximately three times the volume of mononuclear layer.

**Note:** Typically 4 mL of blood/PBS mixture results in about 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step.

7. Discard supernatant and repeat wash step once more.
8. Resuspend cells in complete RPMI 1640 medium. Count an aliquot of cells and determine viability using trypan blue exclusion.
9. If cell viability is  $\geq 80\%$ , dilute cells in complete culture media to a concentration of  $3 \times 10^6$  cells/mL and proceed to step 7.1.

## 6.2 Preparation of HL-60 Cells

Grow cells in complete culture media. Avoid a cell density greater than  $1 \times 10^6$  cells/mL. Count cells on the day of experiment. If cell viability is  $\geq 80\%$ , concentrate cells in complete culture media to the concentration of  $3 \times 10^6$  cells/mL and proceed to step 7.1.

## 7. Experimental Procedure

### 7.1 Cell Treatment

1. Aliquot 3.6 mL of cell suspension into each well of a 6 well plate.
2. Add 400  $\mu$ L of test nanoparticle, positive control and negative control to respective wells. The negative control for both cell models is PBS. The positive control for PBMC is LPS at final concentration of 1  $\mu$ g/mL. The positive control for HL-60 cells is doxorubicin at a final concentration of 50  $\mu$ g/mL.  $\text{Ca}^{2+}$  ionophore can also be used as a positive control. Prepare two wells for each sample. See Appendix for example plate maps.



3. Incubate cells with nanoparticles and controls. Incubation times are 5 hr for the HL-60 model and 24 hr for PBMC.
4. Following incubation, remove cells from the incubator, transfer cells into 5 mL falcon tubes, and wash cells two times with 1 mL of PBS. For each wash cycle spin cells at 400xg for 5 min.
5. After last wash, reconstitute cell pellet in 1 mL of Buffer A. This typically results in a cell concentration of  $10 \times 10^6$  cells/mL. Transfer cells into a 20 mL scintillation vial or equivalent. **Note:** Any vial or tube can be used as long as it fits into the coagulometer warming well.
6. Keep cell suspensions at room temperature; place in the incubator (37°C) 5-10 minutes prior to testing. The sample will then be transferred to the 37°C chamber on the coagulometer when ready to start.

## 7.2 Determining Plasma Coagulation Time

1. Set-up instrument test parameters as shown below:
  - Max Time: 360 sec
  - Incubation Time: 120 sec
  - Single/Duplicate: Duplicate
  - Precision: 5%Allow instrument to warm up to 5-10 minutes prior to use.
2. Prepare all reagents and cells, and warm to 37°C prior to use. Note that lyophilized reagents should be reconstituted at least 30 minutes prior to use. It is not advised to keep more than 10 cell samples at 37°C at one time.
3. Place cuvettes into A, B, C and D test rows on coagulometer. (Note: This protocol is based on semi-automatic STart4 coagulometer from Diagnostica Stago; if using a different instrument, please follow operation guidelines recommended by the instrument manufacturer.)
4. Add one metal ball into each cuvette and allow to warm for at least 3 minutes before use.
5. Add 100 µL of control plasma (step 4.8) or test plasma (step 4.9) to a cuvette. Prepare 1 cuvette (1 strip, 4 wells) for each plasma sample.

6. Start timer for each of the test rows by pressing A, B, C or D buttons. Ten seconds before time is up, timer starts beeping. When this happens, immediately transfer cuvettes to PIP row and press PIP button to activate pipettor.
7. When time is up, add 100  $\mu$ L of Neoplastin reagent to control plasma samples or 100  $\mu$ L of cell suspension from step 7.1.6 in lieu of coagulation activation reagent to corresponding cuvettes, and record coagulation time.

## 8. Calculations

1. The percent coefficient of variation should be calculated for each control or test sample according to the following formula:

$$\%CV = (SD/Mean) \times 100\%$$

2. The percent procoagulant activity of a nanoparticle test sample should be calculated according to the following formula:

$$\%PCA = (\text{mean time}_{\text{positive control sample}}/\text{time}_{\text{test sample}}) \times 100\%$$

## 9. Acceptance Criteria

1. The %CV between replicates representing individual wells in the cuvette should be within 5%. This limit is stored in the instrument setting. When the %CV is greater than 5, there will be a \* mark on the print out.
2. The % CV between replicates of test plasma samples should be within 25%.
3. If two duplicates of the same study sample demonstrated results >25% different, this test sample should be re-analyzed.
4. The positive control is considered positive if the coagulation time in this sample is less than 360 sec. Coagulation times induced by HL-60 cells treated with doxorubicin and that induced by PBMC treated with LPS may vary from passage to passage and from donor to donor, respectively. Typical coagulation times observed in our lab with HL-60 cells treated with 50  $\mu$ g/mL doxorubicin have been 60-90 seconds. Typical coagulation times observed in our lab with PBMC treated with 1  $\mu$ g/mL LPS have been 200-300 seconds; that induced by ionophore has been about 150 seconds.

## 10. References

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## 11. Abbreviations

APL	acute promyelocytic leukemia
CV	coefficient of variation
DIC	disseminated intravascular coagulation
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells

PBS phosphate buffered saline  
 PCA procoagulant activity  
 RT room temperature  
 SD standard deviation  
 U units

## 12. Appendix

### Example Culture Plate Maps

	1	2	3
A	Medium (baseline)	NC	PC
B	Medium (baseline)	NC	PC

	1	2	3
A	VC	TS 1 1.0 mg/mL	TS 1 0.2 mg/mL
B	VC	TS 1 1.0 mg/mL	TS 1 0.2 mg/mL

	1	2	3
A	TS 1 0.04 mg/mL	TS 1 0.008 mg/mL	
B	TS 1 0.04 mg/mL	TS 1 0.008 mg/mL	