NANOTECHNOLOGY CHARACTERIZATION LABORATORY

NCL Method ITA-12
Version 1.0

Coagulation Assays

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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.

Method is written by: _____ 5-22-06.

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_____ 5-22-06.

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Method and instrument performance qualification* performed: 01-31-2006 — 05-08-2006.

Date

Testing facility: NCL, NCI-Frederick, Bldg 469, Rm 250.

*-full validation was not conducted for this assay; performance qualification included analysis of normal and abnormal plasma standards, three sets of pooled plasma untreated or treated with various preparations of nanoparticles.

1. Introduction

This document describes a protocol for assessing an effect of nanoparticle formulation on plasma coagulation. The plasma coagulation is assayed in four tests, i.e. prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and reptilase time (RT). This assay requires 270 μL of a test-nanomaterial.

2. Reagents

2.1. Human blood from at least 3 donors anti-coagulated with sodium citrate

2.2. Neoplastine Cl, Diagnostica Stago, cat#00666

2.3. Thrombin, Diagnostica Stago, cat#0000611

2.4. CaCl1 0.025M, Diagnostica Stago, cat#00367

2.5. Owren-Koller, Diagnostica Stago, cat#00360

2.6. PTT-A, Diagnostica Stago, cat#00595

2.7. Reptilase, Diagnostica Stago, cat#00614

2.8. CoagControlN+ABN, Diagnostica Stago, cat#00676

2.9. SystemControl N+P, Diagnostica Stago, cat#00678

Note: Equivalent reagents from other vendor can be used

3. Equipment

3.1. Pipettors covering range from 0.05 to 10 mL

3.2. Coagulometer

3.5. Refrigerator, 2-8 °C

3.6. Centrifuge

4. Preparation of Study Samples.

This assay requires 270 μL of nanoparticles dissolved/re-suspended in complete culture medium. For the original screen we recommend to use as high concentration of nanoparticles in the sample as possible. The following questions have to be considered when selecting the concentration: i) solubility of nanoparticles in a biocompatible buffer; ii) pH within physiological range; iii) availability of nanomaterial, and iv) stability.

5. Preparation of control and test plasma samples.

Test-Plasma:

Use freshly collected whole blood within 1hour after collection. Spin the blood 10 min at 2500x g at 20-22°C; collect plasma and pool. Pooled plasma is stable for 8h at RT. Do not refrigerate or freeze.

Analyze 2 duplicates of test-plasma in each of coagulation assays, run one duplicate before nanoparticles treated plasma samples and second duplicate at the end of each run.

Nanoparticle-treated test-plasma:

In a microcentrifuge tube combine 90 μL of nanoparticles preparation and 900 μL of test plasma, mix well and incubate 30 minutes at 37◦C. Prepare three tubes for each nanoparticles preparation.

SystemN+P and CoagN+ABN controls:

Reconstitute lyophilized control plasmas with 2mL of distilled water. Let the solutions stand at room temperature 30 minutes prior to use. Mix thoroughly before use. Keep unused portion refrigerated and use within 48h after reconstitution. These plasma samples are used as instrument controls.

7. Experimental Procedure.

7.1. Set-up the instrument test parameters for each of four assays. Refer to Appendix 1 for a quick list of instrument settings and reagent volumes, and let the instrument to warm up for 5-10 minutes prior to use.

7.2. Prepare all reagents and warm them up to 37 C prior to use. Note, that lyophilized reagents should be reconstituted at least 30 minutes prior to use.

7.3. 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 different instrument please follow operation guideline recommended by instrument manufacturer).

7.4. Add one metal ball into each cuvette and let cuvette with ball warm for at least 3 minutes before use.

7.5. Add 100 µL of control or test plasma to a cuvette when testing PT and thrombine time, and 50 µL when testing APTT and reptilase (refer to Appendix 1 for a reference). Prepare three duplicate cuvetts for each plasma sample.

7.6. Only APTT and Reptilase test – add 50 µL of PTT-A reagent (APTT test) or Owren-Koller reagent (reptilase test) to plasma samples in cuvettes.

7.7. Start timer for each of the test rows by pressing A,B,C or D timer buttons. Ten seconds before time is up, the timer starts beeping. When this happens, immediately transfer cuvettes to PIP row and press PIP button to activate pipettor.

7.8. When time is up, add coagulation activation reagent to each cuvette and record coagulation time. Refer to Appendix 1 for the type of coagulation activation reagent and volume for each of four assays.

8. Calculations

A Percent Coefficient of Variation should be calculated for each control or test according to the following formula**:** %CV=SD/Mean x 100%

9. Acceptance Criteria

9.1. %CV for each control and test sample should be within 5%

9.2. If two duplicates of the same study sample demonstrated results different for more then 5%, this sample should be reanalyzed.

10. References

10.1. Start4 Standard Operating procedure and Training manual. Diagnostica Stago, cat#26987, June 2002.

Appendix 1.

ITA-12 Quick Reference Guide

Assay	Instrument Settings/Time/Volumes
PT (Neoplastine)	Control: Coag.Control N+ABN Settings: Max Time: 60sec Incubation Time: 120 sec Single/Duplicate: Duplicate Precision: 5% Volumes: Plasma: 100 µL Neoplastine Reagent: 100 µL (PIP Position 4) Normal Coagulation Time: ≤13.4 sec
APTT	Control: Coag.Control N+ABN Settings: Max Time: 120sec Incubation Time: 180 sec Single/Duplicate: Duplicate Precision: 5% Volumes: Plasma+PTT-A Reagent: 50 µL+50 µL CaCl2: 50 µL (PIP Position 2) Normal Coagulation Time: ≤ 34.1 sec
Thrombin	Control: Coag.Control N+ABN Settings: Max Time: 60sec Incubation Time: 60 sec Single/Duplicate: Duplicate Precision: 5% Volumes: Plasma: 100 µL Thrombine: 100 µL (PIP Position 4) Normal Coagulation Time: ≤ 21 sec
Reptilase	Control: System Control N+ABN Settings: Max Time: 60sec Incubation Time: 120 sec Single/Duplicate: Duplicate Precision: 5% Volumes: Plasma+Owren-Koller: 50 µL+50 µL Reptilase: 100 µL (PIP Position 4) Normal Coagulation Time: ≤20 sec