NCL Method ITA-3

Mouse Granulocyte-Macrophage Colony-Forming Unit Assay

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
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.

Anna N. Ilinskaya, Ph.D.

Marina A. Dobrovolskaia, Ph.D.

1. Introduction

Myelosuppression, a condition where bone marrow activity is reduced, is a common dose limiting toxicity of cytotoxic oncology drugs. Nanoparticles have the potential to distribute to the bone marrow and/or release drug that is delivered to bone marrow. Therefore, understanding the potential toxicity of nanoparticles and the drugs they carry is an important step in preclinical safety evaluation.

Hematopoietic stem cells of the bone marrow (BM) proliferate and differentiate to form discrete cell clusters or colonies. This document describes a protocol for quantitative analysis of granulocyte-macrophage (GM) colony-forming units (CFU), employing murine BM. The protocol can be used for both in vitro and ex vivo analyses. The in vitro protocol involves isolation of bone marrow cells from healthy animals, followed by treatment in vitro with nanoparticle formulations. For ex vivo analysis, the bone marrow is isolated from animals injected with the nanoparticle formulation. The in vitro protocol does not account for nanoparticle biodistribution; however, in cases where dose information is not yet available and/or the nanoparticle formulation is still in the early phases of development, the in vitro protocol allows for a rapid screening of potentially toxic nanoparticle formulations. Furthermore, the in vitro protocol can also provide a quick estimation of the myelosuppressive potential of a cytotoxic oncology drug bound to a nanoparticle surface in comparison to a traditional formulation of the same cytotoxic oncology drug (see Figure 1).

2. Principles

The protocol described herein details the in vitro CFU-GM study. BM cells are isolated from 8-12 week old mice and cultured in methylcellulose-based medium supplemented with cytokines (mSCF, mIL-3 and hIL-6) either untreated (baseline) or treated with nanoparticles (test). The cytokines used in the MethoCult media promote formation of granulocyte and macrophage (CFU-GM) colonies. After a 12 day incubation period of the BM cells, the number of colonies is quantified in both the baseline and test samples. Comparison between the baseline and test samples allows for identification of test materials that can inhibit CFU-GM formation. The basic protocol for BM isolation and culture was adopted from technical manual #28405 developed by StemCell Technologies, Inc. [1-2].

To perform the ex vivo study, steps 4.0, 7.9 and 8.5 are modified. Refer to these sections for additional details.

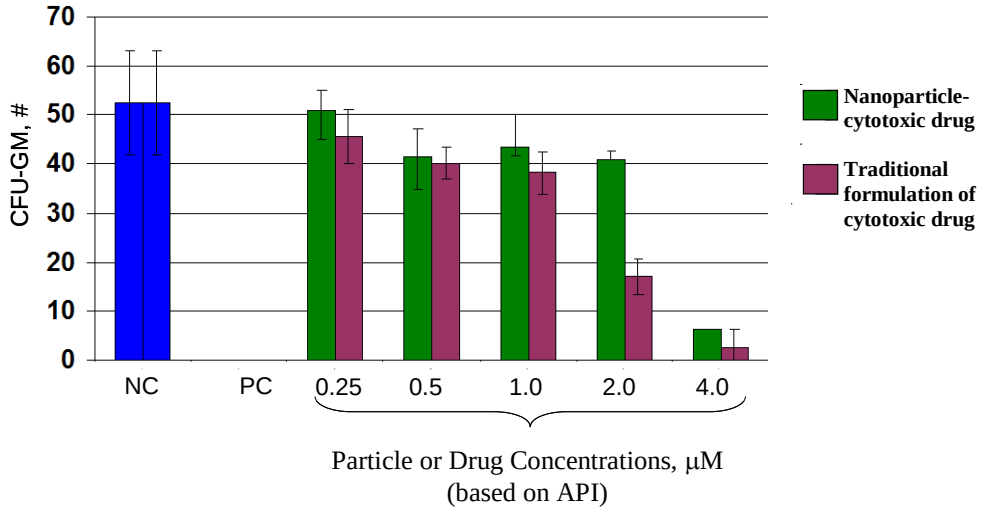

Figure 1. *In vitro* comparison of traditional and nanoformulated cytotoxic oncology drug in CFU-GM assay. Bone marrow cells were treated in vitro with nanoparticle-bound cytotoxic oncology drug or with the traditional formulation of the same cytotoxic oncology drug. The effects of the drug on CFU-GM formation were studied. Cisplatin was used as the positive control. PBS was used as the negative control. Active pharmaceutical ingredient (API) concentration is used to compare the cytotoxic oncology drug at equimolar concentrations.

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. MethoCult medium (Stem Cell Technologies, Inc., 03534)
2. Fetal Bovine Serum prescreened for hematopoietic stem cells (Stem Cell Technologies, Inc., 06200)
3. Iscove's MDM with 2% FBS (Stem Cell Technologies, Inc., 07700)
4. Sterile distilled water
5. Cisplatin (positive control) (Sigma, P4394)

6. Sterile Ca^{2+}/Mg^{2+}-free PBS (GE Life Sciences, HyClone, SH30256.01)
7. 3% Acetic acid with methylene blue (Stem Cell Technologies, Inc., 07060)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Prescreened 35 mm culture dishes (Stem Cell Technologies, Inc., 27100)
3. Blunt-end 16 gauge needles (Stem Cell Technologies, Inc., 28110)
4. 100 mm Petri dishes
5. Plastic beakers
6. Polypropylene tubes, 5 and 15 mL
7. Scissors for tissue dissection
8. Forceps

3.3 Equipment

1. Centrifuge, 700x*g*
2. Refrigerator, 2-8ºC
3. Freezer, -20ºC
4. Cell culture incubator, 5% CO_2 and 95% humidity
5. CO_2 euthanasia box, or appropriate equipment approved by your organization
6. Biohazard safety cabinet approved for level II handling of biological material
7. Inverted microscope
8. Vortex
9. Hemocytometer

4. Animals

This protocol utilizes 8-12 week-old C56BL6 males or females. Use of pooled cells derived from at least two animals is highly recommended. The exception is when the experiment is conducted to support an in vivo study in which the animals have been injected with test nanoparticles. In this case, process each animal separately. Ensure that any animal work is supported by approved institutional protocols.

The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care is

provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols are approved by the FNLAC institutional Animal Care and Use Committee. Any experiments conducted are scientifically justified and do not represent an unnecessary duplication of previous work.

5. Preparation of Reagents and Controls

5.1 MethoCult Medium

MethoCult medium is supplied in 100 mL size batches. This medium can be thawed and used fresh or aliquoted into single 3 mL aliquots in 15 mL conical tubes. It is recommended by the manufacturer that the medium be thawed at room temperature, or in a refrigerator overnight, vortexed to mix well, then kept at room temperature for approximately 5 min to allow air bubbles to dissipate. Use a 16 gauge blunt-end needle to aliquot MethoCult medium. Store the aliquots at a nominal temperature of -20ºC. Before the test, thaw the required number of aliquots at room temperature for approximately 20 min and keep on ice prior to use. Alternatively, they can be thawed on ice or in the refrigerator. Avoid repeated freeze/thaws.

5.2 Positive Control (50 mM Cisplatin)

Cisplatin is supplied in a lyophilized form. Reconstitute the lyophilized powder by adding an appropriate amount of DMSO to make a stock solution with a nominal concentration of 50 mM. Prepare small aliquots and store at a nominal temperature of -20ºC or lower. Prior to use in the assay, thaw an aliquot of the stock solution at room temperature and dilute in IMDM supplemented with 2% FBS to bring the concentration to 1.1 mM. Next, 150 µL of this intermediate solution is then added to 3 mL of MethoCult culture medium. The final concentration of cisplatin in the positive control sample is 50 µM.

Note: This control is not required if the ex vivo study is conducted. The ex vivo study has its own negative and positive controls.

5.3. 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. Dilute this sample the same way as the test nanomaterials.

5.4. Vehicle Control

When nanoparticles are not formulated in saline or PBS, the vehicle should also be tested to estimate the effect of excipients on bone marrow precursors. This control is specific to each given nanoparticle sample. The vehicle control should match the formulation buffer of the test nanomaterial by both composition and concentration. Dilute this sample the same way as the test nanomaterials. This control can be skipped if the vehicle is PBS or saline.

6. Preparation of Study Samples

This assay requires 0.6 mL of nanoparticle solution at a concentration 22X the highest final test concentration. The media for the stock material and subsequent dilutions should be determined for each nanoformulation. Ideally, the nanoparticle should be dissolved/resuspended in IMDM medium. However, this is often not possible due to the limited concentration of the nanoparticle stock solution. In such cases, use nanoparticles directly from the stock and prepare all dilutions in the same buffer used for nanoparticle storage (e.g., if the stock is in PBS, use it directly and prepare dilutions in PBS; if the stock is in a vehicle different than PBS, use directly from stock and use that vehicle to prepare all dilutions).

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 [3] 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 44 mg/mL is prepared. This sample is then diluted 10 fold (4.4 mg/mL), followed by two 1:5 serial dilutions (0.88 and 0.18 mg/mL). When 0.15 mL of each of these sample dilutions is added to the test tube and mixed with 3 mL of MethoCult medium and 0.15 mL of cell suspension, the

final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Three 150 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:

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg}$$ (see reference [5])

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:

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

7. Isolation and Counting of Bone Marrow Cells

1. Position euthanized mouse on its back and rinse fur thoroughly with 70% alcohol. (Euthanize animals according to the protocol approved by your institution.)
2. Cut a slit in the fur just below the rib cage without cutting the peritoneal membrane.
3. Firmly grasp skin and pull back to expose hind limbs.
4. Using sterile sharp dissecting scissors cut the knee joint in the center. Cut through ligaments and excess tissue.
5. Grasp femur with forceps, and cut femur near hip joint.
6. Free tibia by cutting near the ankle joint.
7. Trim the ends of the long bones to expose the interior marrow shaft. Put bones in sterile Petri dish, or in sterile culture medium, and place on ice. Bones can be collected from multiple animals.
8. Using a 3 cc syringe with 21 or 22 gauge needle, draw up to 1-3 mL of cold Iscove's MDM supplemented with 2% FBS.
9. Insert bevel of needle into marrow shaft and flush marrow into 15 mL tube. Repeat this procedure for all bones. The same medium can be used to isolate marrow from 1-3

animals. Once all the marrow has been expelled the bone should appear white. The exception is when this protocol is used to isolate BM from individual animals from an in vivo study. In this case BM from each animal is isolated into 3 mL of medium.

Note: Typically 3 mL of medium is enough to collect BM from 6 femurs (i.e., from 3 animals). However when more animals are used, it is better to collect cells into higher volumes of media (e.g., 3 mL per each 6 femurs). An additional 1-3 mL of fresh medium may be used to flush the BM shaft after the first flush. If such case, the total volume of cell suspension will be more than 3 mL and one may need to concentrate the cells before proceeding with the assay. Concentration by centrifugation is described in the note to step 12 below.

10. Keeping needle below medium surface, gently draw medium with cells up and down with 3 cc syringe and 21 gauge needle 3-4 times to make a single cell suspension.
11. Keep cells in medium, on ice until use.
12. Perform a nucleated cell count. To do so, first dilute the cells 1:100 with 3% acetic acid with methylene blue (e.g., 10 μL cells + 990 μL 3% acetic acid/methylene blue). Then, use either a hemocytometer or automatic cell counter to obtain counts. An average cell count is expected to be $1x10^7$ to $2x10^7$ from the femur and $0.6x10^7$ to $1x10^7$ from the tibia.

    Note: If cells were extracted into larger volumes of media, cells should be concentrated before counting. Spin down isolated cells at 700x*g*. The spinning time is estimated such as to spin 5 minutes for each 3 mL (e.g., if the total volume to be spun is 3 mL, spinning time is 5 min. If the volume of the cell suspension in the tube is 6 mL, spinning time is 10 min, etc.).
13. If cell viability is at least 90% and counts are acceptable, proceed to the next step.

8. Experimental Procedure

1. Label the lids of 35 mm culture dishes at the edge using a permanent, fine-tip, felt marker.
2. Thaw MethoCult medium at room temperature or in refrigerator overnight.
3. Vortex tubes to ensure all components are thoroughly mixed.
4. Dilute cells isolated according to the procedure described in Section 7 with Iscove's medium supplemented with 2% FBS to $4x10^5$ cells/mL.
5. Add 150 μL of cell suspension and 150 μL of either Iscove's medium with 2% FBS (baseline), negative control, positive control, test sample, or vehicle control to 3 mL of MethoCult medium. Test three replicates (n = 3) for each test concentration.

   Note: If the ex vivo study is conducted, add 150 μL of cell suspension from individual animals and 150 μL of Iscove's medium with 2% FBS to 3 mL of Methocult medium. No addition of PBS, nanoparticles or cisplatin is required because animals were previously injected with various formulations representing vehicle control, nanoparticle treatment and/or traditional cytotoxic oncology drug formulation.
6. Vortex tubes to ensure all cells and medium components are mixed thoroughly.
7. Allow tube to stand 5 min to allow bubbles to dissipate.
8. Attach a 16 gauge blunt-ended needle to a 3 cc syringe. Place the needle below the surface of the solution containing Methocult media and cells from steps 5-7 and draw up approximately 1 mL. Gently depress the plunger and expel medium completely. Repeat until no air space is visible.
9. Draw up MethoCult medium with cells into syringe and dispense 1.1 mL per 35 mm dish. All samples are tested in duplicate (n = 2) (i.e., prepare two 35 mm dishes for each sample tested).
10. Distribute the medium evenly by gently tilting and rotating each dish.
11. Place the two covered dishes with cells and one uncovered dish filled with 3 mL of sterile water, into a 100 mm Petri dish.
12. Place cultures in an incubator maintained at 37ºC, 5% CO_2 and 95% humidity.
13. Incubate for 12 days. On the 12^{th} day, remove dishes from incubator, identify and count colonies as described below. Representative values of CFU-GM for C57BL6 mice at 8-12 weeks of age is 64 ± 16.

9. Description of CFU-GM

This classification includes CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M) and CFU-granulocyte macrophage (CFU-GM). The colonies contain 30 to thousands of CFU-G, CFU-M or both cell types (CFU-GM). CFU-GM colonies often contain multiple clusters and appear as a dense core surrounded by cells. The monocytic lineage cells are large cells with an oval to round shape and appear to have a grainy or grey center. The granulocytic lineage cells are round, bright, and are much smaller and more uniform in size than macrophages. It is easy to see individual cells of a CFU-GM colony, especially in the periphery of the colony. See Figure 2 below for a depiction of CFU-GM, CFU-M, and CFU-G colonies.

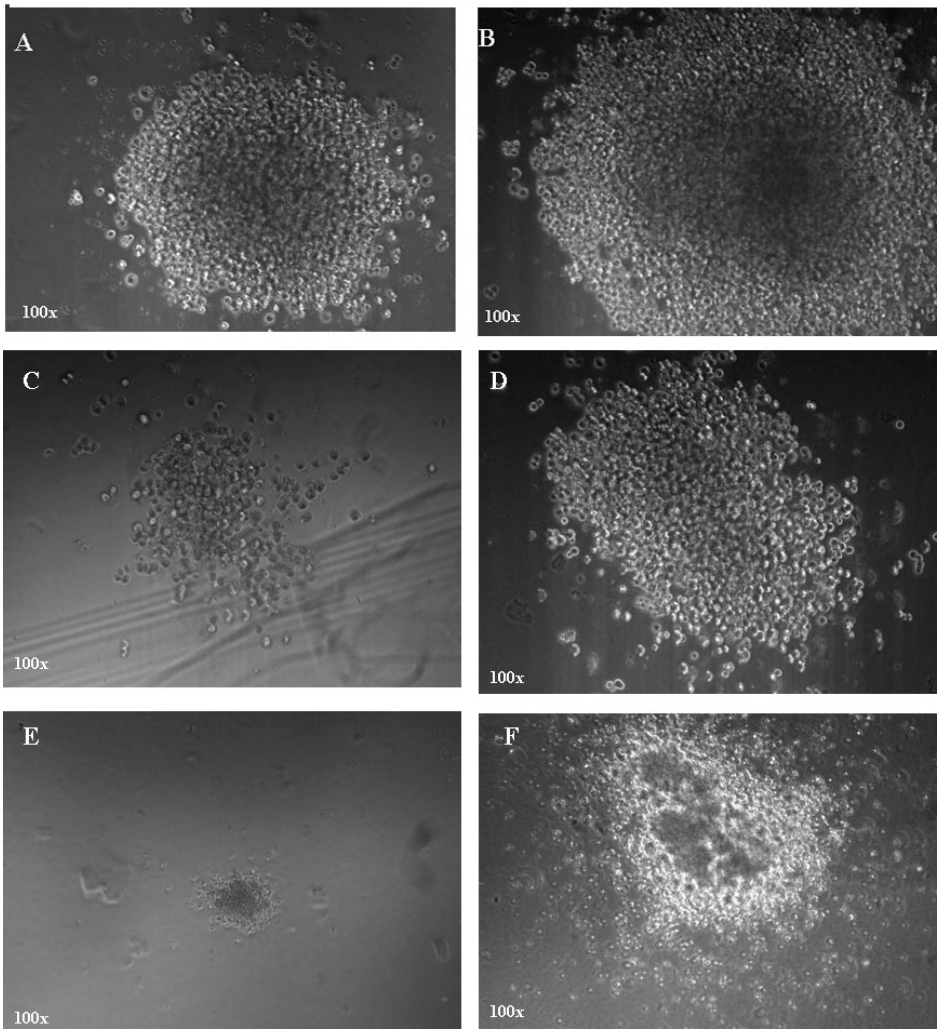

Figure 2. Depiction of CFU-GM, CFU-M, and CFU-G Colonies. A and **B** are CFU-GM colonies. **C** and **D** are CFU-M colonies. **E** depicts a single CFU-G colony. **F** shows a few CFU-G colonies growing together.

10. Calculations

10.1 Percent Coefficient of Variation (%CV)

The % CV should be calculated for each control and test sample:

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

10.2 Percent CFU Inhibition

When analysis is performed to compare traditional and nanoformulated cytotoxic oncology drugs, calculating the percent CFU inhibition may be helpful. The percent CFU inhibition is also helpful in comparing data between experiments and between different strains, if mouse strains other than C57BL6 are needed to address project specific needs.

$$\frac{\text{(Baseline CFU-GM – Test CFU-GM)}}{\text{Baseline CFU-GM}} \times 100\ \%$$

11. Acceptance Criteria

1. The %CV for each control and test sample should less than 30%.
2. If the positive or negative control fails to meet acceptance criterion described in 11.1, the assay should be repeated.
3. Within the acceptable assay, if two of three replicates of the unknown sample fail to meet acceptance criterion described in 11.1, this unknown sample should be re-analyzed.

12. References

1. Mouse Colony-Forming cell Assays Using MethoCult. Technical manual. StemCell Technologies Inc., cat # 28405.
2. Dominique Pifat. Assay Validation. http://www.fda.gov/cber/summaries/120600bio10.ppt - 05-24-2003
3. 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.

13. Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
API	active pharmaceutical ingredient
BM	bone marrow
cc	cubic centimeter
CFU	colony forming units
CFU-G	colony forming unit-granulocyte
CFU-GM	colony forming unit-granulocyte macrophage
CFU-M	colony forming unit- macrophage
CV	coefficient of variation
FBS	fetal bovine serum
hIL-6	human interleukin-6
IMDM	Iscove's modified Dulbecco's medium
mIL-3	mouse interleukin-3
mSCF	mouse stem cell factor
n	number of samples
PBS	phosphate buffered saline
SD	standard deviation