



NCL Method ITA-3

Mouse Granulocyte-Macrophage Colony-Forming Unit Assay

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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

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

Hematopoietic stem cells of 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. This 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. In the *ex vivo* version, 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 when dose information is not available and nanoparticle formulation is in the early phase of development, the *in vitro* protocol allows for rapid screening of potentially toxic nanoparticle formulations. The *in vitro* protocol can also give 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 (for an example, see Figure 1).

2. Principles

The protocol described here 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) and are 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 twelve days 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 the identification of test materials which 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]. If the *ex vivo* study is required, steps 4, 7.9 and 8.5 are modified (please refer to sections 4, 7 and 8 for details).

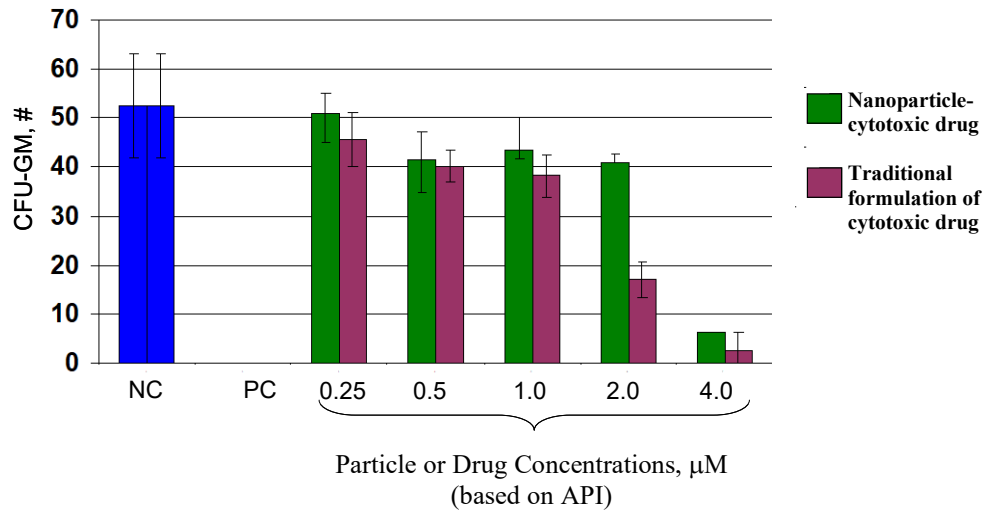


Figure 1. *In vitro* comparison of traditional and nano-formulated cytotoxic oncology drug in CFU-GM assay. Bone marrow cells were treated *in vitro* with nanoparticle-bound cytotoxic oncology drug or with traditional formulation of the same cytotoxic oncology drug. The effects of the drug on CFU-GM formation were studied. Cisplatin was used as positive control. PBS was used as negative control. API (active pharmaceutical ingredient) 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; 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 MethoCult medium (Stem Cell Technologies, Inc., 03534)

3.1.2 Fetal Bovine Serum prescreened for hematopoietic stem cells (Stem Cell Technologies, Inc., 06200)

3.1.3 Iscove's MDM (IMDM) with 2% FBS (Stem Cell Technologies, Inc., 07700)

3.1.4 Sterile distilled water

- 3.1.5 Cisplatin (positive control) (Sigma, P4394)
- 3.1.6 Sterile Ca²⁺/Mg²⁺-free PBS (GE Life Sciences, HyClone, SH30256.01)
- 3.1.7 3% Acetic Acid with Methylene Blue (Stem Cell Technologies, Inc., 07060)
- 3.2 Materials
 - 3.2.1 Pipettes covering the range of 0.05 to 10 mL
 - 3.2.2 Prescreened 35 mm culture dishes (Stem Cell Technologies, Inc., 27100)
 - 3.2.3 Blunt-end, 16-gauge needles (Stem Cell Technologies, Inc., 28110)
 - 3.2.4 100 mm Petri dishes
 - 3.2.5 Plastic beakers
 - 3.2.6 Polypropylene tubes, 5 and 15 mL
 - 3.2.7 Scissors for tissue dissection
 - 3.2.8 Forceps
- 3.3 Equipment
 - 3.3.1 Centrifuge
 - 3.3.2 Refrigerator, 2-8°C
 - 3.3.3 Freezer, -20°C
 - 3.3.4 Cell culture incubator with 5% CO₂ and 95% humidity
 - 3.3.5 CO₂ euthanasia box, or appropriate equipment approved by your organization
 - 3.3.6 Biohazard safety cabinet approved for level II handling of biological material
 - 3.3.7 Inverted microscope
 - 3.3.8 Vortex
 - 3.3.9 Hemocytometer

4. Animals

This protocol utilizes 8-12 week old, C56BL6 males or females. Use of pooled cells derived from at least two (2) 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.

NCI-Frederick 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 NCI-Fredrick institutional Animal Care and Use Committee.

If this procedure is conducted outside NCI at Frederick facilities, one has to ensure that animal work is supported by approved protocols.

5. Reagent and Control Preparation

5.1 MethoCult Medium

The MethoCult medium is supplied in 100 mL size batches. This medium can be thawed and used fresh or aliquoted into single 3 mL volumes 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 16-gauge blunt-end needles to aliquot the MethoCult medium. Store the aliquots at a nominal temperature of -20°C. 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 refrigerator. Avoid repeated freeze/thaws.

5.2 50 mM Cisplatin (Positive Control)

Cisplatin is supplied in a lyophilized form. Reconstitute the lyophilized powder by adding an appropriate amount of DMSO to make a stock solution with nominal concentration of 50 mM. Prepare small aliquots and store at a nominal temperature of -20°C 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. 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, because the ex vivo study has its own negative and positive controls.

5.3. Negative Control (PBS)

Sterile Ca²⁺/Mg²⁺-free PBS is used as a negative control. Store at room temperature for up to 6 months. Dilute this sample the same way you dilute the test nanomaterials.

5.4. Vehicle Control (relevant to each given nanoparticle)

When nanoparticles are not formulated in saline or PBS, the vehicle should be tested to estimate the effect of excipients on the bone marrow precursors. 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.

6. Preparation of Study Samples

This assay requires 0.6 mL of nanoparticle solution at 22X the highest 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, often this is not possible due to the limited concentration of the nanoparticle stock solution. If this is the case use nanoparticles directly from stock and prepare all dilutions in the same buffer as 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). 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 [3] and are summarized in Box 1 below.

The assay will evaluate 4 concentrations: 10X (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 44 mg/mL will be prepared and diluted 10-fold (4.4 mg/mL), followed by two 1:5 serial dilutions (0.88 and 0.18 mg/mL). When 150 µL of each of these samples 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 the 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 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:

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

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.

$$\text{in vitro concentration}_{\text{human matrix}} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

7. Isolation and Counting of Bone Marrow Cells

- 7.1 Position euthanized mouse on its back and rinse fur thoroughly with 70% alcohol. (Euthanize animals according to the protocol approved by your institution.)
- 7.2 Cut a slit in the fur just below the rib cage without cutting the peritoneal membrane.
- 7.3 Firmly grasp skin and pull back to expose hind limbs.
- 7.4 Using sterile sharp dissecting scissors cut the knee joint in the center. Cut through ligaments and excess tissue.
- 7.5 Grasp the femur with forceps and cut the femur near the hip joint.
- 7.6 Free the tibia by cutting near the ankle joint.

- 7.7 Trim the ends of the long bones to expose the interior marrow shaft. Put bones in a sterile Petri dish, or in sterile culture medium, and place on ice. Bones can be collected from multiple animals.
- 7.8 Using a 3cc syringe with a 21 or 22 gauge needle, draw up to 1-3 mL of cold Iscove's MDM supplemented with 2% FBS.
- 7.9 Insert the bevel of the needle into marrow shaft and flush marrow into a 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 3mL of medium in a separate tube.

Note: Typically, 3mL 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 a higher volume of media (e.g. 3 mL per each 6 femurs). Additional 1-3 mL of fresh medium may be used to flush the BM shaft after first flush. If this is the case the total volume of the 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 section 7.12 below.

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

Note: A) If cells were extracted into larger volume of media, one may concentrate the cells before counting. To do so spin down isolated cells at 700xg. 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 6mL, spinning time is 10 min etc.)

B) *While other dyes (e.g., AOPI or trypan blue) can be used to determine cell count and viability, especially when automated cell counters are utilized, manual counting of bone marrow cells is more accurate with the 3% acetic acid methylene blue; this solution eliminates anucleated cells from count. The nuclei of pluripotent stem cells are stained light blue.*

- 7.13 If cell viability (at least 90%) and count are acceptable, proceed to the next step.

8. Experimental Procedure

- 8.1 Label lids of 35 mm culture dishes at the edge using a permanent, fine tip, felt marker.
- 8.2 Thaw MethoCult medium at room temperature or in refrigerator overnight.
- 8.3 Vortex tubes to ensure all components are thoroughly mixed.
- 8.4 Dilute the isolated cells (section 7) with Iscove's medium supplemented with 2% FBS to 4×10^5 cells/mL.
- 8.5 Add 150 μ L of cell suspension and 150 μ L 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 μ L of cell suspension from individual animals and 150 μ L 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.

- 8.6 Vortex the tubes to ensure all cells and medium components are mixed thoroughly.
- 8.7 Let the tubes stand for 5 min to allow bubbles to dissipate.
- 8.8 Attach a 16-gauge blunt ended needle to a 3cc syringe; place the needle below the surface of the solution containing Methocult media with cells in it from steps 8.5-

- 8.7 and draw up approximately 1 mL. Gently depress the plunger and expel medium completely. Repeat until no air space is visible.
- 8.9 Draw up MethoCult medium with cells into the 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).
- 8.10 Distribute the medium evenly by gently tilting and rotating each dish.
- 8.11 Place the two covered dishes with cells and one uncovered dish filled with 3 mL of sterile water, into a 100 mm Petri dish.
- 8.12 Place cultures in an incubator maintained at 37°C, 5% CO₂ and 95% humidity.
- 8.13 Incubate for 12 days. On the 12th 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.

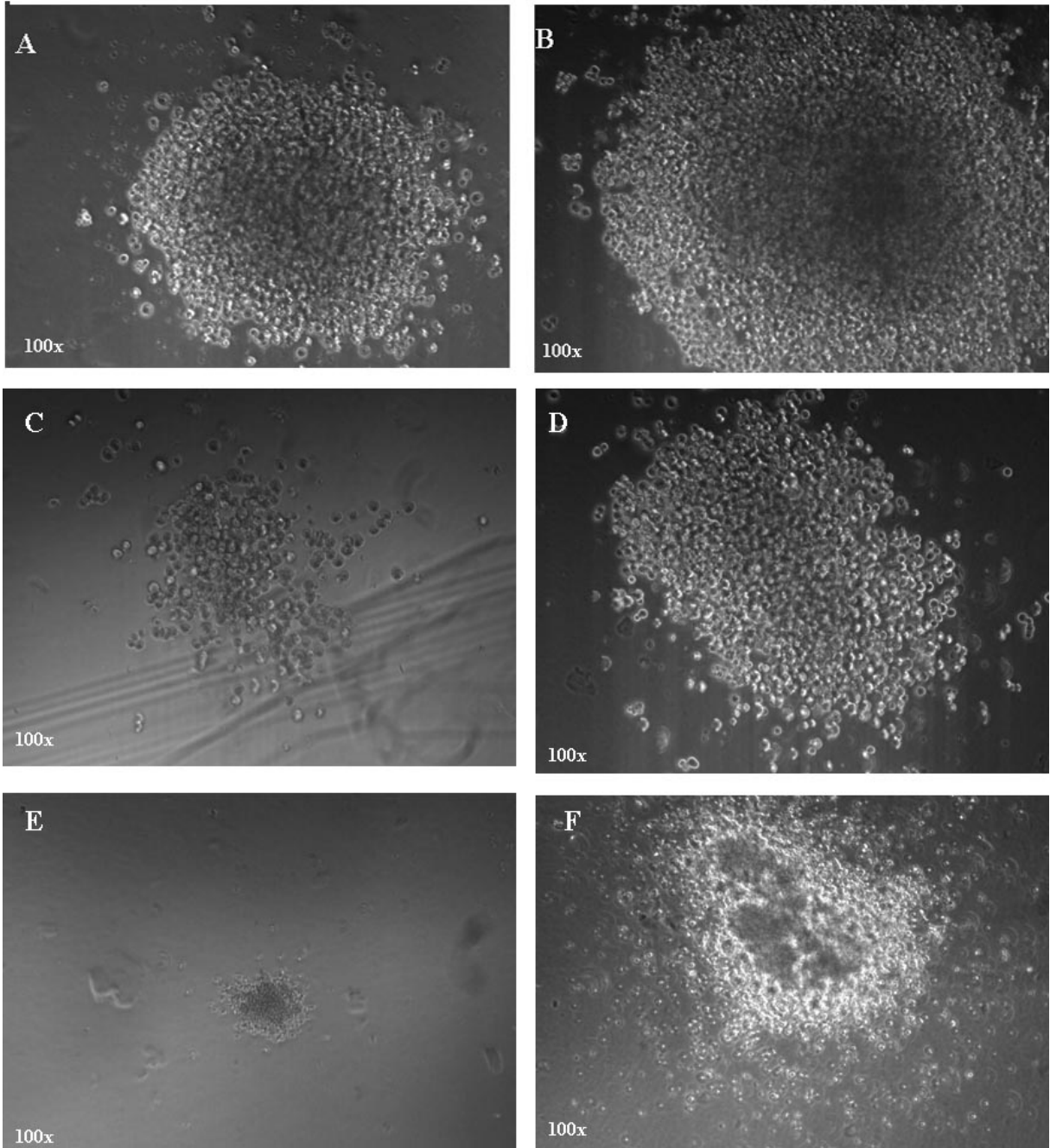


Figure 2. Depiction of CFU-GM, CFU-M, and CFU-G Colonies.

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

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

10.1 Percent Coefficient of Variation

$$\frac{SD}{Mean} \times 100\%$$

10.2 Percent CFU Inhibition

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

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

11. Acceptance Criteria

- 11.1 %CV for each control and test sample should less than 30%.
- 11.2 If positive control or negative control fails to meet acceptance criterion described in 11.1, the assay should be repeated.
- 11.3 Within the acceptable assay, if two of three replicates of 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
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
PBS	phosphate buffered saline
SD	standard deviation