



NCL Method ITA-11

Measurement of Nanoparticle Effects on Cytotoxic Activity of NK Cells by Label-Free RT-CES System

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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

Natural killer (NK) cells are a type of lymphocyte which play a major role in the host-rejection of both cancer cells and cells infected by viruses. NK cells carry small granules in their cytoplasm which contain special proteins, termed perforin and granzymes. When NK cells release perforin in close proximity to target cells (i.e., tumorous or virus-infected cells), it forms pores in the cell membrane of the target cell through which the granzymes and associated molecules can enter, inducing apoptosis. The cytotoxic activity of NK cells is an important component of innate immunity by providing a quick body response to cancerous or virus infected cells before more specialized adaptive immunity can be generated. Understanding a drug's effect on the cytotoxicity of NK cells is thus an important part of immunotoxicity studies aimed at identifying potential immunosuppression.

2. Principles

This document describes a protocol for assessing the effect of nanoparticles on the capacity of human natural killer (NK) cells to lyse tumorous target cells under in vitro conditions. In this method, the NK-92 cell line is used as the model for natural killer cells, and the hepatocellular carcinoma Hep G2 cell line is used as the model for target cells. The viability of the Hep G2 cells following the addition of untreated or nanoparticle-treated NK-92 cells is monitored in real time using the real-time cell electronic system (RT-CES) [1, 2].

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. PBS (GE Life Science, SH30256.01)
2. Fetal bovine serum (GE Life Sciences, Hyclone, SH30070.03)
3. Horse serum (GE Life Sciences, Hyclone, SH30074.03)
4. MEM, Alpha Modification (GE Life Sciences, Hyclone, SH30568.01)
5. RPMI-1640 (GE Life Sciences, Hyclone, SH30096.01)
6. L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

7. Myo-inositol (Sigma, I7508)
8. Folic Acid (Sigma, F8758)
9. β -Mercaptoethanol (Invitrogen, 21985-023)
10. Recombinant Human IL-2 (R&D Systems, 202-IL-010)
11. Trypan blue solution (Invitrogen, 15250-061)

3.2 Materials

1. Pipettes, 0.05 mL to 10 mL
2. Flat bottom 16 well E-plates (**Note:** Certain models of the RT-CES instrument can operate with 96-well E-plates.)
3. Polypropylene tubes, 5 and 15 mL
4. Reagent reservoirs
5. T25 culture flasks

3.3 Equipment

1. Centrifuge, 130xg
2. Refrigerator, 2-8°C
3. Freezer, -20°C
4. Cell culture incubator, 5% CO₂ and 95% humidity
5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemacytometer
9. RT-CES instrument (ACEA Biosciences)

4. Reagent 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 at 56°C in a water bath mixing every five minutes. 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 (to maintain Hep G2 cells)

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

4.3 Complete Alpha MEM (to maintain NK-92 cells)

The complete Alpha MEM medium should contain the following reagents: 2 mM L-glutamine, 0.2 mM inositol, 0.1 mM β-mercaptoethanol, 0.02 mM folic acid, 25 ng/mL recombinant IL-2, 10% horse serum, and 10% heat inactivated fetal bovine serum.

5. Preparation of Study Samples

This assay requires 3 mL of nanoparticle solution dissolved/resuspended in complete Alpha MEM medium, at a concentration 10X the highest final test concentration. 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 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 1.5 mL of each of these sample dilutions is added to a T125 flask and mixed with 13.5 mL of cell suspension, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Additionally, a 200 µL aliquot used for a cell free control.

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. Preparation of Effector and Target Cells

6.1 NK-92 Effector Cells

Grow cells in complete alpha-MEM medium. Split cells when the cell number approaches 1×10^6 cells/mL (i.e., approximately every 2-3 days). Do not allow cells to grow over 1×10^6 cells/mL. Cultures can be maintained by addition or replacement of medium. When replacing media, centrifuge cells at 130xg for 10 min, and resuspend the cell pellet in fresh medium at 2×10^5 to 3×10^5 viable cells/mL. Pipette the cells up and down on the back of the flask every 2-3 days to produce a single cell suspension. NK-92 cells are extremely sensitive to overgrowth and media exhaustion. Replace with fresh medium every 2 to 3 days (depending on cell density).

6.2 Hep G2 Target Cells

Grow cells in complete RPMI medium. Renew growth media twice a week. A subcultivation ratio of 1:4 or 1:6 is recommended. To split the cells, remove and discard culture medium; briefly rinse the cell layer with 0.25% (w/v) Trypsin–0.53 mM EDTA solution to remove all traces of serum (contains trypsin inhibitor). Next, add 2.0 to 3.0 mL of Trypsin-EDTA solution to the flask and observe cells under an inverted microscope until the cell layer is dispersed (usually within 2 to 5 minutes). To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to

facilitate dispersal. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting. Add appropriate aliquots of the cell suspension to new culture flasks and incubate cultures at 37°C in a 5% CO₂ environment.

7. Experimental Procedure (This will require 3 days.)

Day 1

1. Adjust effector cell (NK-92) number to 1×10^6 cells/mL using complete **alpha-MEM**. Prepare 10-15 mL of cell suspension for each sample and control (negative and vehicle).
2. Treat NK-92 cells with test nanoparticles, vehicle or negative control for 24 ± 0.5 hours. Perform treatment in T25 flask.
3. Adjust target cell (Hep G2) number to 0.5×10^6 cells/mL using complete **RPMI**.
4. Plate 50 μ L of media to all wells, attach plate to RT-CES and begin program. Following background measurement, plate 50 μ L of Hep G2 cells from step 3 per each well in RT-CES plates except for nanoparticle-only wells (refer to the template in Figure 1 and remember that 2 E-plates are required per each nanoparticle), attach to RT CES and start data acquisition. Hep G2 cells are in culture for approximately 16–20 hr prior to addition of NK-92 effector cells. Acquisition program is described in Table 1. Either version A or B can be used.

Table 1. RT-CES Acquisition Protocols

Version A

Step #	Step Name	Interval (min)	Sweeps
1	Step 1	1	
2	Step 2	30	1
3	Step 3	2	500
	Step 3	10	30
4	Step 4	Idle	1000

Version B

Step #	Step Name	Interval (min)	Sweeps
1	Step 1	1	1
2	Step 2	30	200
3	Step 3	15	500

Day 2

- Using trypan blue, determine viability of NK-92 cells prepared in step 2.
- Concentrate NK-92 cells from step 5 by centrifugation (5 min, 400xg). Remove and discard supernatants, and reconstitute cells in each sample with fresh alpha-MEM media to a final concentration of 2.5×10^6 viable cells/mL using complete **alpha-MEM (without IL-2)**. This concentration will allow for an effector to target (E:T) ratio of 5:1. An E:T ratio of 2.5:1 or 1.25:1 is also acceptable, and in this case the NK-92 concentration should be adjusted to 1.25×10^6 or 0.625×10^6 viable cells/mL. If the viability of NK cells treated with negative control is $\geq 97\%$, proceed to the next step.

Note: If nanoparticles were cytotoxic to NK-92 cells and resulted in more than 50% cell death, it may not be possible to evaluate the cytotoxicity of NK-92 cells treated with these nanoparticles due to a lack of the required number of effector cells.

- Pause RT-CES data acquisition program, remove RT-CES plates from the instrument and add 100 μ L of NK cells from step 6 to designated wells on RT-CES plate. An example template is provided in Figure 1. Prepare two E-plates for each nanoparticle.
- Return RT-CES plates containing NK-92 effector cells treated with either nanoparticle or negative control and target (Hep G2) cells to the instrument and resume data acquisition for another 24 hr.

Day 3

- Stop acquisition program on the RT-CES instrument and analyze the data.

Hep G2 only (no NK92)	Hep G2 only (no NK92)
Hep G2 Untreated NK-92	Hep G2 Untreated NK-92
Hep G2 NK-92 + NC	Hep G2 NK-92 + NC
Hep G2 NK-92 + NP (2.0 mg/mL)	Hep G2 NK-92 + NP (2.0 mg/mL)
Hep G2 NK-92 + NP (0.2 mg/mL)	Hep G2 NK-92 + NP (0.2 mg/mL)
Hep G2 NK-92 + NP (0.04 mg/mL)	Hep G2 NK-92 + NP (0.04 mg/mL)
Hep G2 NK-92 + NP (0.008 mg/mL)	Hep G2 NK-92 + NP (0.008 mg/mL)
NP (2.0 mg/mL)	NP (2.0 mg/mL)

 This sample represents assay's baseline

Figure 1. Example of RT-CES Plate Template.

In this example, 2.0 mg/mL is the highest tested concentration and the three serial 1:5 dilutions are 0.2, 0.04, and 0.008 mg/mL. Testing of the “nanoparticle only” sample is recommended to identify potential nanoparticle interference with the RT-CES instrument acquisition system. During validation of gold nanoshells, citrate gold colloids, colloidal silver, iron oxide, PAMAM dendrimers, water soluble fullerene derivatives, TiO₂ particles and conductive materials such as gadolinium and ruthenium, no interference has been observed. Since nanoparticles are washed away before addition of treated NK-92 to Hep G2 cells, a potential source of nanoparticles in wells of E-plates is release from NK-92 (assuming NK-92 took up the particles). Only the highest concentration of nanoparticle is tested for interference. If no interference is observed with the highest tested concentration, it is unlikely that lower concentrations will interfere.

8. Calculations

An example of cell index plot is shown in Figure 2. An overview of the instrument and how it functions is shown in Figure 3. Cell index data for each test sample and control is used to calculate area under the curve (AUC). The AUC data from each control and test sample is used to calculate percent cytotoxicity and percent coefficient of variation (CV). The %CV is used to control precision and is calculated according to the following formula:

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

The percent cytotoxicity is calculated to the formula

$$\% \text{ Cytotoxicity} = ((AUC_{\text{baseline}} - AUC_{\text{particles}})/AUC_{\text{baseline}}) \times 100\%$$

where AUC is area under the curve determined for Hep G2 growth from the time of addition of NK-92 effector cells to the time when the data acquisition was stopped (i.e., 24 ± 0.5 hr later) and normalized to the number of cells plated in each individual well.

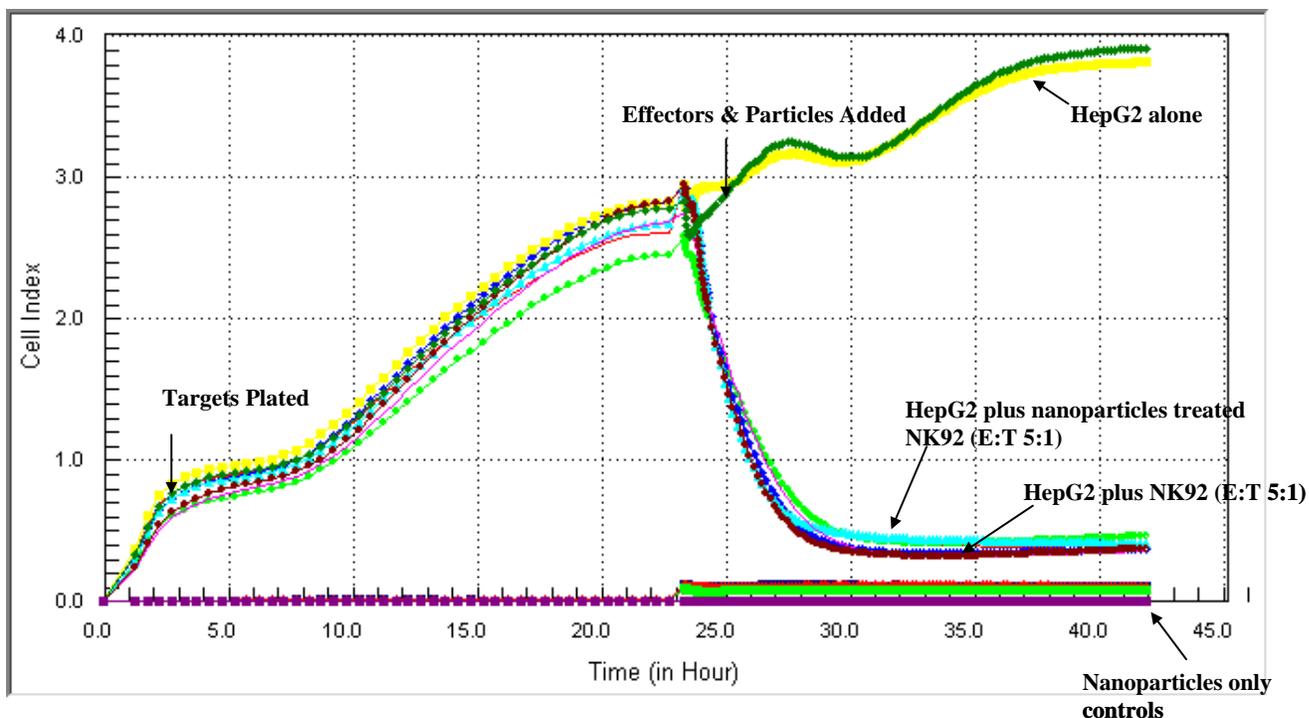
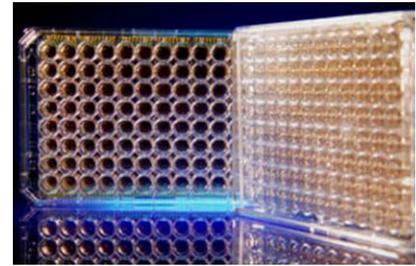
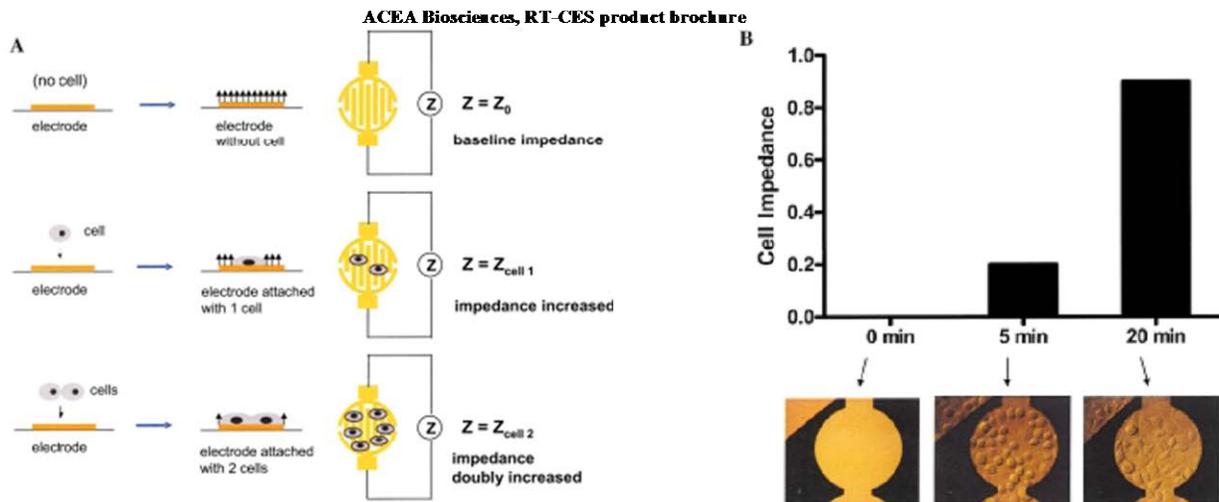


Figure 2. Example of raw data, demonstrating changes in Hep G2 cell index after co-incubation with NK-92 cells.



ACEA Biosciences, RT-CES product brochure



Solly K., et al, ASSAY and Drug Development Technologies, Volume 2, Number 4, 2004

Figure 3. Overview of RT-CES Instrumentation

9. Acceptance Criteria

1. The %CV for each control and test sample should be within 25%.
2. The assay is acceptable if the percent cytotoxicity in the negative control is $\geq 30\%$.

10. References

1. Abassi, Y.; Zhu, J. Label-free assay for NK cell mediated cytotoxicity on the RT-CES system. RT-CES applications. ACEA Biosciences; www.aceabio.com.
2. Zhu, J.; Wang, X.; Xu, X.; Abassi, Y.A. Dynamic and label-free monitoring of natural killer cell cytotoxic activity using electronic cell sensor arrays. *Journal of Immunological Methods* 2006;309:25-33.
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.

11. Abbreviations

AUC	area under the curve
CV	coefficient of variation
E:T	effector to target cell ratio
FBS	fetal bovine serum
HepG2	human hepatocarcinoma cells
IL	interleukin
MEM	minimal essential medium
NK	natural killer
PAMAM	polyamidoamine
PBS	phosphate buffered saline
RT-CES	real-time cell electronic sensing
SD	standard deviation