



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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Please cite this protocol as:

Potter TM, Cedrone E, Neun BW, Dobrovolskaia MA, NCL Method ITA-11: Measurement of
Nanoparticle Effects on Cytotoxic Activity of NK Cells by Label-Free RT-CES System.
<https://ncl.cancer.gov/resources/assay-cascade-protocols> DOI: 10.17917/GMXG-BH29

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 virus. NK cells carry small granules in their cytoplasm which contain special proteins such as 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. Cytotoxic activity of NK cells is an important component of innate immunity which provides quick body response to cancerous or virus infected cells before more specialized adaptive immunity is 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 NK92 cell line is used as the model for natural killer cells, and the hepatocellular carcinoma HepG2 cell line is used as the model for target cells. Viability of HepG2 cells following the addition of untreated or nanoparticle-treated NK92 cells is monitored in real time using a 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; 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 PBS (GE Life Science, Hyclone, SH30256.01)

3.1.2 Fetal Bovine Serum (GE Life Sciences, Hyclone, SH30070.03)

3.1.3 Horse Serum (GE Life Sciences, Hyclone, SH30074.03)

- 3.1.4 MEM, Alpha Modification (GE Life Sciences, Hyclone, SH30568.01)
- 3.1.5 RPMI-1640 (GE Life Sciences, Hyclone, SH30096.01)
- 3.1.6 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)
- 3.1.7 Myo-inositol (Sigma-Aldrich, I7508)
- 3.1.8 Folic Acid (Sigma-Aldrich, F8758)
- 3.1.9 2-Mercaptoethanol (Gibco, 21985-023)
- 3.1.10 Recombinant Human IL-2 (R&D Systems, 202-IL-010)
- 3.1.11 Trypan Blue solution (Gibco, 15250-061)

3.2 Materials

- 3.2.1 Pipettes ranging from 0.05 mL to 10 mL
- 3.2.2 Flat bottom 16 well E-plates
- 3.2.3 Polypropylene tubes, 5 and 15 mL
- 3.2.4 Reagent reservoirs
- 3.2.5 T25 culture flasks

Note : Certain models of RT-CES instrument can operate with 96-well E-plates

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 Biohazard safety cabinet approved for level II handling of biological material
- 3.3.6 Inverted microscope
- 3.3.7 Vortex
- 3.3.8 Hemacytometer
- 3.3.9 RT-CES instrument (ACEA Biosciences)

4. Reagent Preparation

4.1 Heat-inactivation of fetal bovine serum

Thaw a bottle with FBS at room temperature or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 m at 56°C in a water bath, mixing

every 5 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 medium (to maintain HepG2 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 NK92 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 the nanoparticle at a concentration 10 x above the highest final concentration to be tested. Nanoparticles should be dissolved/resuspended in alpha-MEM medium. 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 20 mg/mL will be prepared and 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 samples are combined in a T25 flask with 13.5 mL of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2mg/mL. Each nanoparticle concentration is tested in duplicate. Additional 200 µL is required for cell free control.

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.

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

6. Preparation of Effector and Target Cells

6.1 Preparation of NK92 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. Pipet the cells up and down on the back of the flask every 2-3 days to produce a single cell suspension. NK92 cells are extremely sensitive to overgrowth and media exhaustion. Replace with fresh medium every 2 to 3 days (depending on cell density).

6.2 Preparation of HepG2 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). Then, 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 5% CO₂ environment.

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

Day 1

- 7.1 Adjust effector cell (NK92) 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).
- 7.2 Treat NK92 cells with test nanoparticles, vehicle or negative control for 24 ± 0.5 hours. Perform treatment in T25 flask.
- 7.3 Adjust target cell (HepG2) number to 0.5×10^6 cells/mL using complete **RPMI**.
- 7.4 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 HepG2 cells from step 7.3 per each well in RT-CES plates except for nanoparticles only wells (please refer to the template in Figure 1 and remember that one needs 2 E-plates per each nanoparticle), attach to RT CES and start data acquisition. HepG2 cells are in culture for $\sim 16 - 20$ hr prior to addition of NK92 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	1.00	
2	Step 2	30.0	1
3	Step 3	2.00	500
	Step 3	10.0	30
4	Step 4	Idle	1000

Version B

Step #	Step Name	Interval (min)	Sweeps
1	Step 1	1.00	1
2	Step 2	30.0	200
3	Step 3	15.00	500

Day 2

- 7.5 Using Trypan Blue, determine viability of NK92 cells prepared in step 7.2.
- 7.6 Concentrate NK92 cells from step 7.5. by centrifugation (5 min, 400xg); remove and discard supernatants, and reconstitute cells in each sample with fresh alpha-MEM media to the 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. E:T ratio of 2.5:1 or 1.25:1 is also acceptable to use, and in this case the NK92 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 NK92 cells and resulted in more than 50% cell death, it may not be possible to evaluate the cytotoxicity of NK92 cells treated with these nanoparticles due to a lack of the required number of effector cells.

- 7.7 Pause RT-CES data acquisition program; remove RT-CES plates from the instrument and add 100 μL of NK cells from step 7.6 to designated wells on RT-CES plate. An example of a template is provided in Figure 1. Prepare two E-plates for each nanoparticle.
- 7.8 Return RT-CES plates containing NK92 effector cells treated with either nanoparticle or negative control and target (HepG2) cells to the instrument and resume data acquisition for another 24 hr.

Day 3

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

HepG2 only (no NK92)	HepG2 only (no NK92)
HepG2 Untreated NK92	HepG2 Untreated NK92
HepG2 NK92 + NC	HepG2 NK92 + NC
HepG2 NK92 + NP (2.0 mg/mL)	HepG2 NK92 + NP (2.0 mg/mL)
HepG2 NK92 + NP (0.2 mg/mL)	HepG2 NK92 + NP (0.2 mg/mL)
HepG2 NK92 + NP (0.04 mg/mL)	HepG2 NK92 + NP (0.04 mg/mL)
HepG2 NK92 + NP (0.008 mg/mL)	HepG2 NK92 + 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. Nanoparticles are tested at 4 concentrations. Testing of the “nanoparticles only” sample is recommended to identify potential nanoparticle interference with the RT-CES instrument acquisition system. During validation of gold nanoshells, citrated 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 NK92 to HepG2 cells, potential source of nanoparticles in wells of E-plates is the release from NK92 (assuming NK92 took up the particles); only 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

Example of cell index plot is shown in Figure 2. 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:

$$\%CV = \frac{\text{standard deviation}}{\text{mean}} * 100\%$$

$$\% \text{ Cytotoxicity} = \frac{(AUC_{\text{baseline}} - AUC_{\text{particles}})}{AUC_{\text{baseline}}} * 100\%$$

where AUC is area under the curve determined for HepG2 growth from the time of addition of NK2 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.

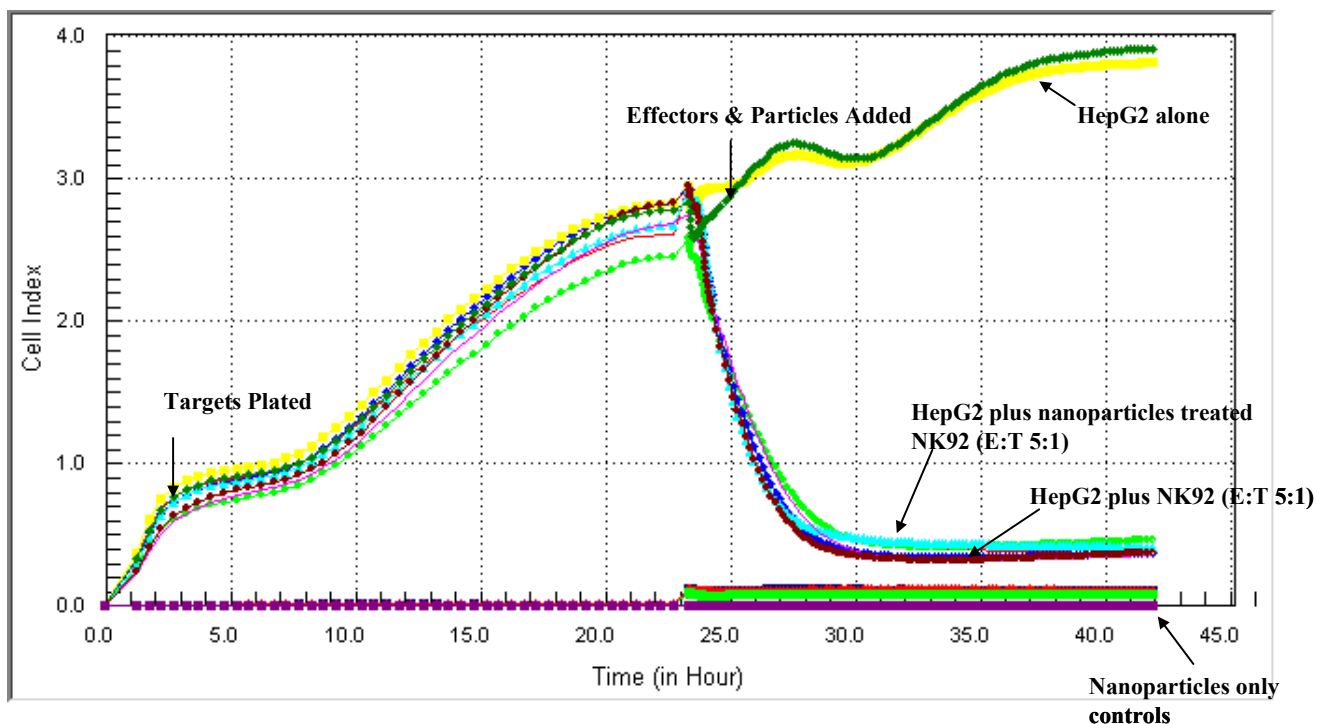
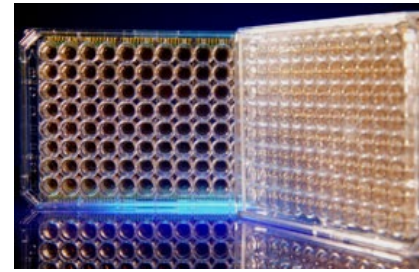
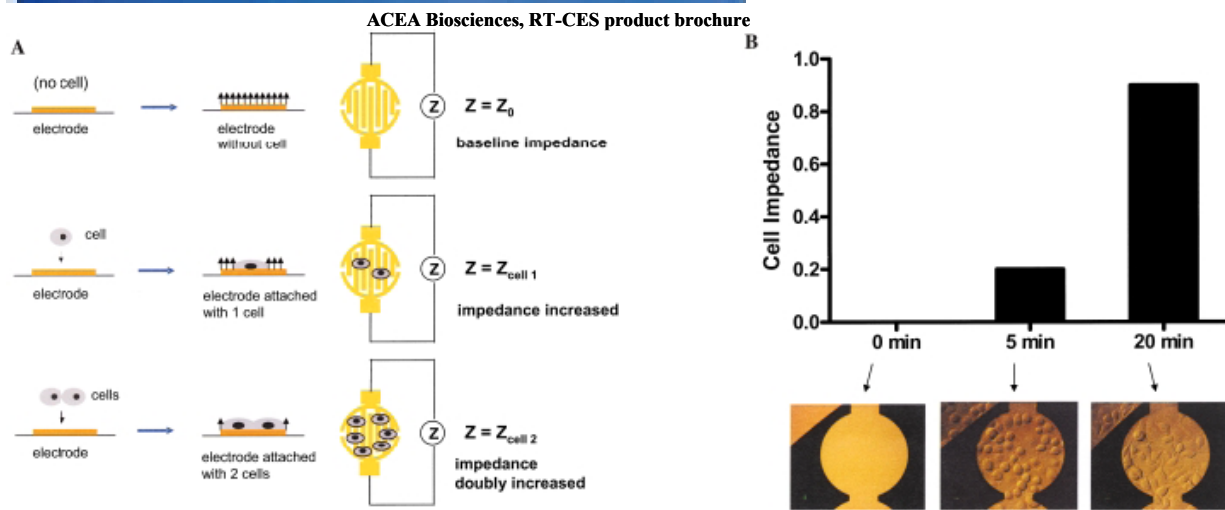


Figure 2. Example of raw data demonstrating changes in HepG2 cell index after co-incubation with NK92 cells.



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. The images above are reproduced from the reference 4, also named on the image, and are used for the informational purposes only.

9. Acceptance Criteria

- 9.1 CV of test samples and control should be within 25%.
- 9.2 Assay is acceptable if percent cytotoxicity in 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 Dec10; 172(2): 456-66
4. Solly K, Wang X, Xu X, Strulovici B, Zheng W. Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev Technol*. 2004;2(4):363-372. doi:10.1089/adt.2004.2.363

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