EXPERIMENTAL PROTOCOL: ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC) IN CO-CULTURE OF NK CELLS AND CELL LINES

Author/s: Navarrete-Sirvent C, Guil-Luna S, Aranda-Aguilar E, Rodríguez Ariza A.
Contact information: b32nasic@uco.es; v22gulus@uco.es

Introduction

Co-culture of NKs cells and cell lines is used when we want to perfom the antibody-dependent cell-mediated cytotoxicity assay (ADCC) and analyze the effect of Cetuximab, an IgG1, or other immunoglobulins, in the interaction between both cellular types. In particular, this assay quantitatively measures the production of lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis by the conversion of a tetrazolium salt (INT) into a red formazan product. For this, the amount of color formed is proportional to the number of lysed cells.

Reagents

- Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780)
- NK MACS Basal Medium and NK MACS Supplement (*see below*)
- Human IL-2 IS premium grade (*see below*)
- Human IL-15 premium grade (*see below*)
- Cetuximab (Erbitux 5 mg/ml)
- Panitumumab (Vectibix 20 mg/ml)
- PBS (tablets, *see below*)
- Bovine serum albumin (*see below*)

Instruments and tools

- Neubauer chamber
- Laminar flow cabinet
- 96-well flat-bottom culture plate compatible with a standard plate reader
- Round- or V-bottom 96-well tissue culture plates
- Multichannel pipettor
- Centrifuge with plate-bucket
- Plate reader capable of recording absorbance 490 nm

Before start

Preparation of Reagents:

Complete NK MACS Medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
NK MACS Basal Medium	Miltenyi Biotec/ 130-114-429	500 ml	100 ml	100 ml
NK MACS Supplement	Miltenyi Biotec/ 130-114-429	5 ml	1 ml	1%
Fetal Bovine Serum (FBS)	Biowest/S1810	500 ml	5 ml	5%
ZellShield®	Minerva Biolabs/ 13-0050	50 ml	1 ml	1%

Expansion NK MACS Medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
Complete NK MACS Medium	*See above*	100 ml	100 ml	100 ml
Human IL2S premium grade	Miltenyi Biotec/ 130-097-746 (200 µg)	200µg	2 ml	0.1 mg/ml
Human IL15 premium grade	Miltenyi Biotec/ 130-095-764 (25 µg)	25 µg	250 µl	0.1 mg/ml

CytoTox 96® Reagent (CytoTox 96® Non-Radioactive Cytotoxicity Assay)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
Assay Buffer	*See below*	60 ml	12 ml	-
Substrate Mix	*See below*	5 vials	-	-

Thaw Assay Buffer, remove 12 ml and stored the unused portion at -20ºC. Warm this 12 ml to room temperature protected from light and add to a bottle of Substrate Mix.

LDH Positive control (CytoTox 96® Non-Radioactive Cytotoxicity Assay)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
PBS (tablets)	Applichem/A9202	100 tablets	10 ml	1X
A1310 Albumin, Bovine Serum, Cohn Fraction V, pH7 (BSA)	US Biological/ 9048-46-8	100 g	For 10 ml	1%
LDH Positive Control	*See above*	-	2 µl	1:5000

PROCEDURE

Priorities:

1) Optimization of Target Cell Number
2) Antibody-dependent cell-mediated cytotoxicity assay (ADCC)
3) Calculation of results

Optimization of Target Cell Number

1. Prepare different target cells dilutions (i.e., 0, 1000, 2000, 3000, 4000, 5000, 7000, 10000, 20000/100 µl) in the same culture medium that will be used for cytotoxicity assays (i.e., Expansion NK MACS Medium).
2. Add 100 µl of cells per well to a round-bottom 96-well plate according to the plate order.
3. Lyse cells by adding 10 µl of Lysis Solution 10X per well and centrifugate plate at 250 g for 4 minutes.
4. Transfer 50 µl of supernatant from all wells to a new flat-bottom plate using a multichannel pipettor.
5. Add 50 µl of CytoTox 96® Reagent to all wells, cover the plate with foil to protect it from light and incubate for 30 minutes at room temperature.
6. Add 50 µl of Stop Solution to all wells, pop any large bubbles using a needle and record the absorbance at 490 nm.

NOTE: for select the Target Cell Number, it is important that the absorbance of the culture medium background (0 cells) is fewer that the absorbance of the selected dilution to achieve the best results, but not greater than the plate reader will be unable to record the absorbance.

Antibody-dependent cell-mediated cytotoxicity assay (ADCC)

We used Cetuximab (Erbitux 5 mg/ml) and Panitumumab (Vectibix 20 mg/ml) as monoclonal antibodies for the assays at a final concentration of 10 and 100 µg/ml.

Controls to prepare before to perform the assay:

- Culture Medium Background (CMB): add 100 µl of fresh culture medium used in the assay (Expansion NK MACS Medium).
- Volume Correction Control (VCC): add 100 µl of fresh culture medium used in the assay (Expansion NK MACS Medium) and 10 µl of Lysis Solution 10X.
- Target Cell Spontaneous LDH Release (TSR): add 100 µl of target cells at dilution determined in the last step to triplicate set of wells.

- Target Cell Maximum LDH Release (TMR): add 100 µl of target cells at dilution determined in the last step to triplicate set of wells. Forty-five minutes prior to the end of experiment (harvesting the supernatants), add 10 µl of Lysis Solution 10X.
- LDH Positive Control (LDH+): prepare a fresh dilution of PBS + 1% BSA + 2 µl of LDH Positive Control and add 50 µl of this for triplicate in the enzymatic plate (flat-bottom 96-well plate).
- Effector Cell Spontaneous LDH Release (ESR): add effector cells (i.e., NK cells) at each concentration used in experimental setup (i.e., Efector:Target cell 1:1, 10:1, 20:1 ratios) to a quadruplicate set of wells. The final volume must be the same as that in the experimental wells (i.e., 100 µl).
- Experimental wells: add 50 µl of target cells at dilution determined previously pre-treated (or not) with Cetuximab or Panitumumab.

1. Add the corresponding controls to a round-bottom 96-well tissue culture (*see below an example*).
2. Prepare a dilution of target cells like every time you take 50 µl, you are taking the total number cells you selected in the "optimization of target cell number" (i.e., for 5000 total cells per well, prepare a dilution of 100000 cells/ml).
3. For the selected treatments, prepare different stocks for Cetuximab and Panitumumab and add the corresponding volume to each well (or make a mix with the cells and the treatment).
4. Incubate target cells with their treatments during 1 hour in a humidified chamber at 37ºC, 5% CO_2.
5. Prepare different dilutions of effector cells (i.e., Efector: Target cell 1:1, 10:1, 20:1 ratios) and add 50 µl to appropriate well.
6. Centrifugate the assay plate at 250 g for 4 minutes to ensure effector and target cell contact.
7. Incubate the cytotoxicity assay plate for 4 hours in a humidified chamber at 37ºC, 5% CO_2.
8. Centrifugate the plate at 250 g for 4 minutes.
9. Transfer 50 µl of each well to a fresh flat-bottom 96-well compatible with standard plate reader using a multichannel pipettor.
10. Add 50 µl of CytoTox 96® Reagent to all wells, cover with foil to protect it from light and incubate for 30 minutes at room temperature.
11. Add 50 µl of Stop Solution to all wells, pop any large bubbles using a needle and record the absorbance at 490 nm.

Calculation of results

1. Subtract the average absorbance value for the CMB from all absorbance values for Experimental, TSR and ESR.
2. Subtract the average absorbance for the VCC from the absorbance values obtained for the TMR.
3. Use the corrected values to calculate percent cytotoxicity for each effector:target cell ratio.

$$\%Cytotoxicity = \frac{Experimental - ESR - TSR}{TMR - TSR} * 100$$

Example of an ADCC assay

- Effector cells: human NK cells obtained by isolation from human PBMCs (healthy donor) activated overnight with IL-2 (500 UI/ml) and IL-15 (140 UI/ml).
- Target cells: HT29 cell line.
- Culture Medium in Assay: Expansion NK MACS Medium.
- Target Cell dilution: 5000 cells/well in 50 µl medium.
- Effector cell ratios: 1:1, 10:1 and 20: 1
- Incubation: for hours at 37ºC, 5% CO_2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CULTURE BACKGROUND (CMB)			VOLUMEN CORRECTION (VCC)			TARGET SPONTENEOUS RELEASE (TSR)			TARGET MAXIMUM RELEASE (TMR)		
B	NKs			NKs			NKs					
C	HT29 + NKs			HT29 + NKs			HT29 + NKs					
D	HT29 + NKs + 10 µg/ml Ctx			HT29 + NKs + 10 µg/ml Ctx			HT29 + NKs + 10 µg/ml Ctx					
E	HT29 + NKs + 100 µg/ml Ctx			HT29 + NKs + 100 µg/ml Ctx			HT29 + NKs + 100 µg/ml Ctx					
F	HT29 + NKs + 10 µg/ml Pab			HT29 + NKs + 10 µg/ml Pab			HT29 + NKs + 10 µg/ml Pab					
G	HT29 + NKs + 100 µg/ml Pab			HT29 + NKs + 100 µg/ml Pab			HT29 + NKs + 100 µg/ml Pab					
H												
	Ratio 1:1			Ratio 10:1			Ratio 20:1					