Institut für HIV Forschung
SOP# 15-00 **(April 2015)**

NK Cell Degranulation Assay

Reagents

Reagent	Vendor	Catalogue #	Stock Conc.
FACS Tubes	BD Falcon	352054	-
P815 Ab=rabbit anti-mouse lymphocyte	Accurate Chemical	AIA3940	-
GolgiPlug (Brefeldin A (BFA))	BD	555029	-
GolgiStop (Monensin)	BD	554724	-
Cytofix/Cytoperm Kit	BD	554722	-
PMA	Sigma	1585	1mg/mL
Ionomycin	Sigma	141128	1mg/mL
RPMI-1640	Sigma	R0883	-
Pen/Strep	Cellgro	30-001-Cl	Pen: 5000 IU/mL Strep: 5000ug/mL
L-glutamine	Cellgro	25-002-Cl	200mM; 29.2mg/mL
HEPES (1M; 238.3mg/mL)	Cellgro	25-060-Cl	1M
FBS, Heat-inactivated	Sigma	F4135	-
PBS	Sigma	D8537	-

Note: addition of GolgiStop is necessary AND sufficient to look at CD107a as a marker for degranulation. A combination of GolgiStop and BFA improves intracellular staining for cytokines such as IFN-gamma. Please keep in mind that CD69 expression is blocked by BFA, and that expression of some surface markers is affected by these inhibitors.

Media:

R10

Reagent	Stock Concentration	Volume to Add	Final Concentration
RPMI 1640	-	500	-
FBS	100% (v/v)	55ml	10% (v/v)
Penicillin/Strep	Pen: 5000 IU/mL Strep: 5000ug/mL	5.5mL	Pen: 50 IU/mL Strep: 50ug/mL
L-glutamine	200mM	5.5mL	2mM
HEPES	1M	5.5ml	10mM

PMA

Working solution: 1mg/mL. Reconstitute in DMSO (1mg in 1mL)
Prepare 20uL aliquots and keep at -20C
Final concentration: 100ng/mL. Prepare a 1:10 dilution and add 1uL/mL culture

Ionomycin

Working solution: 1mg/mL. Reconstitute in DMSO (1mg in 1mL)
Prepare 10uL aliquots and keep at -20C

Final concentration: 1ug/mL. Add 1uL/mL culture

Assay

Note: *The assay should ideally be performed using fresh PBMCs that have been isolated within 6h following collection in ACD tubes. However frozen PBMCs can be used.*

1. Isolate PBMCs as described in SOP #08-00

Note: *If necessary, this assay can be performed using enriched PBMC subpopulations such as CD3-depleted PBMCs or NK cells. In this case, use the relevant RosetteSep cocktail from StemCell Technologies according to manufacturer instructions before the ficoll procedure.*

2. Count PBMCs and resuspend the pellet in R10 at $1x10^6$/mL.

3. Count target cells from stock in culture

Note: *Here is a list of target cells commonly used to test NK cell function. These can be substituted by any other relevant cell line.*
K562 cells: MHC-I-deficient, express NKG2D ligands, maintained in R10
221 cells: MHC-I-deficient, express NCR ligands, maintained in R10
P815 cells: murine cell line expressing Fc receptors, can be either coated with p815 antibodies in order to assess ADCC function, or coated with any specific Ab to perform a redirected-lysis assay. To coat p815 cells, add 10ug/mL Ab (10uL of stock) to 1 million p815 cells in 1 mL R10, incubate for 1h at 37C, wash and resuspend pellet in 1mL R10. In parallel, prepare 1 million of uncoated p815 to use as control.

4. Put 1 million target cells in a FACS tube or a 15mL conical (adjust amount of cells if more than 10 patients)

5. Spin 350xg, 10min. Discard supernatent

6. Resuspend pellet at $1x10^6$/1mL R10

7. Put 1mL PBMCs in each reaction tube (FACS tube) and set up your experiment as follows:
    a. Unstimulated control: no target cells
    b. Stimulation control: add 1uL of PMA and 1uL of ionomycin
    c. Add 100uL of 221 cells
    d. Add 100uL of K562 cells
    e. Add 100uL of uncoated p815
    f. Add 100uL of Ab-coated p815 cells
    g. Any other cell line to be tested……..

Note: *100uL of target cells results in an E:T ratio of 10:1 which is optimal when using whole PBMCs and the abovementioned target cells. If testing other stimuli or cell lines, it is recommended to try other ratios (5:1 or 20:1). If using pure NK cells, start with 1:1 or 2:1 E:T ratios.*

8. In each tube, add 1uL of GolgiStop/mL and a CD107a conjugated antibody. If looking at cytokine-producing cells by ICS, add 1uL BFA/mL. If more than 3 tubes, prepare a master mix.

9. Incubate the PMA-ionomycin reaction tube for 2h at 37C, then put at 4C protected from light. Longer incubation time results in massive cell death.

10. Incubate other reaction tubes for 4-6H at 37C, then proceed with the staining or put at 4C O/N protected from light. **If stimulation requires more than 16h of incubation, add the CD107a Ab and GolgiStop/BFA only for the last 6h of incubation**.