aCella–TOX™

Bioluminescence Cytotoxicity assay

U.S. Patent #: 6,811,990 (2004)

ATTENTION:

Added CDC and Stop Reaction in January 2012.

FAQ's added in October 2010.

PROTOCOL VERSION 1.3 – NK cell protocol added in Feb 2009.

Please contact techsupport@celltechnology.com or call us at 650-960-2170 with any questions.

Acknowledgements:

We would like to thank Cambridge Antibody Technology (http://www.cambridgeantibody.com/), Milstein Bldg, Granta Park, Cambridge, UK, for their continued support and assistance in optimizing our published ADCC protocol.

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I: Introduction

Cell Technology introduces ***aCella-TOX***, a new and highly sensitive assay that employs our recently patented Coupled Luminescent Technology for the detection of cytotoxicity. This assay can also be used to detect cytotoxicity in **primary cells**. The principle of the assay is quantitative measurement of the release of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) from mammalian cell lines or bacterial cells [1,2,3,4]. This enzyme is abundantly present in all known living cells. Other enzyme release assays, such as the Lactate Dehydrogenase (LDH) release assay [5,6,7,8], suffer from low sensitivity as a result of interference by serum or phenol red present in the media. ***aCella-TOX*** can work in the presence of both of these media constituents and allows overnight assay protocols while retaining sensitivity.

II: Assay Principle

GAPDH is an important enzyme in the glycolysis pathway. This homotetrameric enzyme catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. In the aCella-TOX scheme, the release of GAPDH from dying cells leads to ATP production, which is then coupled with the luciferase/luciferin Bioluminescence methodology producing light.

III: Measurement Modes

Cytotoxicity. aCella-TOX may be added directly to either a cell culture or supernatant to detect enzyme released from cells that have lost membrane integrity. It is not necessary to remove live cells prior to measurement. No form of pre-treatment is needed. The luminescent readout begins to rise immediately and may be read as early as a few seconds after addition of the reagent, or up to 2 hours later. In this mode the assay is non-destructive and continuous, allowing monitoring of additional parameters such as gene expression.

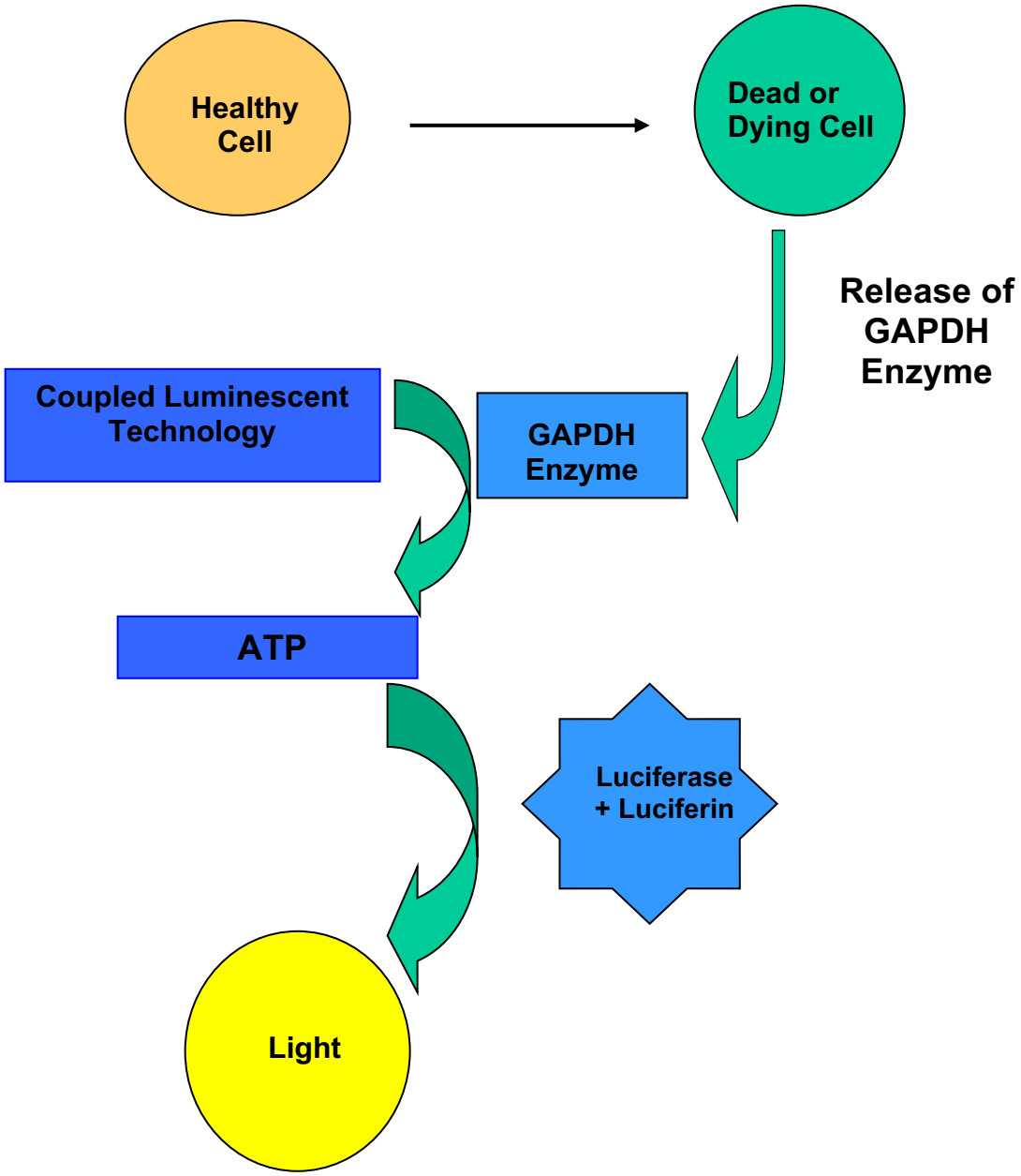
Note: When setting up cellular mediated cytotoxicity assays, it is important to include controls to distinguish from spontaneous **(1)** effectors cell death vs. **(2)** effector cell mediated target cell death. This can be accomplished by including control wells of effector cells alone at their various effector: target cell ratios to measure their spontaneous cell death. Target cell death is represented by the difference between the two measurements (**2-1**).

Cytotoxicity/Proliferation (Dual Mode). aCella-TOX may be used to measure both cytotoxicity and cell viability (or proliferation). Cells are grown in 200µL of media.

1. For cell viability: spin down plate and pipette 100µL of cell free supernatant in a white opaque 96 well plate. Add aCella-TOX reagents as described in the protocol.
2. For cell proliferation: to the remaining 100µL of cells add the lytic reagent as described below and proceed with the aCella-TOX reaction. Viability is represented by the difference between the two measurements.

Time-Point and Kinetics Methods The generation of ATP being an ongoing process, readings can be measured at specified time intervals and the kinetics of the reaction plotted. A 1:1 ratio of sample (supernatant) and Enzyme Assay Reagent (see below) should be maintained in order to maximize signal to noise ratios.

Assay Principle



IV: Procedures

4.1 Kit Contents:

Catalog # - CLATOX100-3 (500 tests):

Component Description	Part #	Volume	Storage
1. **Component 1:** 4X Enzyme Assay Reagent	6001	26 ml	-20°C to -40°C
2. **Component 2:** 1X Enzyme Assay Diluent	3008	60 ml	2-8°C
3. **Component 3:** Glyceraldehyde 3-Phosphate (G3P)***check label for conc.	6003	≈0.20 ml	-20°C to -40°C
4. **Component 4:** 50X Detection Reagent (Luciferase/Luciferin)	6002	0.55 ml	-20°C to -40°C
5. **Component 5:** 5.5X Detection Assay Diluent	3009	5.5 ml	-20°C to -40°C
6. **Component 6:** Lysis Buffer	3035	5.0 ml	2-8°C

CLATOX100-4 (1000 tests) is supplied as 2 X CLATOX100-3 kits.

CLATOX100-3 L: 5 x 96 well white luminescence plates included with the above 500 test kit.

CLATOX100-3 P: 5 x 96 well white luminescence plates included + 5 x 96 well Tissue Culture plates also included with the above 500 test kit.

4.2 Equipment Required:

96 well plate Luminometer.
Centrifuge with micro plate rotor attachment.

4.3 Materials and Reagents Required but not supplied:

1. **Opaque White** 96 well plates for luminometer*. (Perkin Elmer Optiplates#6005290 or Corning #3605 with cover, #3099)
   IMPORTANT: Please refer to luminometer guide for appropriate plate specifications.
2. Leukopack, LRS chamber or Buffy pack. (source of fresh blood)
3. ADCC Culture media (RPMI 1640 supplemented with 10% low IgG FBS, GIBCO #16250, NEAA and PSG).
4. Histopaque, Sigma cat.#1077 or Ficoll-Paque Plus from GE Healthcare, cat.# 17-1440-02
5. Sterile-filtered solutions of 0.2% NaCl and 1.6% NaCl for RBC Lysis.
6. Sterile filtered solution of 1XPBS with 2mM EDTA
7. Miltenyi Biotec MidiMACS Starting kit, (Cat.# 130-042-301) including MidiMACS separation unit, 1 MACS Multistand, 25 LS columns and CD56 Microbeads (Cat.#130-050-401). Pre-separation filters (Cat.# 130-041-407LS) are not supplied, but may be required if cell pellet is clumpy.
8. Target cells such as Daudi, Raji, Ramos or breast cancer cell lines such as SKBR3 or MCF7 etc.
9. An antibody such as Commercial Rituximab (humanized Anti CD20), Herceptin (anti humanized HER2/neu) or other test antibody for ADCC.
10. Trypan Blue
11. 96 well U-bottom, sterile tissue culture plate for ADCC or CMC reaction. (TPP cat.# TP92097 available from Mid-Sci)

4.4 Schematic Representation of the aCella-TOX assay:

50µL of reaction supernatant from ADCC or CMC reaction

+

50µL of Component 2 (Part # 3008: Enzyme Assay Diluent)

⇩

100µL of 2X Enzyme Assay Reagent (2X Component 1: Part# 6001+ Component 3: Part #6003:G3P)

⇩

50µL of 1X Detection Reagent (1X Component 5: Part #3009 + Component 4: Part # 6002: 50X Detection reagent)

⇩

Read on luminometer.

4.5 Reagent set up:

1. The **4X Enzyme Assay Reagent:(Component 1): Part #6001** (**Green label**)
For 100 tests (one 96 well plate), 10 ml of the 2X reagent is required.
Component 1 is a frozen 4X liquid concentrate. To thaw the contents, the vial should be placed in a lukewarm water bath, or allowed to equilibrate to room temperature by leaving on the bench or on ice for about half hour. **DO NOT thaw in incubator or subject contents to high temperatures.** Upon thawing, immediately aliquot and freeze the rest between
-20^{0}C and -40°C in polypropylene tubes; if you are not using the complete contents of the vial. (Short term storage: Ice)
10 ml of the 2X cocktail is required for 100 tests (one 96 well plate).
To make a 2X cocktail, dilute the 4X concentrate 1:1 with the Enzyme Assay Diluent (**Component 2)**. For example, to 5mL of 4X enzyme assay reagent, add 5mL of Assay Diluent and **add G3P (Part # 6003; Component 3) just prior to use**. For dilution, see step 2.

Cautionary Notes:
Avoid repeated freeze thaw cycles.
If 5 plates of experiments are planned, dilute the entire contents of Part#6001 1:1 with Part#3008. Make 5 aliquots of 10 ml each for 500 reactions. Store aliquots at 4°C. Bring each aliquot to room temperature about 10 minutes prior to addition. The G3P (Part# 6003) should be added to the 2X solution IMMEDIATELY before the reaction.

Preparation of 2X Enzyme Assay Reagent:

For 250 reactions on 4 plates,
13ml of 4X Enzyme Assay Reagent (Component 1: Part# 6001)

+

13 ml of Enzyme Assay Diluent (Component 2: Part # 3008) makes 26ml of 2X Enzyme Assay Reagent

⇩

Aliquot 6ml per tube and store at 4°C or on ice

⇩

Equilibrate each tube for 10 minutes at RT

⇩

Add 21µL G3P (Component 3: Part #6003) just before adding to reaction wells.

2. **Glyceraldehyde 3-phosphate (Component 3): Part # 6003 (Green label)**
Next **quickly** thaw **Component 3** (G3P). Check vial label for concentration in mg/ml.

KIT LOT #	G3P concentration on vial label in mg/ml	Volume in **µL** to be added **per mL** of 2X cocktail
	45	3.9
KN21110	46	3.8
KN10306, KN20406, KN20606, KN50507, KN20112	47	3.7
	48	3.6
	49	3.6
KN40906, KN20906, KN40108, KN30708, KN21008, KN20409, KN20909, KN20210, KN30210	50	3.5
	51	3.4
	52	3.4
KN11218	53	3.3
	54	3.2
	55	3.2

Aliquot G3P into single use polypropylene vials, and store between -20°C and -40°C.
Use the above table to determine the volume of G3P to be added to the 2X Enzyme Assay Reagent prepared above.
For example, if G3P concentration on vial is 50 mg/ml, add 3.5µL of Component 3 (G3P) to each mL of 2X Enzyme Assay Reagent (from step 1 above). Therefore, to 10 ml of 2X Reagent prepared, add 35 µL of G3P to make sufficient cocktail for 100 reactions. Prepare sufficient amount of this Enzyme Assay Reagent for a full day of use (short term storage: on ice).
Add 100µL of this Enzyme Reaction Cocktail containing G3P to every 100µL of sample.
Cautionary Notes:
Avoid repeated freeze thaw cycles. Any left over 2X Enzyme assay Reagent should be stored at -20°C WITHOUT G3P to avoid high background.

3. The 5.5X Detection Assay Diluent: (Component 5): Part # 3009 (Red label)
Thaw Component 5. This is a 5.5x concentrate. Dilute the contents 1: 5.5 with reagent grade Di water to make it a 1X diluent. For example, for 100 reactions, make 5.5 ml of 1X diluent by adding 1ml of the 5.5X Detection assay diluent to 4.5mL of reagent grade Di water. Any unused material can be aliquoted and stored frozen between -20^{0}C and -40°C in polypropylene tubes.
Note: Prepare as much of the 1X Detection Reagent Diluent (Part # 3009) as needed for the day's experiments. Prepare aliquots of required volume per plate and store at 4°C. Bring to room temperature about 10 minutes prior to addition. Add the 50X Detection Reagent (Part # 6002) to the diluent **IMMEDIATELY** before the reaction.

Preparation of 1X Detection Reagent:

For 250 reactions on 4 plates,
2.75ml of 5.5X Detection reagent Diluent (Part # 3009: Component 5) + 12.375ml of di water makes 1X Detection Reagent Diluent

⇩

Aliquot 3.5ml per tube and store at 4°C or on ice.

⇩

Equilibrate each tube for 10 minutes at RT

⇩

Add 70µL of 50X Detection Reagent (Component 4: Part # 6002) just before adding to reaction wells.

4. The 50X Detection Reagent: (Component 4): Part # 6002 (Red label)

This is a frozen 50X concentrate containing Luciferase and Luciferin. Prior to use, thaw the vial and keep on ice. Aliquot as single-use portions in polypropylene vials and freeze the rest between -20^{0}C and -40°C, if you are not using the complete contents of the vial.
Just before adding the detection reagent to your sample, dilute it 1:50 with the 1X **Detection Assay Diluent** prepared in step 3 above. For 100 reactions, 100µL of the 50X Detection Reagent is added to 4.9ml of the 1X Detection Assay Diluent to make 1X Detection Reagent.
Add 50µL of the 1X Detection Reagent to each sample in the well containing 100µL of cells +100µL of the 2X Enzyme Reaction Cocktail.

Cautionary Notes:

1. Keep contents protected from direct light at all times.
2. Avoid repeated freeze thaw cycles.
3. Luminometer sensitivity: If the RLU values are low, it may indicate that the luminometer is not very sensitive. In such a case, we recommend titrating the Detection reagent (Part #6002) to a 1:25, 1:10 and a 1:5 dilution in Step 4 above to determine a suitable strength. Please note that this will reduce the number of reactions that can be performed using one aCella-TOX kit to 250, 100 or 50 tests depending on the dilution used.
We have found that different luminometers have different sensitivities. We have tested the BD Monolite, Berthold, PE Victor, PE Top Count and the Veritas. The assay has been optimized to work on the Veritas luminometer from Turner Biosystems.

5. Lysis Buffer: Component 6: (Part #3035) (Yellow label).

Add 10µL of lysis buffer as a positive control for total release of GAPDH. Store lysis buffer at 4-8^{0}C. Equilibrate to room temperature before use.

Note: For some cell lines, it may be necessary to perform a titration of the lysis buffer to optimize cell lysis. Use the minimum amount of lysis buffer that gives the maximum signal in a titration with 10,000 cells. (Substituting with Triton X 100 as the lytic agent will severely reduce the assay signal.)

V. Assays

General Guidelines:

Cell Technology's non-radioactive aCella-TOX kit can be used to efficiently and accurately measure Antibody Mediated Cellular Cytotoxicity (ADCC), Cellular Mediated Cytotoxicity (CMC) and Complement dependent cytotoxicity (CDC). The following guidelines will help to ensure the success of assays using aCella-TOX. Since the aCella-TOX kit measures the release of the GAPDH enzyme as an indicator of ADCC/CMC, it is highly recommended to use fresh effector cells when using whole blood. Peripheral blood lymphocytes (PBL's) contain a heterogeneous population of cells, many of which are not involved in the ADCC/CMC reactions.

We highly recommend using freshly isolated PBL's or fresh immunoaffinity purified effector cells (for example NK or $CD8^+$ T lymphocytes). Cytokine-activated PBL's [9,10] may also be employed in this assay. The use of cytokines, however, may cause the assay to exhibit a higher donor-to-donor variation (CMC) as each donor's NK cells may be stimulated to a different extent by the cytokine. The use of 10% low IgG serum helps reduce background and increase sensitivity of the ADCC assay. Avoid using serum with high hemoglobin contamination (Hb levels <6.5 mg% desirable) as this will increase background and reduce assay sensitivity. Avoid using wells on the edge of the plate, as this will increase assay signal variance due to the "edge effect". These edge wells should be filled with media or PBS.

Before starting ADCC/CMC experiments, we recommend performing the following preliminary assays:

1. Cell titration on target cells to determine the optimal number of cells to be plated.

2. Determination of the optimal effector: target (E:T) ratio by using varying ratios of effector cells (for example, 5 to 25) keeping the number of target cells and antibody concentration constant. (for ADCC Assays)

3. For ADCC Assays: Antibody titration to determine the dynamic range required to obtain an optimal dose-response in the ADCC assay.

4. We recommend adapting the target cells in low IgG media for a few passages before the assay.

5.1 Target cell titration:

GAPDH expression will vary between target cell lines. It is important to determine the linear response range of aCella-TOX within your particular target cell line. This can be accomplished by titrating the target cells in the assay media (we recommend 20,000 to 1000 cells/well), adding lysis buffer to each well (as described in maximum lysis control below). Next add enzyme assay reagent and detection reagent as described below and measure luminescence. For further experiments, use the cell concentration that falls in the linear range of the assay.

See example below:

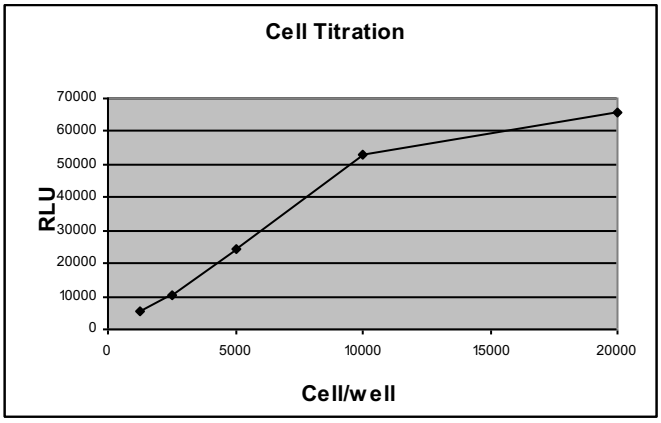


Figure: Cell titrated in media, lysed and run as described above. For further assays, use cell concentration at or below 10,000 cells/well. 20,000 cells/well is out of linear range in this particular cell line.

Linearity: Prolonged incubation with aCella-TOX can cause your assay to fall out of linear range. The enzyme reagents are constantly producing ATP until one of the components becomes limiting. Take several time point readings 5 min to 25 min to get an optimized time point linear readout.

5.2 Determination of optimal E:T ratio:

Effector cells should be titrated from 5:1 to 25:1 or greater (depending on the purity). If purified NK cells are used, E:T of 10:1 or 15:1 could work well. If PBMC's are used, the E:T ratio would have to be higher, possibly 25 or 30:1. This should be determined empirically, and would vary depending on the target cells used.

As an example, an antibody concentration of 1µg/ml is appropriate for this experiment.

Appropriate controls would be media alone, target cells alone and control including the maximum lysis of the target cells. For each effector: target (E:T) ratio, controls with effectors alone in the appropriate number and a zero antibody control (for CMC) have to be included as well.

A sample template would be as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		2E	ADCC 2E	CMC 2E	5E	ADCC 5E	CMC 5E	10E	ADCC 10E	CMC 10E		
C		2E	ADCC 2E	CMC 2E	5E	ADCC 5E	CMC 5E	10E	ADCC 10E	CMC 10E		
D		2E	ADCC 2E	CMC 2E	5E	ADCC 5E	CMC 5E	10E	ADCC 10E	CMC 10E		

E		15E	ADCC 15E	CMC 15E	20E	ADCC 20E	CMC 20E	targets	Max lysis	media		
F		15E	ADCC 15E	CMC 15E	20E	ADCC 20E	CMC 20E	targets	Max lysis	media		
G		15E	ADCC 15E	CMC 15E	20E	ADCC 20E	CMC 20E	targets	Max lysis	media		
H												

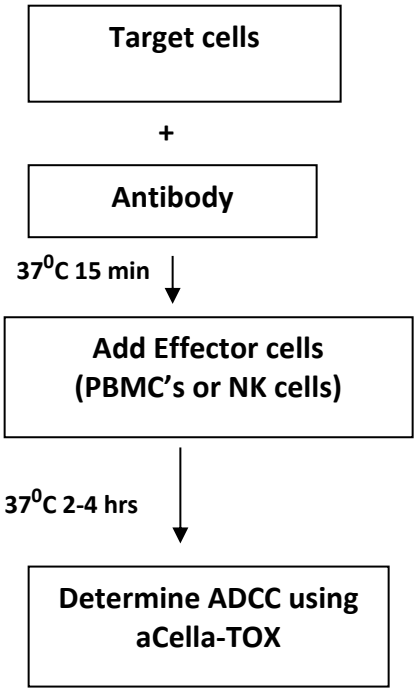
5.3 Antibody titration:

Prepare 1:4 or 1:5 serial dilutions of the antibody to be tested to cover a dynamic range over four orders of magnitude. As an example, a starting point could be 10 µg/ml.

The E:T ratio as determined from the experiment above should be used. All appropriate controls should be included in triplicates. (targets alone, effectors alone, media and maximum lysis of targets).

VI. Antibody Dependent Cellular Cytotoxicity (ADCC) PROCEDURE

6.1 Schematic Representation of an ADCC Assay:



Target to Effector cell ratios:

As outlined in section V above, we recommend using 5,000-10,000 target cells per well with a effector:target cell ratio between 10:1 to 20:1 (E:T ratio as determined by the experiments detailed in Section V above). Since the aCella-TOX assay is very sensitive, it is recommended to use lower ratios of effector:target cells; however, each investigator should determine this empirically.

6.2 For target cells in Suspension:

A. ADCC protocol overview:

1. Plate the target cells at 5000 cells per well in 25µL.
2. Add 25 µL of antibody to the target cells and incubate in a 37°C incubator for 15 minutes.
3. Add 25µL of effector cells and incubate the plate for 2-4 hours in a 37°C incubator.
4. Proceed with the aCella-TOX reaction.

B. Preparation of target cells:

1. On the morning of the experiment, spin down 8-10 ml of target cells. Wash once in PBS containing EDTA. Resuspend in ADCC culture media containing 10% low-IgG serum.

2. Check cell count with Trypan Blue stain and adjust cells to a concentration of $2X10^5$ cells/ml. Plating 25µL of this will give 5,000 cells/well. Cell concentration can be adjusted to plate cells at a lower or higher concentration.

C. Separation of PBMC's from leukopack or LRS chamber:

If using an LRS chamber, the blood should be diluted with 2X volume of PBS/BSA/EDTA before loading onto an equal volume of the density gradient.

1. Pipette 20-25ml of Histopaque (or Ficoll Paque or other suitable density gradient medium) into a 50 ml tube. Layer 20-25 ml of diluted leukopack on the Histopaque. Centrifuge at 500xg for 30 minutes. (slow stop; no brakes)

2. Carefully pipette the top serum layer into a separate tube. Aspirate the middle layer containing the PBMCs into another tube. Wash the PBMC's with 1-2 volumes of 1X PBS containing EDTA twice. Centrifuge at 500xg for 15 minutes.

3. Repeat step 2. After the final wash, decant the supernatant and gently vortex the cells pellet. Proceed to step D (Lysis of Red Blood Cells) only if using PBMC's for the assay. If using NK cells, you may skip this step.

D. Lysis of Red Blood Cells. (please skip this step if isolating NK cells)

1. Resuspend cell pellet in 10-20ml of 0.2% NaCl for **30 seconds** to lyse RBCs (gently agitate the tube to ensure proper mixing) and immediately add 10-20 ml (equal volume) of 1.6% NaCl to neutralize the osmolarity of the cells. Do not exceed 30 seconds in 0.2% NaCl. After the addition of the 1.6% NaCl, proceed to the centrifugation step immediately.

2. Centrifuge at 500xg for 5-10 minutes and resuspend pellet in 2 to 5ml of ADCC culture media containing 10% low-IgG serum.

3. Count cells with Trypan blue at a 1:100 dilution. At this time point isolated PBMC's can be used directly in the ADCC assay or incubated overnight in the ADCC culture media.

Note: Depending on the donor, it is possible that the RBC lysis step may have to be repeated if the RBC count is too high. If the supernatant is cloudy, repeat the wash step with PBS/EDTA to remove the remaining platelets.

E. NK cell isolation:

We recommend the use of NK cells isolated using CD56 beads by Positive selection in ADCC assays (using Miltenyi Biotec kit Cat# 130-050-401). Isolated NK cells can be used immediately or cultured overnight. The use of isolated NK cells gives a better dose-response curve due to a lower background, and therefore, a higher signal-to-noise ratio.

http://www.miltenyibiotec.com/download/datasheets_en/58/DS130_050_401.pdf

Adjust effector cell concentration to deliver, in 25 µL, the optimized E:T ratio. If culturing cells overnight wash the cells in ADCC media at the time of the assay.

F. Preparation of Antibody (*e.g.*, Rituximab (Humanized Anti-CD20)):

1. Dilute the antibody to 3µg/ml in ADCC culture media. Next serially dilute the antibody 5-fold 7 times across the plate. Plating 25µl of each antibody concentration per well will yield a range from 1ug/ml to $6.4X10^{-5}$ µg/ml (final concentrations). Starting antibody concentrations are 3X since they will be subsequently diluted 1 in 3 by the addition of target and effector cells.
2. For the zero antibody control, plate 25 µL of media only (see section G. Control 5 below).
   Note: Perform reactions in triplicate.

G. Controls:

1. **Control 1:** Spontaneous release of GAPDH from target cells: Plate 25µL containing 5,000 target cells in triplicate and add 50µl of ADCC media.
2. **Control 2:** Spontaneous release of GAPDH by effector cells: Plate 25µL containing effector cells (the same ratio as used in the experiment) and add 50 µL of ADCC media in triplicate.
3. **Control 3:** Maximum lysis of target cells: Plate 25µL containing 5,000 target cells in triplicate and add 50 µL of ADCC media.
4. **Control 4:** Media only control: Plate 75µl of the ADCC media in triplicate.
5. **Control 5:** As mentioned above, a zero Antibody control, 25uL of media+ effector cells + target cells. This reaction represents cell-mediated cytotoxicity (CMC) that occurs due to the killing of the targets by the effectors without the mediation of the antibody.

H. Preparation of aCella-TOX reagents:

The reagents supplied in the aCella-TOX kit are light sensitive. Avoid direct light, as in a bio-safety hood. Indirect lab light is acceptable for short exposures (15-20 minutes). Reduce light levels in the lab if possible. Follow the step-by-step directions described in the section "**aCella-TOX: Reagent Set Up**" (**Section 4.5** above) of the protocol supplied with the kit to make working stocks of reagents. The reagents should be at equilibrated to room temperature before adding them to the ADCC assay.

I. ADCC Reaction:

1. In setting up the plate, plan to avoid using the outer rows and columns to reduce any "edge effects". Add only 200µL of ADCC culture media or PBS to these wells.
2. The target and effector cells should be counted and set up at required cell counts. The serial dilutions of the antibody should be ready for use in the required volumes.
3. Plate the target cells at 5000 cells/well in 25µL of media in triplicate in a sterile, U-bottom, 96-well tissue culture plate.
4. Set up all controls as mentioned above.
5. Plate 25 µL of each dilution of the antibody in the wells containing the target cells.
6. Incubate the plate at 37°C for 15 minutes to allow opsonization of antibody to occur. (This optional step should be included based on the target cell line.)

7. Add 25µL of the effector cells to start the reaction. Spin down plate for 1 minute at 500g to bring targets and effectors in close contact.
8. Cover the plate with a 96-well plate cover. Incubate the plate at 37°C for 2-4 hours. The time of the reaction should be determined empirically.
9. At the end of the incubation, allow the plate to equilibrate to RT for 15 minutes.
   Add 10µL of lysis buffer (Component 6, Part #3035) to the wells indicated for maximum lysis of target cells. Incubate for 5-10 minutes at RT. (monitor lysis under microscope)
10. Add 125µL of low-IgG serum media to all the reaction wells to bring the volume to 200µL. Spin down plate in a micro plate centrifuge for 1' at 750Xg.

11. In an opaque plate for the luminometer (Perkin Elmer Optiplates#6005290), add 50µL of Enzyme Assay Diluent (Component 2, Part #3008) to all corresponding reaction wells. Now carefully transfer 50µL of the supernatant to 50µL of Component 2 using a multichannel pipettor, changing tips each time.
12. Add 100µL of the Enzyme Assay reagent containing G3P (from step IV: Procedures above) to all reaction wells.
13. Immediately, add 50µL of the Detection reagent (from step IV: procedures above) to each well.
14. Read the plate on the luminometer **immediately** (without the cover) and take several time point readings 5 minutes apart to determine the optimal reading time-point.

J. Calculations:

To determine ADCC, first calculate the mean of the triplicate luminescent values for each of the reaction conditions.
First subtract the mean media only luminescent value (control 4) from all calculated mean values. Then use the following formula to calculate % ADCC.

$$\% \text{ ADCC} = \frac{\text{(Sample)-(Control 1 Target spontaneous release)-(Control 2 effector alone)}}{\text{(Control 3 maximum release) - (Control 1 Target spontaneous release)}} \times 100$$

The equation above normalizes the data for %ADCC by subtracting the spontaneous target release from the maximum lysis control.

For the dose-response curve, plot the log of the Antibody concentrations in mg/ml vs. the % ADCC as calculated by the formula above.

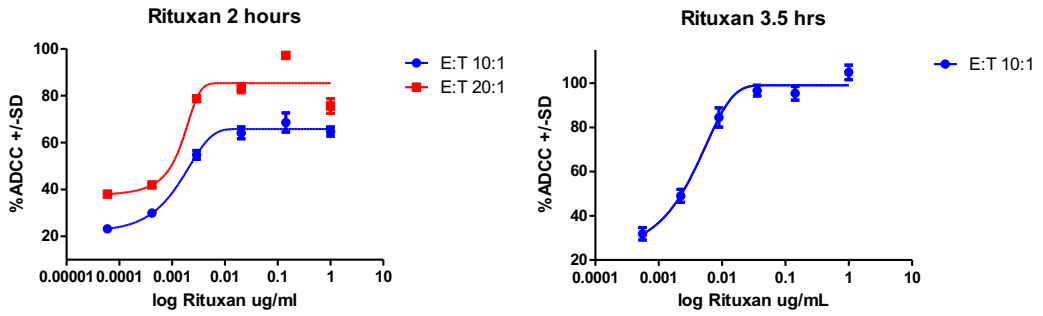


Figure: 5000 Ramos cells/well were incubated with serially diluted Rituxan antibody for 15 minutes prior to the addition of purified NK cells stimulated overnight with 10 ng/ml IL-2. The ADCC reaction was further incubated for 2 hours (top graph) or 3.5 hours (bottom

graph) at the specified E:T ratios. % Cytotoxicity was measured using the aCella-TOX assay. 3.5hour time point has been optimized with the 10:1 E:T ratio. Graph with 20:1 E:T ratio is flat.(100% cytotoxicity)

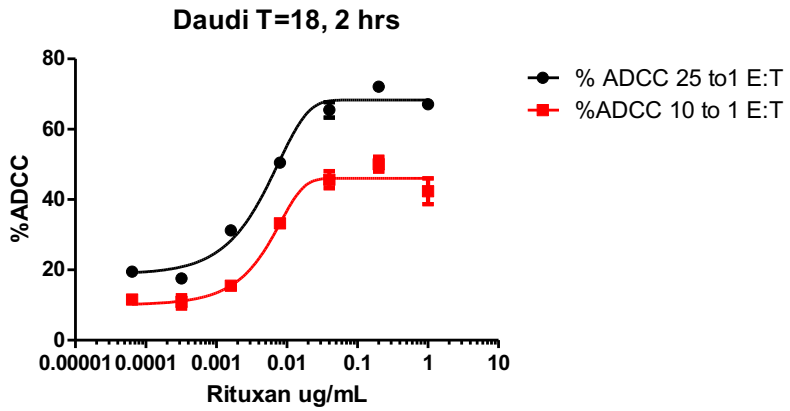


Figure: 5000 Daudi cells/well were incubated with serially diluted Rituximab for 15 minutes prior to the addition of purified NK cells stimulated overnight with IL-2. The ADCC reaction was further incubated for 2 hours at the specified E:T ratios. % ADCC was measured using the aCella-TOX assay. Log(EC50) value for E:T 25:1 was -2.344 and for E:T 10:1 was -2.213.

K. Technical Notes:

1. The volume of lytic agent per 75µL of cells to obtain 100% lysis has to be determined empirically, by checking under a microscope. We recommend starting at 10 L and titrating down (lysis buffer can be titrated in di water). The incubation time could also vary from 5-15 minutes. The plate should be allowed to equilibrate to RT for about 15 minutes before the addition of the lysis buffer to the maximum lysis wells.
2. It is important to use freshly separated PBMC's from blood in order to reduce death of effector cells, which will increase effector-only background. Also high RBC contamination in the PBMC fraction will increase background and compromise assay sensitivity.
3. For NK cell isolation, we recommend avoiding the RBC lysis step. This may help improve the final yield of the NK cells isolated per donor.
4. The optimal target to effector cell ratio also has to be determined prior to performing the ADCC assay. Plating two or three different target cell concentrations and varying the effector cell ratios across the plate can accomplish this.
5. There is considerable variation in the effector cells isolated from different donors. This effect is amplified if using cytokines to maintain the PBMC's or NK cells. We recommend using the ADCC culture media if effector cells have to be maintained overnight.
6. We strongly recommend the use of NK cells in ADCC assays (isolated using Miltenyi Biotec kit Cat# 130-050-401). Isolated NK cells can be used immediately or cultured overnight in a CO_2 incubator. The use of isolated NK cells gives a better dose-response curve due to a lower background, and therefore, a higher signal-to-noise ratio.
7. Target cells should not be extensively sub cultured (follow guidelines specific to cell line), as they may exhibit changes in levels of surface antigen expression, which may make them resistant to cytotoxicity by the antibody.

Note: When choosing an optimized time point for your calculations, it is important to choose a time point where the maximum lysis control is increasing in RLU's. (Typically, we use T=15'). Do not choose a time point where the maximum lysis has decreased from the previous time point.

A schematic representation of an ADCC reaction plate is shown below:

Using two previously determined effector:target cell ratios, set up an antibody titration across the plate with the appropriate controls. Reactions are run in triplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 10:1 Control 2	Control 1		
C		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 10:1 Control 2	Control 1		
D		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 10:1 Control 2	Control 1		
E		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 20:1 Control 2	Control 3	Control 4	
F		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 20:1 Control 2	Control 3	Control 4	
G		Antibody 1ug/ml	Antibody 0.2ug/ml	Antibody 0.04 ug/ml	Antibody 0.008 ug/ml	Antibody 0.0016 ug/ml	Antibody 0.00032 ug/ml	Antibody 0 ug/ml Control 5	E:T 20:1 Control 2	Control 3	Control 4	
H												

6.3 ADCC with Adherent Target Cell Lines

A. Protocol Overview:

1. Plate the target cells at 5000 cells/well the night before the assay.
2. Wash with fresh media and decant. Add 50µL of the antibody directly on the cells and allow opsonization for 15 minutes.
3. NK cells purified from a leukopack. They are washed the morning of the assay and added to the target cells in a volume of 50µL. The plate is incubated for 2-4 hours.
4. Perform the aCella-TOX reaction.

B. Preparation of target cells:

1. The night before the assay, the cells are trypsinized, washed and plated at the required cell density (5000 cells/well) in 100µL of low IgG-serum containing media on a U bottom 96-well Tissue culture plate.
   NOTE: All reactions should be done in triplicates.
   For certain cell lines, it may be required to use a gentler Trypsin replacement such as TrypLE Express (catalog # 12604 from GIBCO).
2. Incubate overnight at 37ºC.
3. On the morning of the assay, wash the cells with about 100µL of fresh warm media once before the addition of the antibody. This can be accomplished my gently inverting the tissue culture plate over a paper towel and gently tapping it to decant the media.

4. Next gently add 100µL of fresh media and decant the media as described above.

C. Preparation of antibody dilutions:

1. Starting with a concentration of about 1µg/ml (determined from titration performed in earlier experiment), prepare serial 2X dilutions of the Antibody (as desired) to range over three orders of magnitude. These dilutions are prepared in the low-IgG serum media.
2. Decant the media from the plate and add the antibody dilution in 50µl volume directly to the washed cells.
3. Incubate at 37ºC for 15 minutes for opsonization to occur.
4. For the zero Antibody control (CMC) reaction, 50µL of media is added to the target cells (see section E. Control 5 below)

D. Preparation of Effector cells:

Refer to **Section C: Separation of PBMC's from Leukopack** and **Section D: Lysis of Red Blood Cells** above. PBMC's may be used immediately or maintained in the ADCC culture media overnight at 37°C. PBMC's can be further sorted and specific populations; such as NK cells may be isolated using Miltenyi Biotec's MACS systems. We find this reduces the effector cell background significantly.

http://www.miltenyibiotec.com/download/datasheets_en/58/DS130_050_401.pdf

1. On the morning of the assay, the effectors must be spun down, washed with fresh warm media, and counted. Effectors should be resuspended in ADCC media at the appropriate cell concentration to deliver the optimal E:T ratio in 50µL.
2. Incubate the plate for 2-4 hours at 37ºC for the ADCC reaction to occur.

E. Controls:

1. **Control 1:** Spontaneous release of GAPDH from target cells: To wells containing 5,000 target cells in triplicate, add 100µl of ADCC media.
2. **Control 2:** Spontaneous release of GAPDH by effector cells: Plate 50µL of effector cells (the same ratio as used in the experiment) and add 50µL of ADCC media in triplicate.
3. **Control 3:** Maximum lysis of target cells: To 5,000 target cells in triplicate, add 100 µL of ADCC media.
4. **Control 4:** Media only control: Plate 100µl of the ADCC media in triplicate.
5. **Control 5:** As mentioned above, a zero Antibody control, 50µL of media+ 50µL of effector cells should be added to target cells, which are already present in the well. This reaction represents cell-mediated cytotoxicity (CMC) that occurs due to the killing of the targets by the effectors without the mediation of the antibody.

F. Preparation of aCella-TOX reagents:

The reagents supplied in the aCella-TOX kit are light sensitive. Avoid direct light, as in a bio-safety hood. Indirect lab light is acceptable for short exposures (15-20 minutes). Reduce light levels in the lab if possible. Follow the step-by-step directions described in the section "**aCella-TOX: Reagent Set Up**" (above) of the protocol supplied with the kit to make working stocks of reagents. The reagents should be at equilibrated to room temperature before adding them to the ADCC assay.

G. ADCC Reaction:

1. In setting up the plate, plan to avoid using the outer rows and columns to reduce any "edge effects". Add 200µL of ADCC culture media or PBS to these wells.
2. The target and effector cells should be counted and set up at required cell counts. The serial dilutions of the antibody should be ready for use in the required volumes.
3. Washed target cells are incubated with the antibody (50µL) for 15 minutes, allowing opsonization to occur.
4. Effector cells (NK cells or PBMC's) are added in a volume of 50µL. The ADCC reaction is allowed to proceed for 2-4 hours at 37ºC.
5. At the end of the incubation, the reaction plate is allowed to equilibrate to RT for 15 minutes. Lyse the target cells in the Maximum Lysis control wells with 10µL of Lysis buffer. Incubate 5-10 minutes at RT. (monitor lysis under microscope)
6. Add 100µL of low IgG-serum media to all reaction wells to bring volume to 200µL.
7. Spin down plate in a micro plate centrifuge for 1' at 750Xg.
8. In the opaque plate for the luminometer, (Perkin Elmer Optiplates#6005290) add 50µl of Component 2 (Enzyme Assay Diluent, Part #3008) to all reaction wells.
9. Now carefully transfer 50µL of the supernatant to the 50µL of Component 2 using a multichannel pipettor, changing tips each time.
10. Add 100µL of the 2X Enzyme Assay reagent containing G3P (from step IV: Procedures) to each reaction.
11. Immediately, add 50µL of 1X Detection reagent (from step IV: Procedures above) to each well.
12. Read the plate on the luminometer **immediately** (without the cover) and take several time point readings 5 minutes apart to determine the optimal reading time-point .
13. Analyze and graph data.

H. Calculations:

To determine ADCC, first calculate the mean of the triplicate luminescent values for each of the reaction conditions.

First subtract the mean media only luminescent value (control 4) from all calculated mean values. Then use the following formula to calculate %ADCC.

$$\%\ \text{ADCC} = \frac{\text{(Sample)-(Control 1 Target spontaneous release)-(Control 2 effector alone)}}{\text{(Control 3 maximum release)-(Control 1 target spontaneous release)}} \times 100$$

The equation above normalizes the data for %ADCC by subtracting the spontaneous target release from the maximum lysis control.

For the dose-response curve, plot the log of the Antibody concentrations in mg/ml vs. the % ADCC as calculated by the formula above.

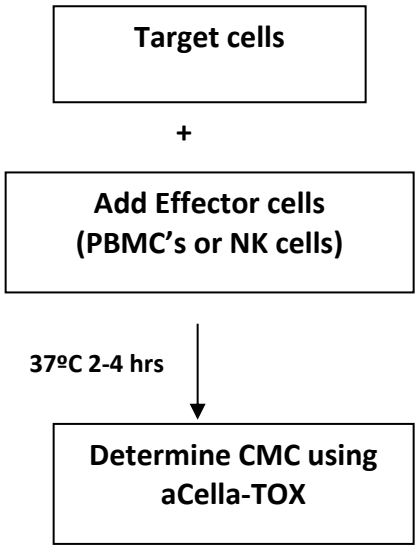
Note: When choosing an optimized time point for your calculations, it is important to choose a time point where the maximum lysis control is increasing in RLU's. Do not choose a time point where the maximum lysis has decreased from the previous time point. (Typically, we use T=15')

A schematic representation of an ADCC reaction plate for two antibodies (positive and negative) is shown below: (1:4 serial dilutions of antibodies)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		20µg/ml	5µg/ml	1.25	0.3125	0.078	0.0195	0.0049	0	T	E	
C		+antibody							0	T	E	
D		triplicates							0	T	E	
E		20µg/ml	5µg/ml	1.25	0.3125	0.078	0.0195	0.0049	0	Max	Media	
F		-antibody							0	Max	Media	
G		triplicates							0	Max	Media	

VII. Cell Mediated Cytotoxicity (CMC) Procedure

7.1 Schematic Representation of a CMC Assay



7.2 CMC Assay Description

A. Cells, Reagents and instruments required:

1. Target cells such as K562, Jurkats or other cell line.
2. Leukopack, LRS chamber or Buffy pack (source of fresh blood)
3. Cell Culture media (RPMI 1640 supplemented with 10% FBS, Non-Essential Amino acids and Penn/Strep + L-Glutamine).
4. Histopaque, Sigma# 1077
5. aCella-TOX Kit (Cell Technology, Inc.)
6. Luminometer
7. White opaque 96-well Optiplates (Perkin Elmer, #6005290 or Corning #3605 with cover, #3099)
8. U bottom 96 well tissue culture plates for CMC reation.
9. Sterile-filtered solutions of 0.2% NaCl and 1.6% NaCl for RBC lysis.
10. Sterile-filtered solution of !X PBS containing 2mM EDTA.
11. Miltenyi Biotec MidiMACS Starting kit, (Cat.# 130-042-301) including MidiMACS separation unit, 1 MACS Multistand, 25 LS columns and CD56 Microbeads (Cat.#130-050-401).

B. Preparation of target cells:

1. On the morning of the experiment, centrifuge 8-10 ml of Target cells. Wash once in 1X PBS containing EDTA. Resuspend in culture media.

2. Check cell count with Trypan Blue stain and adjust cells to a concentration of $2X10^5$ cells/ml. Plating 50µL of this will give 10,000 cells/well. Cell concentration can be adjusted to plate a lower cell number.

C. Separation of PBMC's from leukopack:

If using an LRS chamber, the blood should be diluted with 2X volume of PBS/BSA/EDTA before loading onto an equal volume of the density gradient.

1. Pipette 20-25ml of Histopaque (or other suitable density gradient medium) into a 50 ml tube. Layer 20ml of blood on the Histopaque. Centrifuge at 500xg for 30 minutes.

2. Carefully pipette out the top serum layer into a separate tube. Aspirate the middle layer containing the PBMCs into another tube, taking care to minimize RBC contamination. Add 1-2 volumes of PBS containing EDTA to the PBMC's and centrifuge at 500xg for 15 minutes. Next decant the PBMC's and gently vortex the cell pellet.

3. Repeat wash step 2. After the final wash decant supernatant and gently vortex the cell pellet.

D. Lysis of Red Blood Cells:

1. Resuspend cell pellet in 10-20 ml of 0.2% NaCl for **30 seconds** to lyse the RBCs (gently agitate the tube to ensure proper mixing) and immediately add 10-20 ml (equal volume) of 1.6% NaCl to neutralize the osmolarity. Do not exceed 30 seconds in the 0.2% NaCl solution. After the addition of the 1.6% NaCl, proceed to the centrifugation step immediately.

2. Centrifuge for 10 minutes and resuspend the cell pellet in 2 to 5ml of ADCC culture media containing 10% low IgG serum.

Note: Depending on the donor, it is possible that the RBC lysis step may have to be repeated if the RBC count is too high. If the supernatant is cloudy, repeat the wash step with PBS/EDTA to remove the remaining platelets

E. Preparation of Effector cells:

PBMC's may be used immediately or activated with IL-2 overnight. PBMC's can be further sorted and specific populations; such as NK cells may be isolated using Miltenyi Biotec's MACS systems. We find this reduces the effector cell background significantly. We recommend isolating NK cells using Miltenyi Biotec's kit cat# 130-050-401.
http://www.miltenyibiotec.com/download/datasheets_en/58/DS130_050_401.pdf

1. On the morning of the assay, the effectors must be spun down, washed with fresh warm media, and counted with Trypan Blue. Effectors should be resuspended in the appropriate volume of media to deliver the optimal ratio to the target cell number in 50µL. Since aCella-TOX is a very sensitive assay, start with a 1:1 ratio and titrate up.

2. Immediately, spin down the plate for 1 minute at 500Xg to allow surface contact of the Effectors and Targets. (only for suspension cells. Do not spin if target cells are adherent)

3. Incubate the plate for 2-4 hours at 37ºC.

F. Controls:

1. **Control 1:** Target cells alone: spontaneous release of GAPDH. Plate 50µL of target cells + 50 µL of media.
2. **Control 2:** Effector cells alone at the respective E:T ratios.: spontaneous release of GAPDH from effectors. Plate 50µL of effector cells + 50µL of media.
3. **Control 3:** Target cells alone for Maximum GAPDH release. Plate 50µL of target cells + 50 µL of media.
4. **Control 4:** Media only. Plate 100µL of media.

G. Preparation of aCella-TOX reagents:

The reagents supplied in the aCella-TOX kit are light sensitive. Avoid direct light, as in a bio-safety hood. Indirect lab light is acceptable for short exposures (15-20 minutes). Reduce light levels in the lab if possible. Follow the step-by-step directions described in the section "**aCella-TOX: Reagent Set Up**" (above) of the protocol supplied with the kit to make working stocks of reagents. The reagents should be equilibrated to room temperature before adding them to the ADCC assay

H. CMC Reaction: Plate all reactions in triplicate.

1. Plate 5000 target cells in 50µL+ 50µL of the various effector cell numbers.
   Plate the various controls as shown in the template.
1. In the case of suspension target cells, the plate may be spun down for a minute to establish contact between the targets and effectors. We do **not** recommend adherent cells be spun down.
2. Cover the plate and incubate the CMC reaction at 37°C for 2-4 hours. This length of time should be determined empirically.
3. Allow the plate to equilibrate to RT for 15 minutes. Add 5-10µl of lytic agent to the wells indicated for maximum lysis of target cells (control 3).
   Incubate 5-10 minutes at RT. (monitor lysis under microscope)
4. Add 100µL of media to all reaction wells to bring volume to 200µL. Spin down for a minute at 750Xg.
5. Transfer 50µL supernatant into corresponding wells on a white plate containing 50µL of Enzyme assay diluent.
6. Add 100µL of the Enzyme Assay reagent containing G3P to all reaction wells.
7. Immediately, add 50 µL of the Detection reagent to each well.
8. Read the plate on the luminometer (without the cover) **immediately** and take several time point readings 5 minutes apart, to determine optimal reading time-point.

I. Calculations:

In order to calculate % cytotoxicity, calculate the mean of the triplicate luminescent values for each reaction condition and controls and use the following procedures:

a. First subtract the mean media only luminescent value (control 4) from all calculated mean values for each reaction condition.
b. Next for each calculated mean value of E:T ratio, subtract the respective mean calculated value of effector spontaneous release control 2.
c. This is represented by the following formula:

$$\% \text{ cytotoxicity} = \frac{(\text{Sample}) - (\text{Control 1 Target spontaneous release}) - (\text{Control 2 Effectors alone})}{(\text{Control 3 maximum release}) - (\text{Control 1 Target spontaneous release})} \times 100$$

For the dose-response curve plot the E:T ratio vs. the % cytotoxicity as calculated by the formula above.

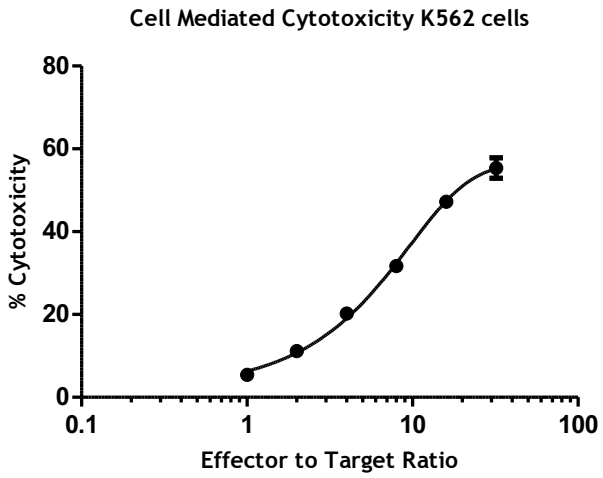


Figure shows CMC assay with K562 as target cells incubated with different E:T ratios of IL-2 stimulated (overnight) NK cells isolated using Miltenyi Biotec's kit cat# 130-050-401. Target cells were plated at 5,000 cells/well and increasing effector cell concentrations from 1:1 to a ratio of 32:1. The assay was incubated for 2 hours at 37°C and % cytotoxicity measured with the aCella-TOX kit.

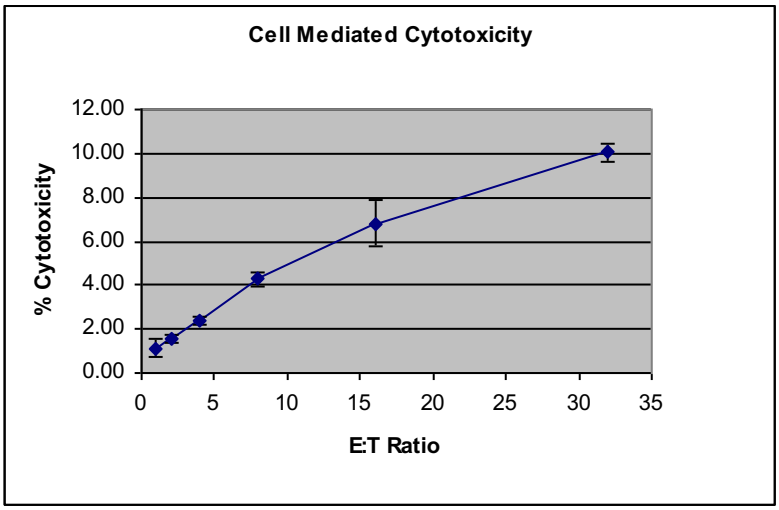


Figure: CMC: 10,000 Jurkat cells/well were mixed with freshly isolated Peripheral Blood Lymphocytes at various Effector:Target cell ratios. The CMC assay was incubated for 4 hours and % cytotoxicity detected using the aCella-TOX assay.

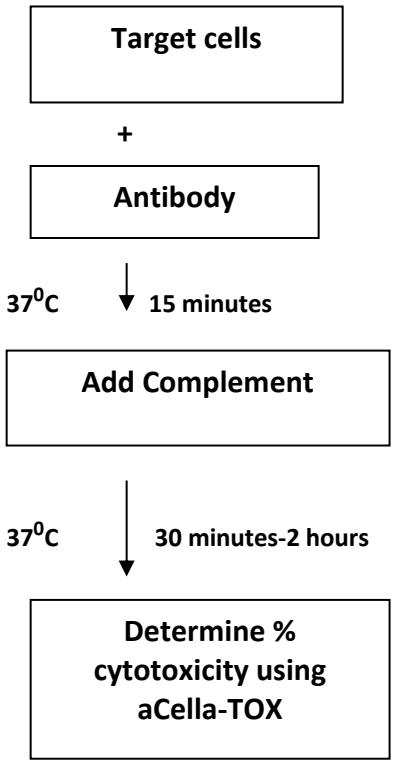
Sample CMC set up

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		E:T 1:1	E:T 1:1	E:T 1:1	Control 2 1 E	Control 2 1 E	Control 2 1 E	Control 1	Control 4			
C		E:T 2:1	E:T 2:1	E:T 2:1	Control 2 2 E	Control 2 2 E	Control 2 2 E	Control 1	Control 4			
D		E:T 4:1	E:T 4:1	E:T 4:1	Control 2 4 E	Control 2 4 E	Control 2 4 E	Control 1	Control 4			
E		E:T 8:1	E:T 8:1	E:T 8:1	Control 2 8 E	Control 2 8 E	Control 2 8 E	Control 3				
F		E:T 16:1	E:T 16:1	E:T 16:1	Control 2 16 E	Control 2 16 E	Control 2 16 E	Control 3				
G		E:T 32:1	E:T 32:1	E:T 32:1	Control 2 32 E	Control 2 32 E	Control 2 32 E	Control 3				
H												

TECHNICAL NOTES:

1. High RBC contamination in the PBMC fraction (effector cells) will increase background and compromise assay sensitivity.
2. Substituting regular FBS with low IgG serum (Gibco, Cat. # 16250) may help to lower the standard deviations.
3. Different donors react differently to IL-2 and other cytokines. The use of cytokines with the effector cells may therefore contribute to a higher CMC response, while adding to the donor-to-donor variability.
4. We recommend using purified NK cells instead of PBMC's as effector cells to increase the signal-to-noise ratio. If NK cells are used in the assay, a lower E:T ratio (typically 10:1) than is used for PBMCs is recommended.

VIII. Complement Dependent Cytotoxicity (CDC)



Antibody Dependent Complement Mediated Cytotoxicity Protocol:

Materials Required:
1. 96 will tissue culture plates, White opaque 96 well plates.
2. Media of choice.
3. Lab instruments, pipettes, tips etc.

Assay:
1. Plate target cells between 5,000-10,000, in 25-50µL, cells per well in complete media (heat- inactivated 10% FCS + Penn/Strep/L-Glutamine). Use 96 well tissue culture plates.
2. Titrate serial dilutions of antibody, in 25µL, onto the target cells (include zero antibody control) and incubate 15 minutes at 37°C in CO_2 incubator for opsonization to occur.
3. Add complement in 25µL, to a final concentration of 5-25% (this needs to be determined empirically as per your complement source.)
4. Controls:
   A. Maximum lysis (Target cells + complement+ media). 5-10µL of lysis buffer (Part # 3035) will be added to this control.
   B. Target spontaneous release (no complement). No lytic agent will be added to this control.
   C. Media control with complement.

5. Set up samples and controls and incubate the reaction plate for 30-120 minutes in incubator at 37°C. This incubation time will depend on antibody and complement source and must be determined empirically.
6. After required incubation time, remove the 96 well plate from the incubator and allow it to equilibrate to room temperature (15 minutes).

7. Add 5-10µL of lysis buffer (Part # 3035) to the maximum lysis control wells (control A). Pipette up and down several times to ensure complete lysis. Incubate for 5 minutes at RT.
8. Next add 100µL or 125µL of media to all wells to bring the final volume to 200µL.
9. Spin down the assay plate (1 minute at 750Xg).
10. Transfer 50µL supernatant into corresponding wells on a white plate containing 50µL of Enzyme assay diluent. (Part # 3008)
11. Add 100µL of the 2X Enzyme Assay reagent (Part # 6001 + Part #3008) containing G3P (Part #6003) to all reaction wells.
12. Immediately, add 50µL of the 1X Detection reagent (Part # 6002 + diluted Part # 3009) to each well.
    Note: Follow the step-by-step directions described in the section "**aCella-TOX: Reagent Set Up**" (**Section 4.5** above) to make working stocks of reagents.
13. Read the plate on the luminometer (without the cover) **immediately** and take several time point readings 5 minutes apart, to determine optimal reading time-point. (typically T=15)

Calculations:

In order to calculate % cytotoxicity, calculate the mean of the triplicate luminescent values for each reaction condition and controls and use the following procedures:

a. Average controls A, B and C.
b. First subtract the mean media only luminescent value (control C) from all sample wells and from controls A and B.
c. This is represented by the following formula:

$$\% \text{ cytotoxicity} = \frac{(\text{Sample RLU's}) - (\text{Control B Target spontaneous release})}{(\text{Control A maximum release})} \times 100$$

For the dose-response curve plot the E:T ratio vs. the % cytotoxicity as calculated by the formula above.

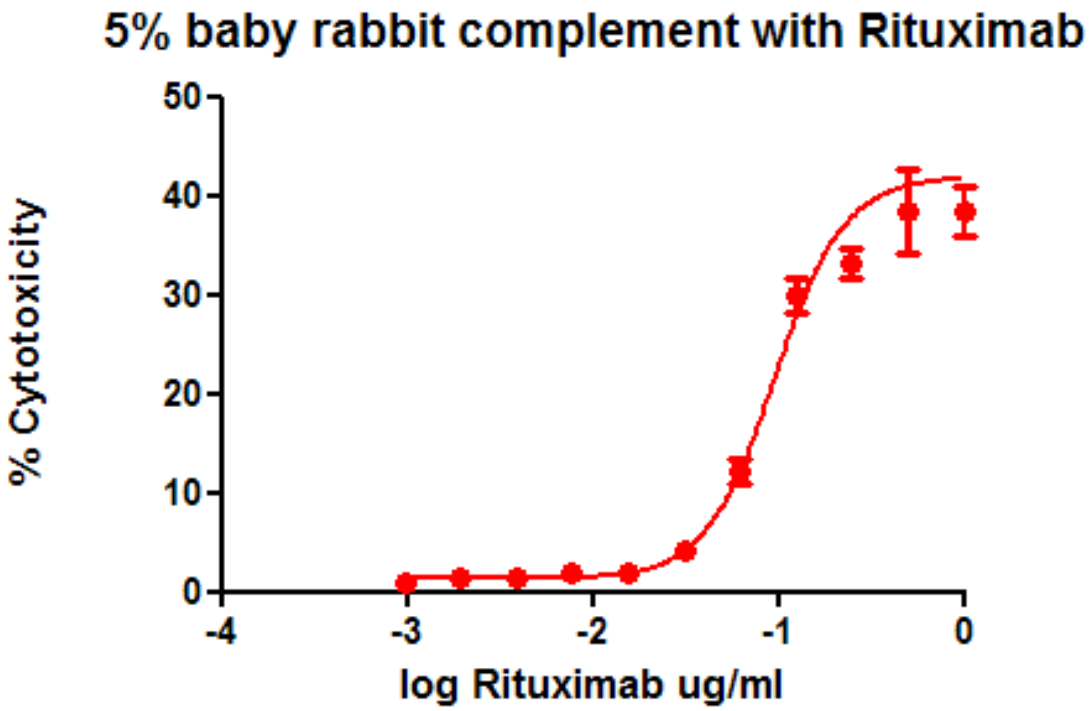


Figure: The above graph shows an antibody titration of Rituximab from 0.000977 to 1µg/ml incubated with 5% baby rabbit complement and 5000 Daudi target cells for 30 minutes at 37°C.

IX. Drug Induced Cytotoxicity

A. Recommended controls:

1. Negative Control: In 100 µL:

A. Plate in triplicate cell culture media alone to measure background signal.

AND / OR

B. Untreated cells or cells treated with solvent/vehicle to dissolve test compound.

2. Positive Control: Maximum Release of GAPDH**:**

A. Plate in triplicate cells alone. At the end of the experimental protocol add 5-10µL of the lysis buffer to 100 µL of maximum release of GAPDH control.

Note: Use the optimized volume of lysis buffer for your cell line as determined in StepIV: Procedures step 5 reagent set up (above).

B. Assay:

Note:

1. **Washing the cells into fresh media prior to starting the assay could result in lowering the background signal.**
2. **We have found that the background signal in this assay greatly depends on the type of serum used in the culture of cells. We recommend the use of USDA approved, US origin, Fetal Bovine sera with Hemoglobin levels <6.5 mg%, in order to get lowest background signals.**

1. In opaque white plates, plate in triplicate, test sample cells at 1000-10,000 cells per well in 100 µL. Then plate all controls in 100 µL. Cells may be plated in reduced serum or 10% serum media. We have determined the linear range of the assay in the presence of 10% FCS to be between 1000 and 20000 cells/well. In serum-free media, aCella-TOX can detect as few as 7 cells/well. Therefore, the presence of serum compromises the assay sensitivity. It is important to determine the range of cell concentration in order to maintain the linear response for a particular cell line.

2. Add test compounds to your sample cells.

3. After incubation according to your experimental protocol:

   A. Maximum Lysis (control 3): To 100µL of the positive control sample, add 5-10 µL of lysis buffer. Incubate 5-15 minutes before proceeding to step B.
   B. Reaction: Then add 100µL of 2X Enzyme Reaction Cocktail (from Step IV: Procedures: Reagent set up step 2) to each well, including positive control and negative control.

4. Detection: Immediately, add 50µL of the 1X Detection Reagent (as diluted 1:50 in Step IV: Procedures: reagent set up step 4) to each well, and read in a luminometer. We recommend taking multiple readings at 5-minute intervals to determine the optimal readout time point.

C. Calculation of Results:

$$\text{Cytotoxicity} = 100 \times \frac{\text{(Experimental sample – Negative control (media or solvent/vehicle)}}{\text{(Maximum GAPDH Release) – (Negative control (media or solvent/vehicle))}} \text{ Percent}$$

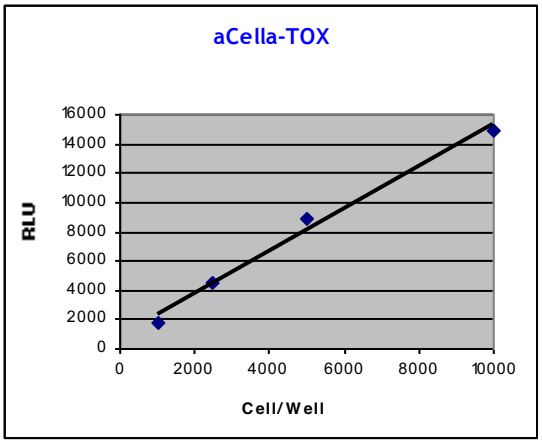


Figure: Demonstrates linear response of the aCella-TOX kit: Jurkat cells were plated at various cell concentrations per well. The lysis buffer was added to each well. aCella-TOX kit was used to detect GAPDH enzyme release of the lysed cells. Data points show samples run in triplicate with background subtraction.

X. STOP REACTION FOR aCella-TOX: (For High-Throughput/ Batch Processing applications)

Additional materials required:

1. Water bath set at 75°C to be able to accommodate 4-6 plates at a time, to enable batch processing.
2. Plate sealers (e.g. Corning Cat# 3095).

Procedure:

1. After the ADCC, CMC or CDC incubation reaction at 37°C, the reaction plate is equilibrated to RT for 15 minutes.
2. Add 5-10µL of lysis buffer (Part # 3035) to the maximum lysis wells and incubate for 5 minutes. (monitor lysis under microscope).
3. Bring total volume of the reaction to 200µL by adding 100µL or 125µL of ADCC culture media to all wells.
4. Spin down plate for 1 minute at 750Xg.
5. Transfer 50µL of supernatant from all wells to 50µL of Enzyme assay diluent (Part #3008) in corresponding wells on a white luminescence plate.
6. Add 100µL of the 2X Enzyme assay reagent (Part #6001 + diluted Part # 3008) containing G3P (Part #6003) to all reaction wells.
7. Seal the plate with plate sealer.
   Incubate at RT for 15 minutes in the dark.
8. Place plate securely in a water bath at 75°C for 30 minutes.
9. You may proceed to step 10 below or freeze plate overnight at -20°C. (with plate sealer).
10. The plate is allowed to equilibrate to RT. (may be an hour or so)
11. 50µL of 1X Detection reagent (Part # 6002 diluted 1:50 in 1X Detection reaction diluent) is added to all wells.
12. Read the plate on the luminometer (without the cover) **immediately** and take 2-3 time point readings 5 minutes apart. Typically, the T=5 (second reading) will be the optimal time point to be analyzed. (the reading just before the RLU values start decreasing)

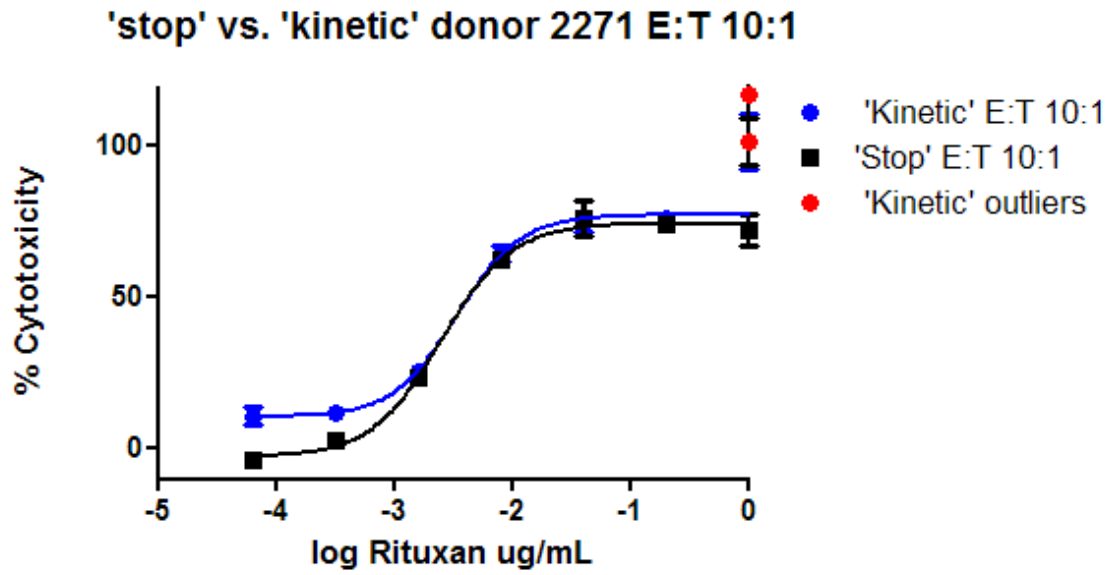


Figure: The above graph overlays the dose response curves at E:T of 10:1 generated using Rituximab on Daudi cells and NK cells purified from donor 2271. The 'kinetic' reaction refers to the reaction run at T=15' after the aCella-TOX reagents were added to the ADCC reaction wells immediately. The 'stop' reaction refers to the reaction that was stopped after the addition of the enzyme assay reagent and heated at 75°C for 30 minutes and frozen overnight. The detection reagent was added just prior to reading on the luminometer.
IC50 values with 95% confidence intervals range from 0.002608 to 0.004371 for the 'kinetic' and 0.001885 to 0.003310 for the 'stop' reaction. (4PLC using Graph Pad Prizm)

XI. References:

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XII. Frequently Asked Questions (F.A.Q.)

PROBLEM:	POSSIBLE FIX:
No luminescence signal or low RLU values	**Check Luminometer settings** 1. Check luminometer gain setting. Read luminometer instruction manual and adjust settings accordingly. 2. Ensure the proper working of the instrument by checking the RLU value of a 1µM ATP solution as a positive control, if required. This number would be close to the maximum RLU reading for your luminometer. 3. Are you using opaque white plates on the luminometer? Clear plates will lead to the scattering of light and a low signal. Please contact the instrument manufacturer for related technical assistance. **Assay-related:** 1. Upon arrival, check if contents of the kit were stored at appropriate temperatures. Part#'s 6001, 6002 and 6003 are extremely temperature-sensitive and need to be frozen between -20 and -40°C. 2. Have the Enzyme Assay Reagent (Part #6001) and Detection Reagent (Part# 6002) gone through repeated freeze-thaw cycles? It is recommended to store the frozen components as single-use aliquots. 3. Was the G3P (Part # 6003) added to the Enzyme Assay Reagent (Part #6001)? 4. Check the cells in the maximum lysis wells under the microscope to make sure you see 100% lysis. If not, you may have to perform a cell titration with the lysis buffer and incubation time.
High background issues causing flattening of the dose-response curve.	1. Are you using low-IgG FBS as recommended? 2. Have the target cells been adapted to the low-IgG FBS media for a few passages? 3. Are you isolating NK cells? Using PBMC's can contribute to high background due to the large number of cells. 4. Was the PBMC pellet isolated clear of RBC's and platelets? A cloudy supernatant may indicate the presence of platelets. Typically, a wash with 1X PBS/EDTA should clarify the pellet. 5. Were the target and effector cells washed and resuspended in fresh low-IgG FBS culture-media just

	prior to the start of the ADCC reaction? If not, the dying/dead cells will increase the background signal. 6. Did you add the G3P (Part# 6003) to the 2X Enzyme Assay reagent (diluted Part #6001) just before it was added to the supernatant? 7. Following the ADCC reaction, is the plate being spun down and the supernatant used in the assay? Using the lysate as a whole without spinning may contribute to the high background luminescence. 8. Have the NK cells been freshly isolated? Use of frozen-and-thawed effector cells in the assay may also contribute to a high background.
High donor-to-donor variation	1. Use of NK cells rather than PBMC's as effector cells should minimize this problem to a great extent. 2. Cytokines such as IL-2 may stimulate effector cells to different degrees in different donors. This may contribute to different CMC responses in different donors, and decrease the signal-to-noise ratio of the dose-response curve.