aCella –TOX™

Bioluminescence Cytotoxicity assay

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

ATTENTION:

PROTOCOL VERSION 1.2 – All kits shipped on or after April 6th 2007 should use this protocol.

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

KIT LOT #'s KN20906 and KN40906: The concentration of G3P (Component 3) in these lots is at 50 mg/ml. Please use 3.5

Step 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.

StepII: 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.

StepIII: Measurement Modes

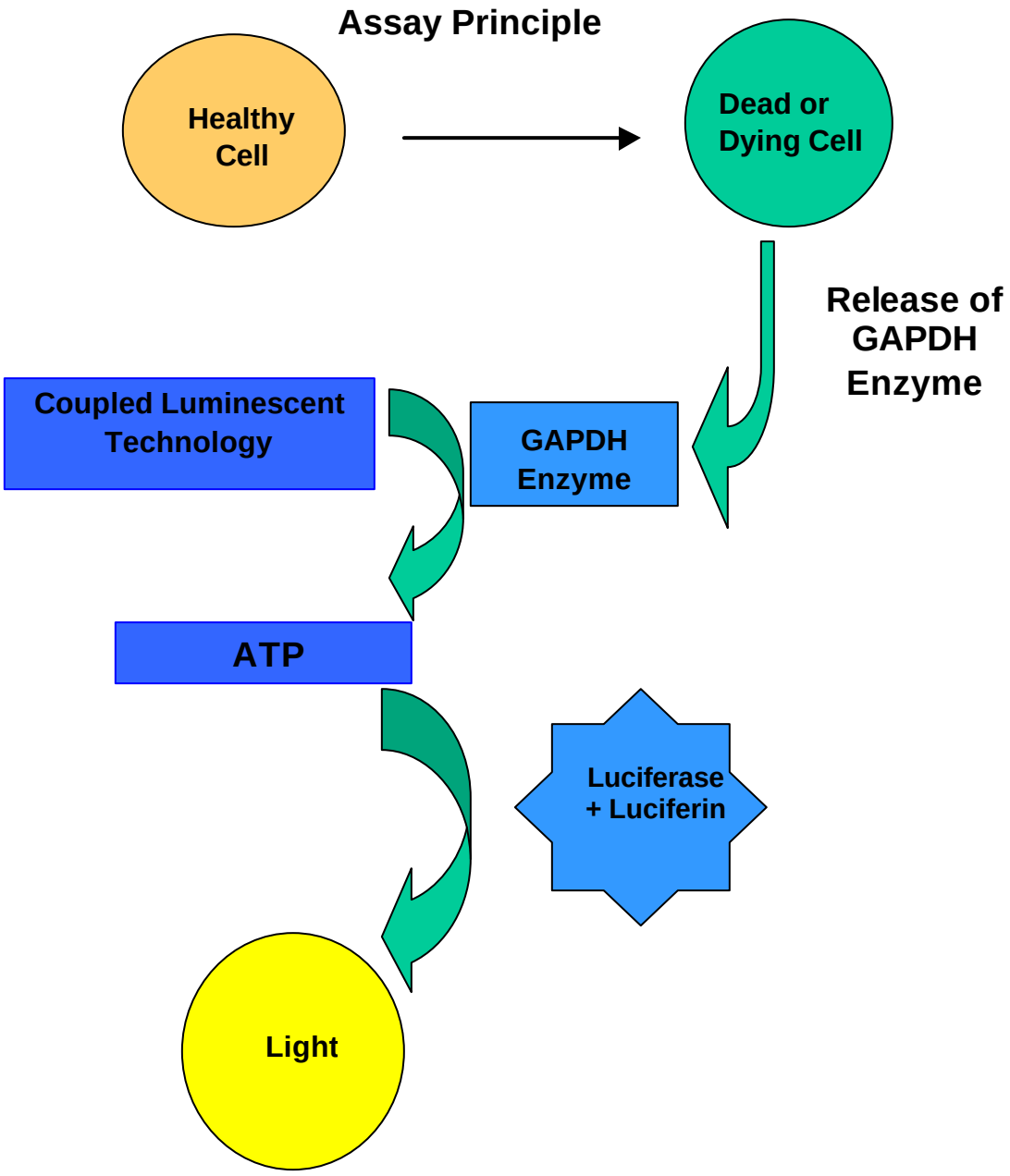
Cytotoxicity. aCella-TOX may be added directly to either a cell culture or supernatant to detect enzyme released from cells that have lost membrane integrity. It is not necessary to remove live cells prior to measurement. No form of pre-treatment is needed. The luminescent readout begins to rise immediately 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 200 µL of media.

1. For cell viability: spin down plate and pipette 100µL of cell free supernatant in a white opaque 96 well plate. Add aCellaTOX 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 aCellaTOX 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
Healthy Cell
Dead or Dying Cell
Release of GAPDH Enzyme
Coupled Luminescent Technology
GAPDH Enzyme
ATP
Luciferase + Luciferin
Light

StepIV: Procedures

Kit Contents:

Component Description	Part #	Volume	Storage
1. **Component 1:** 4X Enzyme Assay Reagent	6001	26 ml	≤ -20°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
4. **Component 4:** 50X Detection Reagent (Luciferase/Luciferin)	6002	0.55 ml	≤ -20°C
5. **Component 5:** 5.5X Detection Assay Diluent	3009	5.5 ml	≤ -20°C
6. **Component 6:** Lysis Buffer	3035	5 ml	2-8°C

Materials and Equipment Needed:

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. Luminometer.

Reagent set up:

1. 4X Enzyme Assay Reagent:(Component 1): Part #6001 (**Green label**)
For 100 tests (one 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 at or below -20°C, 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:
1. Avoid repeated freeze thaw cycles.

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 mL to be added **per mL** of 2X cocktail
	45	3.9
	46	3.8
KN10306,KN20406,KN20606, KN50507	47	3.7
	48	3.6
	49	3.6
KN40906, KN20906, KN40108	50	3.5
	51	3.4
	52	3.4
	53	3.3
	54	3.2
	55	3.2

Aliquot G3P into single use vials.
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 35µL 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). Should you have left over material of the 2X Enzyme Assay Reagent mixture, from this step, it can be stored for 2 weeks at -20^0C or below, without significant loss of activity.
You will add 100 µL of this Enzyme Reaction Cocktail containing G3P to every 100µL of sample.

Cautionary Notes:

1. Avoid repeated freeze thaw cycles.

3. 5.5X Detection Assay Diluent: (Component 5): Part # 3009 (Magenta 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 4.5 mL of reagent grade Di water. Any unused material can be aliquoted and stored frozen at or below -20^0C.

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

This is a frozen 50X concentrate containing Luciferase and Luciferin. Prior to use, thaw the vial and keep on ice. Aliquot and freeze the rest at or below -20^0C, 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 50 µL 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 sensititvity: 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 aCellaTOX kit to 250, 100 or 50 tests depending on the dilution used.

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 4-8^0C. 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.)

Step V: Assays:

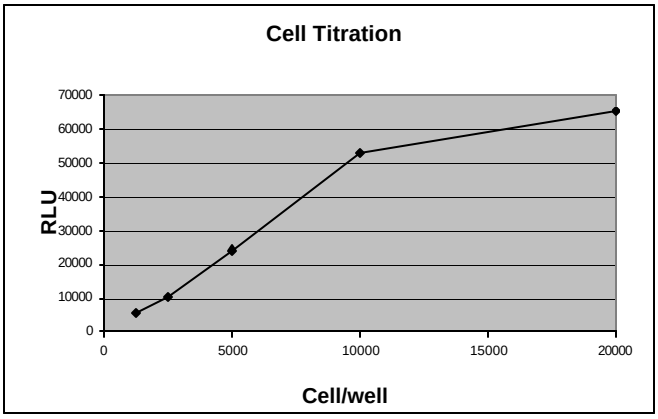
General Guidelines:
Cell Technology's non-radioactive aCella-TOX kit can be used to efficiently and accurately measure Antibody Mediated Cellular Cytotoxicity (ADCC) and Cellular Mediated Cytotoxicity (CMC). The following guidelines will help to assure the success of assays using acella-TOX. Since the aCella-TOX kit measures the release of the GAPDH enzyme as an indicator of ADCC/CMC, it is necessary 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. Left unstimulated, these cells would die during the course of the ADCC/CMC reaction and release the GAPDH enzyme, yielding a higher background signal.

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 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". Do fill these edge wells with media only.

Before starting experiments:
GAPDH expression will vary between cell lines. It is important to determine the linear response range of aCella-TOX within your particular cell line. This can be accomplished by titrating your cells in your 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:



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 aCellaTOX 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.

1.ADCC PROCEDURE: Sample protocol [11]

Target to Effector cell ratios:
We recommend using 5,000-10,000 target cells per well with a effector:target cell ratio between 10:1 to 20:1. Since the aCella-TOX assay is very sensitive, it is better to use lower ratios of effector:target cells; however, each investigator should determine this empirically.

ADCC protocol overview:

1. Plate the target cells at 5000 to 10,000 cells per well in 25µL.
2. Add 25 µL of antibody to the target cells and incubate in an incubator for 15 minutes.
3. Add 50µL of effector cells and incubate the plate for 2-4 hours.
4. Proceed with the aCella-TOX reaction.

A. Cells,Reagents and instrument required:

1. Target cells such as: Daudi, Raji, Ramos or breast cancer cell lines such as SKBR3 o rMCF7 etc.
2. Leukopack or Buffy pack.
3. ADCC Culture media (RPMI 1640 supplemented with 10% low IgG FBS, GIBCO #16250, NEAA and PSG).
4. An antibody such as: Commercial Rituximab (humanized Anti CD20), Herceptin (anti humanized HER2/neu) or other suitable antibody for ADCC.
5. Histopaque, Sigma# 1077.
6. aCella-TOX Kit (Cell Technology, Inc.).
7. Luminometer.
8. 96-well Optiplates (Perkin Elmer, #6005290 or Corning #3605 with cover, #3099)
9. Sterile-filtered solutions of 0.2% NaCl and 1.6% NaCl for RBC Lysis.

B. Preparation of target cells:

1. On the morning of the experiment, spin down 8-10 ml of target cells. Wash once in PBS. 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 $4X10^5$ cells/ml. Plating 25µL of this will give 10,000 cells/well. Cell concentration can be adjusted to plate cells at a lower concentration.

C. Separation of PBMC's from buffy pack:

Note: Should be prepared on the morning of the ADCC assay.

1. Pipette 20ml of Histopaque (or other suitable density gradient medium) into a 50 ml tube. Layer 20ml of blood on the Histopaque. Centrifuge at 400xg for 30 minutes.

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. Centrifuge at 400xg 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.**

D. Lysis of Red Blood 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 400xg 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. Bring cells to $3.4X\ 10^6$ cells/ml. Plating 50µl of this will give 170,000 cells/well and a effector:target cell ratio of 17:1 in the wells.

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.

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

1. Dilute the antibody to 4µg/ml in ADCC culture media. Next serially dilute the antibody 4-fold 7 times across the plate. Plating 25µl of each antibody concentration per well will yield a range from 1ug/ml to 0.00024 µg/ml (final concentrations). Starting antibody concentrations are 4X since they will be subsequently diluted 1 in 4 by the addition of target and effector cells.

2. For the zero antibody control, plate 25 µl of media only (control 5 see **F. Control 5** below). Note: Perform reactions in triplicate.

F. Controls:

3. **Control 1:** Spontaneous release of GAPDH from target cells: Plate 25µL containing 10,000 target cells in triplicate and add 75µl of ADCC media.
4. **Control 2:** Spontaneous release of GAPDH by effector cells: Plate 50µL containing effector cells (the same ratio as used in the experiment) and add 50 µL of ADCC media in triplicate.
5. **Control 3:** Maximum lysis of target cells: Plate 25µL containing 10,000 target cells in triplicate and add 75 µL of ADCC media.
6. **Control 4:** Media only control: Plate 100µl of the ADCC media in triplicate.
7. **Control 5:** As mentioned above, a zero Antibody control, 25uL of media+ effector cells + target cells.

G. Preparation of aCella-TOX reagents:

The reagents supplied in the aCellaTOX 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.

H. ADCC Reaction:

1. In setting up the plate, plan to avoid using the outer rows and columns to reduce any "edge effects". Add only 100µL 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 10000 cells/well in 25µL of media in triplicate.
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^{o}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 50µL of the effector cells to start the reaction. Cover the plate with a 96-well plate cover. Incubate the plate at 37^{o}C for 2-4 hours. The time of the reaction should be determined empirically.
8. After the incubation remove the plate from the incubator and equilibrate the plate to room temperature for about 15 minutes.
9. Add 5-10µL of lytic agent to the wells indicated for maximum lysis of target cells.
10. Incubate 5-15 minutes at room temperature.
11. Add 100µL of the Enzyme Assay reagent containing G3P (from step IV: procedures above) to all reaction wells.
12. Immediately, add 50µL of the Detection reagent (from step IV: procedures above) to each well.
13. 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.

I. Calculations:

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

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

For the dose-response curve, plot the Antibody concentrations in µg/ml vs. the % cytotoxicity as calculated by the formula above.

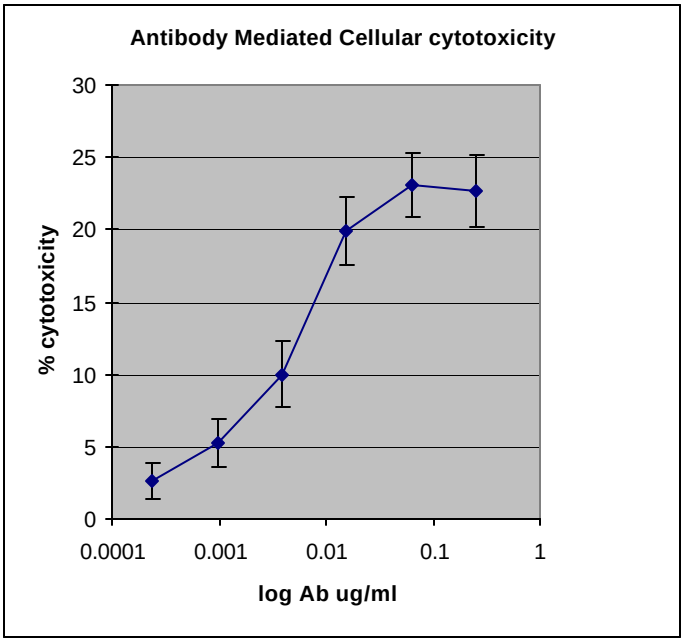


Fig. 1 ADCC: 10,000 Ramos cells/well were incubated with serially diluted Rituxan antibody (humanized anti CD20) for 15 minutes prior to the addition of freshly isolated effector cells. The ADCC assay was further incubated for a total of 4 hours and % cytotoxicity detected using the aCella TOX assay. Effector:Target ratio 17:1.

J.TECHNICAL NOTES:

1. If desired, the reactions can be set up in twice the volume, i.e. 200µl and the plate can be centrifuged to pellet cells and debris, whereupon 100µl of the supernatant is used to perform the aCella-TOX reaction in a separate 96 white plate. This may help lower the background.

2. The volume of lytic agent per 100µL of cells to obtain 100% lysis has to be determined empirically. We recommend starting at 5µL and titrating down (lysis buffer can be titrated in Di water). The incubation time could also vary from 5-15 minutes.

3. 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.

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.

6. Target cells should not be extensively subcultured (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.

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

Using a previously determined effector: target cell ratio, 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 0.25ug/ml	Antibody 0.0625 ug/ml	Antibody 0.0156 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.00098 ug/ml	Antibody 0.00024 ug/ml	Antibody 0 ug/ml Control 5		
C		Antibody 1ug/ml	Antibody 0.25ug/ml	Antibody 0.0625 ug/ml	Antibody 0.0156 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.00098 ug/ml	Antibody 0.00024 ug/ml	Antibody 0 ug/ml Control 5		
D		Antibody 1ug/ml	Antibody 0.25ug/ml	Antibody 0.0625 ug/ml	Antibody 0.0156 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.0039 ug/ml	Antibody 0.00098 ug/ml	Antibody 0.00024 ug/ml	Antibody 0 ug/ml Control 5		
E		Control 1	Control 1	Control 1	Control 2	Control 2	Control 2	Control 3	Control 3	Control 3		
F		Control 4	Control 4	Control 4								
G												
H												

2. CMC PROCEDURE: Sample protocol

A. Cells, Reagents and instruments required:

1. Target cells such as K562, Jurkats or other cell line.
2. Leukopack or Buffy pack
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. 96-well Optiplates (Perkin Elmer, #6005290 or Corning #3605 with cover, #3099)
8. Sterile-filtered solutions of 0.2% NaCl and 1.6% NaCl for RBC lysis.

B. Preparation of target cells:

1. On the morning of the experiment, centrifuge 8-10 ml of Target cells. Wash once in 1X PBS. 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 buffy pack:

Note: Should be prepared on the morning of the CMC assay.

1. Pipette 20ml of Histopaque (or other suitable density gradient medium) into a 50 ml tube. Layer 20ml of blood on the Histopaque. Centrifuge at 400xg 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 to the PBMC's and centrifuge at 400xg 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.

3. Count cells with Trypan blue and adjust cell concentration with cell culture media to deliver the desired number of effector cells in 50 µL. Since aCella-TOX is a very sensitive assay, start with a 1:1 ratio and titrate up.

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.

E. 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: maximum GAPDH release. Plate 50µL of target cells + 50 µL of media.
4. **Control 4:** Media only. Plate 100µL of media.

F. Preparation of aCellaTOX reagents:

The reagents supplied in the aCellaTOX 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. CMC Reaction: Plate in triplicate.

1. To a white opaque 96 well plate add 50 µL of target cells + 50 µL of the various effector cell numbers.
2. Plate the various controls.
3. Cover the plate and incubate the CMC reaction at 37^{o}C for 2-4 hours. This length of time should be determined empirically.
4. After the incubation, remove the plate from the incubator and equilibrate the plate to room temperature for 15 minutes.
5. Add 5-10µl of lytic agent to the wells indicated for maximum lysis of target cells (control 3).
6. Incubate 5-15 minutes at room temperature.
7. Add 100 µL of the Enzyme Assay reagent containing G3P to all reaction wells.
8. Immediately, add 50 µL of the Detection reagent to each well.
9. 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.

G. 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:

1. First subtract the mean media only luminescent value (control 4) from all calculated mean values for each reaction condition.
2. Next for each calculated mean value of E:T ratio, subtract the respective mean calculated value of effector spontaneous release control 2.
3. Then use the following formula:

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

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

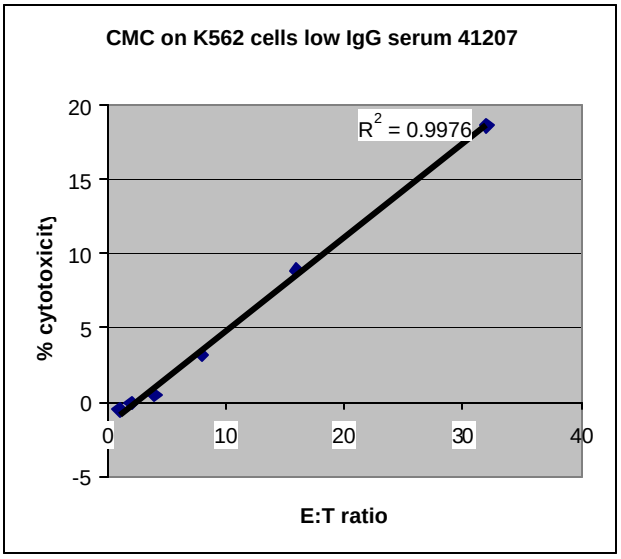


Figure 2 shows CMC assay with K562 as target cells with freshly isolated PBMC's. Target cells plated at 10,000 cells/well and increasing effector cell concentrations to a ratio of 32:1. The assay was incubated for 4 hours at 37°C and % cytotoxicity measured with the aCella-TOX kit .

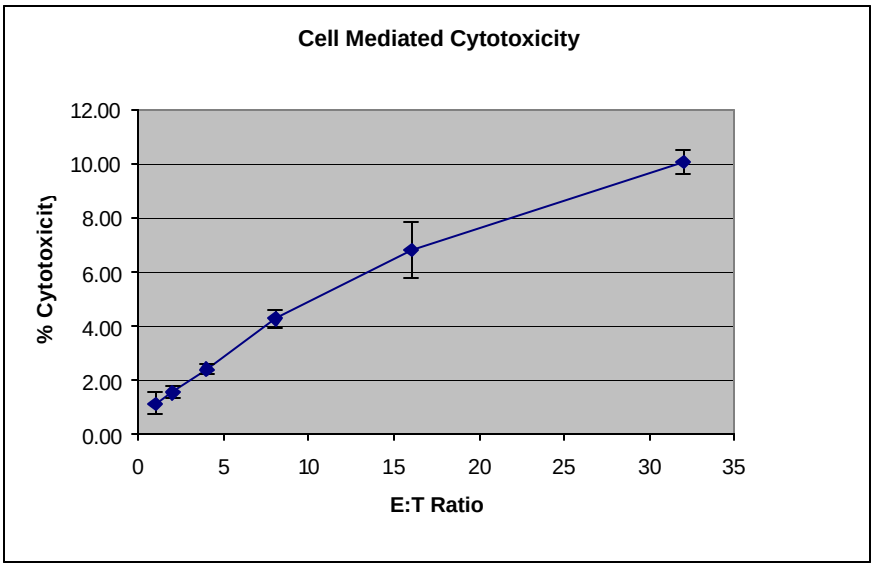


Figure 3 CMC: 10,000 Jurkat cells/well were mixed with freshly isolated Peripheral Blood Lymphocytes at various Effector:Target cell ratios. The CMC assay was incubated for 4 hours and % cytotoxicity detected using the aCella-TOX assay.

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. Drug induced Cytotoxicity Sample protocol

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.

4. 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.

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.

C. Calculation of Results:

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

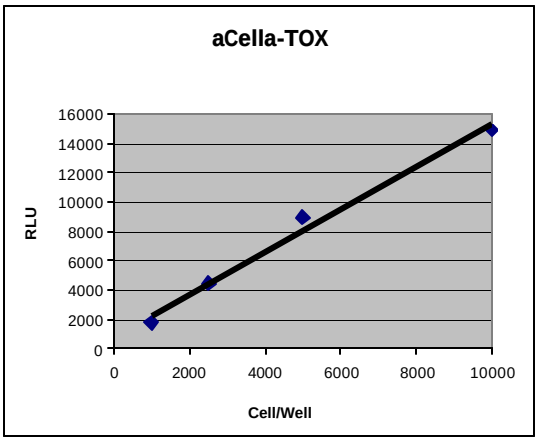


Figure 1: Demonstrates linear response of the aCella-TOX kit: Jurkat cells were plated at various cell concentrations per well. The lysis buffer was added to each well. aCella-TOX kit was used to detect GAPDH enzyme release of the lysed cells. Data points show samples run in triplicate with background subtraction.

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