Assay for Cell Death: Chromium Release Assay of Cytotoxic Ability

Overview

Source: Frances V. Sjaastad1,2, Whitney Swanson3,4, and Thomas S. Griffith1,2,3,4

1 Microbiology, Immunology, and Cancer Biology Graduate Program, University of Minnesota, Minneapolis, MN 55455
2 Center for Immunology, University of Minnesota, Minneapolis, MN 55455
3 Department of Urology, University of Minnesota, Minneapolis, MN 55455
4 Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

One of the main functions of the cells of the immune system is to remove target cells that have been infected with viruses or have undergone transformation into a tumor cell. In vitro assays for measuring the cytotoxic capacity of immune cells have been a staple in laboratories for many years. These assays have been used to determine the ability of T cells, NK cells, or any other immune cell to kill target cells in an antigen-specific or -nonspecific manner. Death ligands (e.g., Fas ligand or TRAIL), cytokines (e.g., IFNg or TNF), or cytotoxic granules (i.e., perforin/granzyme B) expressed by effector cells are some ways in which target cell death can be induced. With the explosion in tumor immunotherapy research in recent years, there is growing interest in finding agents to increase the cytotoxic activity of immune cells to improve patient outcomes. Conversely, some diseases are hallmarked by the overexuberant activity of immune cell cytotoxic activity, resulting in efforts to identify agents to temper these responses. Thus, having an assay in which the user can easily integrate any number of different effector cells, target cells, and/or response modifiers into the experimental design can serve as a valuable means of quickly assessing the cytotoxic capacity of effector cells and/or the responsiveness of the target cell.

These in vitro assays involve the mixing of different cell populations, as well as using a relatively low number of both effector and target cells. Thus, one necessity of the assay is to label the target cells in a manner that can easily be detected and quantitated, allowing the user to then determine the ‘percent specific lysis’ mediated by the effector cells. Radioactivity - especially, chromium 51 (51Cr) in the form of Na251CrO4- is an inexpensive way to rapidly and nonspecifically label cellular proteins within the target cells (1). The short labeling and total assay times reduces the potential for significant changes in the number and/or phenotype of the target cells, which could influence the outcome of the assay. Upon the loss of membrane integrity of the target cells as a result of the cytotoxic activity of the effector cells, the 51Cr-labelled cellular proteins within the target cells are released into the culture supernatant, becoming available for quantitation. As with any assay examining the function of immune cells in vitro, there are a number of important considerations to consider improving the performance of the experiment. One of the most critical features is to use healthy effector (for maximal cytotoxic activity) and target (for maximal responsiveness and minimal spontaneous death/51Cr release) cells. Effector and target cell contact is required (leading to the common use of round-bottom 96-well plates to encourage cell-cell contact) (2). Finally, data analysis is dependent on the inclusion of positive and negative control target cell populations.

The following protocol will outline the steps for performing a standard 51Cr release assay to measure the cytotoxic ability of a population of effector cells, though a nonradioactive version using Europium has recently been developed. 51Cr is a powerful y-radiation emitter. Consequently, the use of this assay requires proper radiation safety training, dedicated laboratory space, a gamma counter, and disposal of radioactive samples.

The general sequence of events in this assay are: 1) prepare 51Cr-labeled targets; 2) prepare effector cells and add to plate while target cells are labeling; 3) add labeled targets to plate; 4) incubate plate; 5) harvest supernatants; and 6) analyze data after running samples on counter. Samples are commonly prepared in triplicate, and then averaged to account for any subtle pipetting differences.

Proper PPE is important for this assay. Specifically, the user should wear a lab coat and gloves. Safety glasses may be required based on the laboratory or institution. There should be ample lead shielding for safe storage and use of the 51Cr during all steps. Finally, there should be dedicated lab space and equipment set aside for using 51Cr, including all the proper signage to indicate where samples with 51Cr are being kept and a Geiger counter equipped with gamma probe to survey the space for possible contamination.

In this lab exercise, we will determine the ability human peripheral blood mononuclear cells (PBMCs), (CpG stimulated vs. unstimulated) to kill melanoma cells, using human melanoma cell line WM793 as model and the chromium release assay.

Procedure Overview

The typical 51Cr-release assay for measuring cell death involves the following steps:

1. First, the target cells are labeled with Na2[51Cr]O4. This distinguishes them from the effector cells in the assay.
2. While the target cells are labeling, the effector cells are collected and, using the serial dilution technique, a decreasing titration of the effector cells is generated in a round bottom 96-well assay plate.
3. At the end of the target cell labelling, the cells are first washed and then a fixed number of cells are added to the assay plate that already contains a series of effector cells dilutions.
4. Next, the target-effector cell mix is incubated for a defined period of time to allow for sufficient cell interaction with the target cells, to mediate cell lysis.
5. Finally, the culture supernatants are harvested and collected into tubes. The 51Cr amount is quantitated using a gamma counter.
6. At the end, the data is collected and used to calculate the "percentage specific cell lysis" of the target cells.

1. *Labeling Target Cells with $^{51}$Cr*

1. To begin, prepare the target cells, (here- human melanoma cell line WM793), into a single cell suspension.
2. To do this, first remove the media from the tissue culture flask.
3. Then, wash the cells with 5 mL of 1X PBS.
4. Trypsinize the cells by adding 1 mL of trypsin to the plate for ~2 min.
5. Gently tap the flask to loosen the cells from the flask surface.
6. Add 5 mL of RPMI media to the flask and pipet the media up and down to detach the cells.
7. Collect the cell suspension into a 15 mL conical tube and centrifuge the cell suspension for 5 min at 1200 rpm. Decant the supernatant.
8. Add 10 mL of media to the pellet and gently pipet the media up and down to bring the cells into suspension.
9. Determine the cell concentration using a hemocytometer.
10. Transfer 1x10$^6$ cells into a new 15 mL conical tube.
11. Centrifuge the cell suspension for 5 min at 1200 rpm and decant the supernatant.
12. Briefly vortex the tube to resuspend the cell pellet in the small volume of medium left behind.
13. Add 100 µCi of $^{51}$Cr directly to the WM793 target cell suspension.

   **Note:** There should be a dedicated lab space set up for the particular radioactivity. In addition, there should be ample lead shielding for safe storage and use of the $^{51}$Cr during all steps as well as proper signage to indicate where samples with $^{51}$Cr are being kept. A Geiger counter equipped with a pancake probe is also necessary, for surveying the space for possible contamination.
14. Add a small piece of radioactive tape to the tube to indicate that the tube is now radioactive.
15. Place the tube in a 37°C incubator with a lead shield and incubate for 1 h. Flick the tube every 15-20 minutes to increase target cell uptake of the chromium.
16. After the incubation period, wash the target cells with 5 mL of FBS to remove any excess $^{51}$Cr.
17. Centrifuge the cells at 1200 rpm for 5 min. Decant radioactive FBS wash into appropriate waste container.
18. Resuspend the pellet and wash a second time with FBS. Check the pellet for incorporated radioactivity using a Geiger counter.
19. Resuspend the pellet in 10 mL complete medium, achieving a cell concentration of 10$^5$ cell/mL.

   **Note:** The cell counting step after the $^{51}$Cr labelling is omitted here largely for the safety reasons. It can be assumed that the WM793 cell concentration is same as it was prior to the $^{51}$Cr labelling.

2. *Preparing Effector Cells*

1. A variety of effector cells can be used, such as, human or mouse T cells and NK cells. In this example, PBMCs, isolated from whole blood by standard density gradient centrifugation (to a concentration of 5x10$^6$), were used.
2. To begin, add 100 µL of tissue culture medium to every well of one of the rows (here row A, See Table 1) of a 96-well round-bottom plate. Here, no effector cells are added, and they will serve as the 'blank' for determining the "minimum/spontaneous $^{51}$Cr release" from the target cells.

<table>
<thead>
<tr>
<th>Condition 1</th>
<th>Condition 2</th>
<th>Condition 3</th>
<th>Condition 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Min” – spontaneous release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50:1 E:T</td>
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<td></td>
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<tr>
<td>25:1 E:T</td>
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<tr>
<td>12.5:1 E:T</td>
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<tr>
<td>6.25:1 E:T</td>
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<tr>
<td>3.12:1 E:T</td>
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<tr>
<td>1.56:1 E:T</td>
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<table>
<thead>
<tr>
<th>H</th>
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<tbody>
<tr>
<td>“Max” – total release</td>
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</table>

3. Next, generate a 2X serial cell dilution of the PBMCs (in triplicate for each experimental condition), to obtain an effector cell concentration ranging from 5x10$^5$ to 15,625 cells/100 µL of media (here rows B to G).

   **Note:** In this example, the starting effector: target cell (E: T) ratio is be 50:1. However, this ratio can be adjusted depending on the experimental specifics.
4. Leave the last row empty (here row H) by not adding any effector cells to these wells. (This row will be used to generate the "total counts/min, or (c. p. m)" or the "maximum $^{51}$Cr release").
5. Place the plate into 37°C incubator until the target cells are ready to be added.
3. Adding \(^{51}\)Cr Labeled WM793 Target Cells to the Assay

1. After the incubation period, remove the target cells from the incubator and wash with 5 mL of FBS to remove any excess \(^{51}\)Cr.
2. Centrifuge the cells at 1200 rpm for 5 min, using a designated centrifuge.
3. Remove the FBS wash (radioactive supernatant) into an appropriate waste container.
4. Repeat the wash step by resuspending the pellet in a fresh 5 mL of FBS.
5. Centrifuge the cells again at 1200 rpm for 5 min.
6. Finally, resuspend the pellet in 10 mL of complete medium.
7. Pour the \(^{51}\)Cr-labelled WM793 cell suspension (10^5 cells/mL) into a disposable reagent reservoir.
8. Then, add 100 µL of these labeled target cells, to each well of the 96-well effector cell plate, using a multi-channel pipette.
9. Next, add 100 µL of 1% NP-40 (in water) to the row of wells that are devoid of any effector cells (here row H). 1% NP-40 will lyse the target cells, and thus these wells will serve as controls to determine the "total counts/min, or (c. p. m)" or the maximum \(^{51}\)Cr release.
10. Secure lid to plate by adding a small piece of gas-permeable tape to each side of the plate and place a piece of radioactive tape on the lid to indicate it contains \(^{51}\)Cr.
11. Briefly, centrifuge the plate at 1200 rpm. If only one experimental plate is being used, add a balance plate to the centrifuge.
   **Note:** It is important to use a centrifuge marked to handle radioactive samples.
12. Remove the plate from the centrifuge.
13. Place the plate in a 37°C incubator with a small piece of lead shielding over the plate for additional safety. Incubate for 16 h to allow for target cell lysis.
   **Note:** The incubation period can vary from 4 to 18 h depending on the effector cells used and potential mechanism of killing employed.

4. Harvesting the Supernatants

1. At the end of the incubation period, carefully remove the tape around the edge of the plate and remove the lid.
2. Next, place the harvesting frame on the plate, making sure to confirm that the small filter discs are in place for each of the cotton plugs. This will ensure the collection of a cell-free supernatant.
3. Now, slowly and gently press the cotton plugs into the wells.
4. After approximately 10 seconds, release the pressure on the cotton plugs and then transfer the cotton plugs to tube strips.
5. Place each of these tubes into a secondary FACS tube.
6. Finally, load FACS tubes onto gamma counter and run the samples to quantitate the amount of \(^{51}\)Cr released in each condition. Samples are typically measured for 1 minute, allowing for an easy determination of the "counts/minute".
7. Carefully, record the order in which the tubes were loaded into the counter.

5. Data Analysis

1. Here, unstimulated PBMCs were added to the first three columns, and CpG-stimulated PBMCs (CpG ODN, (1 µg/mL) for 24 h) were added to columns 4-6. See Table 2.
Table 2: $^{51}$Cr release assay data: ‘Counts per minute’ / ‘c. p. m’ data values, average c. p. m values, and calculated percent specific lysis values.

2. The collected data (counts per minute, i.e. c.p.m) were entered into the cells of a spreadsheet in the same manner as the samples were laid out in the original plate.

3. First, the averages of the triplicates were calculated. Table 2 - cells I3 to P3, for unstimulated PBMCs and cells I6 to P6, for CpG-stimulated PBMCs.

4. Once the averages were determined, the percent of specific lysis for each condition was calculated using the following formula-

   \[
   \text{% specific lysis} = \frac{\text{experimental c.p.m} - \text{spontaneous c.p.m.}}{\text{total c.p.m.} - \text{spontaneous c.p.m.}} \times 100
   \]

5. The percent of specific lysis was calculated for each condition (Table 2 - cells J4 to O4, for unstimulated PBMCs and J7 to O7, for unstimulated PBMCs).

Results

In this example, effector cells stimulated with CpG (Figure 1, black circles) killed the target cells more effectively, as the ratio of effector cells to target cells increased. This increase was not observed in the unstimulated PBMCs (white circles), indicating that CpG stimulation is necessary for the observed increase in target cell lysis.

Figure 1: $^{51}$Cr assay scatter plot: Tumoricidal activity by human PBMCs, unstimulated (white circles) and after stimulation with CpG (black circles), tested at different effector: target cell ratios (E: T) ratios (ranging from 50:1 to 1.5:1).
Applications and Summary

The assay described here has considerable flexibility, as a variety of effector and target cells can be used depending on the question being asked. For example, effector cell specificity can be determined by using different target cells or the mechanism of effector cell killing can be determined by using cells deficient in specific proteins or using protein specific inhibitors. A major problem with the $^{51}$Cr release assay is the potential for a high spontaneous release rates by the target cells. When cultured alone (without effector cells), the spontaneous release of $^{51}$Cr by the target cells should ideally be no more than 30% of the total (“maximal”) release by the target cells immediately lysis. Higher spontaneous release rates may be due to using unhealthy target cells, either due to poor health (e.g., extended culture of a cell line) or an overly long labelling period.

References