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ab270780 Cytotoxicity Assay Kit (CFSE, 7-AAD)

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For the measurement of cytolytic activity in cultured cells.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Cytolytic activity is an important process for eliminating intracellular pathogens and cancer cells. Cytotoxicity Assay Kit (CFSE, 7-AAD) (ab270780) allows you to assess cytolytic activity in cell culture.

Older methods to assess NK cytolytic activity include measuring the release of lactate dehydrogenase, and more commonly, the release of radioactive ^{51}Cr from lysed target cells. Unfortunately, these techniques have several drawbacks. Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells. Problems associated with ^{51}Cr release methods include high spontaneous leakage resulting in high backgrounds, high cost, short half-life, and the health risks due to exposure to radioactive material.

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. One such early version involved the detection of NK cytotoxicity activity by staining target cells with the green fluorescent dye, F-18, in combination with the DNA intercalating dye, propidium iodide . Since then, a red fluorescent membrane dye, PKH-26, has been used in preference to F-18, and in combination with the viability probe, TO-PRO-3 iodide4-7. However, despite correlations of greater than 95% when compared with the ^{51}Cr release assay, the PKH-26 method is problematic. It is difficult to use at a constant concentration, leading to unreliable staining, and the staining procedure requires multiple steps, often decreasing the viability of the target cells.

Cytotoxicity Assay Kit (CFSE, 7-AAD) includes two fluorescent reagents: CFSE and 7-AAD. CFSE, a green fluorescing cellular stain, is utilized to identify the target cell population. The unstained effector cells are added and incubated with the target cells. 7-AAD, a red fluorescing live/dead stain, is then added to stain all necrotic cells red by binding to the DNA of membrane-compromised cells. The target cell and effector cell populations can then be easily distinguished, and flow cytometry easily identifies live and necrotic cells.

2. Materials Supplied and Storage

Store kit at -20°C immediately on receipt. However, some components may be stored at +4°C.

Δ Note: Once reconstituted with DMSO, use CFSE and 7-AAD immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

Δ Note: 10X Assay Buffer may be stored frozen or refrigerated. 1X Assay Buffer may be stored at 4°C for 1 week, or frozen and used within 6 months.

Item	Quantity		Storage temperature
	125 tests	250 tests	
CFSE, green cellular stain, 250 tests	1 vial	1 vial	-20°C
7-AAD, red live/dead stain, 125 tests	1vial	2 vials	+4°C or -20°C
10X Assay Buffer	1 x 30 mL	1 x 60 mL	+4°C or -20°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- DMSO, up to 1 mL to reconstitute reagents
- DiH_2O, 270-540 mL to dilute 10X Assay Buffer
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- FBS and/or BSA to add to buffer when handling cells
- Cultured cells treated with the experimental conditions ready for labelling
- 15% ETOH (in PBS or 1X Assay Buffer) to create live/dead controls for 7-AAD staining
- Reagents to induce cytotoxicity and create controls
- Hot water bath, to create live/dead controls for 7-AAD staining
- 37°C incubator
- Ice bath
- Centrifuge at 200 *x g*
- Hemocytometer
- 15 mL polypropylene centrifuge tubes (1 per sample)
- FACS tubes
- Flow cytometer

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Experimental Overview

Quantifying cell death with this kit can be completed within a few hours. However, the experiment is performed on living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or cytotoxic process, or to induce necrosis, and create controls. Each investigator should adjust the amount of the reagents and incubation times to accommodate their particular cell line(s) and research conditions.

Control populations must be made for the experimental conditions. If the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc., prepare a control of non-infected target cells combined with effector cells to determine cell death which normally occurs within the healthy target cells. In addition, several control tubes must be prepared for compensation and gating of the flow cytometer (Section 6).

The kit includes 10X Assay Buffer, and two lyophilized fluorescent reagents: CFSE and 7-AAD, which must be reconstituted and diluted prior to use. First dilute and filter the 10X Assay Buffer, as it is used to dilute the other reagents. Then reconstitute the lyophilized reagents with DMSO to create the stock concentrates and store on ice. Once it is time to use the reagent, prepare the working solution by diluting the stock. Here is a quick overview of the procedure.

5.1 Dilute 10X Assay Buffer 1/10 with diH_20, forming 1X Assay Buffer and sterile filter (Section 9).

5.2 Reconstitute CFSE with 200 µL DMSO, forming a 2500X stock concentrate (Section 11).

5.3 Dilute 2500X CFSE stock 1:250 in sterile 1X Assay Buffer, forming a 10X working solution (4 µL into 996 µL).

5.4 For each sample and control, adjust target cells to 1-2 x 10^6 cells/mL, wash, and resuspend each in 1.8 mL 1X Assay Buffer (Section 10).

5.5 Prepare control tubes (Section 6).

5.6 Add 200 µL diluted 10X CFSE to target cells, and all controls except B (at 1.8 mL).
5.7 Incubate 15 mins at room temperature.
5.8 Wash cells: add 1 mL media, centrifuge, remove supernatant, and add 2-3 mL media.
5.9 Incubate 30 mins at 37°C.
5.10 Adjust stained target cells to 1 x 10^5 cells/mL and make 200 µL aliquots (2 x 10^4 cells/aliquot).
5.11 Adjust unstained effector cells to the desired concentration, such as 50 times the concentration of the target cells, in 200 µL aliquots.
5.12 Add 200 µL unstained effector cells to the CFSE stained target cells. This forms the 'E:T' mixture at 400 µL.
5.13 Incubate 'E:T' mixture 4-6 hours at 37°C.
5.14 Create a positive control of killed cells to generate Control E (Section 6).
5.15 Reconstitute 7-AAD with 260 µL DMSO, forming a 210X stock concentrate (Section 15).
5.16 Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer, forming a 21X working solution (40 µL into 360 µL).
5.17 Add 20 µL diluted 21X 7-AAD to Controls D, E, & F (at 400 µL).
5.18 Incubate controls 10 mins on ice.
5.19 Run the instrument controls to set up gating and compensation on the flow cytometer (Section 6).
5.20 Add 20 µL diluted 21X 7-AAD to samples (at 400 µL).
5.21 Incubate samples 10 mins on ice.
5.22 Read samples and analyze (Section 16).

6. Flow Cytometry Controls

Several control populations are needed to properly gate the flow cytometer and set up compensation on the instrument (Section 13). Follow the table below to create the control tubes and set up the flow cytometer. Examples shown here were generated on a BD FACS Caliber; compensation requirements may differ among instruments. Treated experimental population(s): cells exposed to the experimental condition(s).

Control #	
A	Control A contains target cells stained with CFSE and effector cells. It is used to separate the green target cells from the unstained effector cells. Target and effector cells can also be separated by size using forward and side scatter plots.
B and C	Control B contains unstained target cells. Control C contains target cells stained green with CFSE. They are used to determine the shift of target cells from left to right along FL-1 the green axis.
D and E	Control D contains live target cells stained green with CFSE and red with 7-AAD. Control E contains killed target cells stained green with CFSE and red with 7-AAD (Section 7). They will determine the shift of 7-AAD from bottom to top along FL-3 the red axis. Control D will also act as an experimental control to measure the basal level of necrosis not caused by the experimental treatment. It will determine the level of spontaneous cell death which normally occurs within the cell line without the influence of the effector cells. Subtract the level of spontaneous cell death from the experimental samples to determine the true level of cytotoxicity.
F	Control F contains non-infected target cells stained with CFSE mixed with effector cells and stained with 7-AAD to determine spontaneous cell death. Prepare Control F if the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc.

7. Prepare Killed Cells

In Section 6, Control E contains target cells labeled green with CFSE that have been killed and then labeled red with 7-AAD. For compensation and gating, it is preferable to use a control that contains a mixture of live and dead cells. Below are 2 methods for preparing such a control.

Method 1: Hot water bath.

- Immerse the tube of cells in a 56°C water bath for 10-20 mins. The optimal heat exposure period may vary with cell type. For best results, determine a reproducible method for killing 30-60% of the cell population prior to commencing the experiment.
- Place on ice.
- Add 7-AAD to stain necrotic cells (Section 13).

Method 2: Ethanol is an effective killer, however, ethanol may decrease the fluorescence output of the CFSE cellular stain, therefore the population may not shift as far to the right along FL-1, the green axis .

- Centrifuge cells at 200 *x g* for 5-10 mins at room temperature (RT).
- Carefully remove the supernatant.
- Resuspend cells in 15% ethanol. For example, add 150 µL ethanol plus 850 µL PBS or 1X Assay Buffer to resuspend cells.
- Incubate 10 mins at RT.
- Add 2-3 mL 1X Assay Buffer.
- Centrifuge at 200 *x g* for 5-10 mins at RT.
- Carefully remove the supernatant.
- Add 400 µL media to resuspend cells.
- Add 7-AAD to stain necrotic cells (Section 13).

8. Preparation of Samples and Controls

All target cells must be stained green with CFSE to distinguish them from non-stained effector cells. If studying the effects of a toxic agent rather than cell mediated cytolytic activity, CFSE staining is optional as the only cells present will be the target cells.

Cultivate the proper number of target and effector cells for the sample and control populations. Allow time for the experimental treatment or cytotoxic process. Do not use target cells that are capable of proliferating more than 4 hours prior to the assay; when a CFSE labeled cell divides, its progeny each inherit half the number of fluorescent tagged molecules as the parent cell. Therefore, proliferation will decrease the average fluorescence intensity of the target cell population.

Δ Note: As cell media will quench CFSE fluorescence, the media must be replaced with 1X Assay Buffer before staining with CFSE.

9. Preparation of 1X Assay Buffer

10X Assay Buffer is used to replace cell culture media, dilute reagents, and wash cells. It is a PBS-based buffer that does not contain any preservatives and should be stored at ≤4°C (precipitates may form in the 10X buffer during cold storage). It is supplied as a 10X concentrate which must be diluted to 1X with sterile/endotoxin free diH_20 prior to use and sterile filtered. 1X Assay Buffer may be stored at 4°C for 1 week or frozen and used within 6 months.

Instead of using 1X Assay Buffer to dilute the reagents, sterile PBS can be used. In some steps, fresh cell culture media can be used in place of 1X Assay Buffer (but not while staining with CFSE).

- 10X Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
- Dilute 10X Assay Buffer 1/10 in diH_20.
  For example:
  a. Add 30 mL 10X Assay Buffer to 270 mL diH_20 (forming a total volume of 300 mL).
  b. Add 60 mL 10X Assay Buffer to 540 mL diH_20 (600 mL total).
- Mix for 5 mins or until all crystals have dissolved.
- Sterilize by filtration.

10. Adjust Target Cells in 1X Assay Buffer

10.1 Adjust target cells to 1-2 x 10^6 cells/mL in 1 mL 1X Assay Buffer Do not use media as it will quench CFSE fluorescence.

10.2 Wash target cells twice with 1X Assay Buffer to remove any media. Centrifuge at 200 x *g* for 5-10 mins at RT and discard supernatant.

10.3 Resuspend target cells with 2-3 mL 1X Assay Buffer.

10.4 Centrifuge at 200 x *g* for 5-10 mins at RT; discard supernatant.

10.5 Resuspend target cells (1-2 x 10^6) in 1.8 mL 1X Assay Buffer (5.56 x 10^5 cells/mL to 1.11 x 10^6 cells/mL).

11. Stain Target cells with CFSE

5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), is used to label cells with a green fluorescence potential stain. In this assay, it is used to label all the target cells green prior to exposure to the effector cells. CFSE is supplied as a highly concentrated lyophilized powder; the amber vial may appear empty as the reagent is lyophilized onto the insides of the vial. Once reconstituted in DMSO, it should have a slight hint of color.

CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1/250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells.

Δ Note: Do NOT dilute CFSE in media, as the reactive properties of the CFSE stain will be neutralized. Store the lyophilized CFSE and 2500X stock at ≤ -20°C protected from light

11.1 Reconstitute CFSE with 200 µL DMSO. This yields a 2500X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the vial until completely dissolved. At room temperature, this should take just a few mins. Protect from light.

11.2 If not using all of the 2500X stock concentrate at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes

11.3 When ready to use in the assay, dilute the 2500X stock 1:250 with sterile 1X Assay Buffer. For example, add 4 µL of 2500X CFSE stock to 996 µL sterile 1X Assay Buffer and mix. This yields 1 mL of 10X CFSE working solution. For best results, the 10X working solution should be used within 2 hours, stored on ice and protected from light.
Δ Note: Do NOT dilute in media, as the fluorescence will be quenched.

11.4 Add 200 µL 10X CFSE working solution to each 1.8 mL suspension of target cells, and to all control tubes except B. Gently vortex.
The optimal concentration of CFSE may vary among cell types. Adjust the concentration of CFSE and the incubation

time to adequately stain the target cells for the experiment. Excessive staining may cause problems when compensating the flow cytometer.

11.5 Incubate 15 mins at room temperature.

11.6 Add 1 mL cell culture media per tube to stop the CFSE binding reaction.

11.7 Centrifuge at 200 *x g* for 5-10 mins at RT; discard supernatant.

11.8 Resuspend in 2-3 mL cell culture media per tube.

11.9 Incubate 30-60 mins at 37°C in a CO_2 incubator (or other conditions appropriate for the experiment). Plan the experiment so the cells incubate no more than 1 hour while setting up the assay.

11.10 Centrifuge at 200 *x g* for 5-10 mins at RT; discard supernatant.

11.11 Resuspend with cell culture media. Adjust the concentration of the target cells to 1 x 10^5 cells/mL, therefore a 200 µL volume will contain 2 x 10^4 target cells. The 200 µL aliquots of target cells will be combined with 200 µL of effector cells, yielding the desired Effector:Target cell ratio (E:T), such as 50:1 (Section 12).

12. Add Effector Cells

12.1 Adjust the concentration of the effector cells so that approximately 200 µL can be added to the target cells yielding the desired E:T ratio, such as 50:1.
For example, if the 200 µL target cell suspension contains 2 x 10^4 cells (Section 11), in order to add 50 times that number of effector cells (50 x (2 x 10^4 target cells) = 1 x 10^6 effector cells) in 200 µL, the concentration of effector cells needed would be 5 x 10^6 cells/mL (1 x 10^6 cells ÷ 0.2 mL = 5 x 10^6 cells/mL). An optimal E:T cell ratio is required to effectively determine cytolytic activity.

12.2 Add effector cells to samples and Controls A and F and adjust the total volume to 400 µL.

12.3 Incubate 4-6 hours at 37°C in a CO_2 incubator. The incubation time may vary depending on the experiment.

13. Label Controls with 7-AAD

Dead target cells can be identified with 7-aminoactinomycin D (7-AAD). 7-AAD is a red vital stain that can be used to identify and quantitate dead and dying target cells resulting from the cytolytic activity of the effector cells or toxic agent. This dye will penetrate the structurally compromised cell membranes of dead and dying cells and complex with DNA. The intercalated 7-AAD dye exhibits a red fluorescence in the FL-3 region with maximum output at 647 nm. Staining with 7-AAD should be done just prior to analysis; it is the last step due to its toxic effect on most cell types.

7-AAD is supplied as a highly concentrated lyophilized powder; the amber vial may appear empty as the reagent is lyophilized onto the insides of the vial. It must first be reconstituted in DMSO, forming a 210X stock concentrate, and then diluted 1/10 in sterile 1X Assay Buffer to form a final 21X working solution. Store the lyophilized 7-AAD refrigerated or frozen; store 210X stock at ≤-20°C.

Danger: 7-Aminoactinomycin D (7-AAD) is fatal if swallowed, may cause cancer if swallowed, and may damage the unborn child if swallowed. See SDS for further information.

13.1 Reconstitute lyophilized 7-AAD with 260 µL DMSO. This yields a 210X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the amber vial until completely dissolved. At room temperature, it should be dissolved within a few mins.

13.2 If not all of the 210X 7-AAD stock concentrate will be used at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.

13.3 When ready to use in the assay, dilute the 210X stock 1/10 with sterile 1X Assay Buffer or media. For example, add 40 µL of the 210X stock to 360 µL 1X Assay Buffer and mix. This yields 400 µL of the 21X working solution. For best results, the 21X working solution should be used within 2 hours, stored on ice and protected from light.

13.4 Add 20 µL 21X 7-AAD to Controls D, E, and F (at 400 µL) just prior to analysis and mix or gently vortex.

Δ **Note:** Do not add 7-AAD to the experimental samples until the flow cytometer has been set up.

13.5 Incubate 10 mins on ice protected from light.

14. Run Controls to set up Flow Cytometer

Set up the proper instrument gating and compensation adjustments based on the controls (Section 6).

14.1 Run Control A to separate live, green fluorescing target cells from unstained effector cells.

14.2 Run Controls B and C to ensure live, green fluorescing target cells fall in the proper region.

14.3 Run Controls D and E to ensure dead, red fluorescing target cells fall in the proper region. Control D will also determine the level of spontaneous cell death that normally occurs within the cell line without the influence of effector cells.

14.4 Run Control F if the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc. It contains non-infected target cells stained with CFSE mixed with effector cells and stained with 7-AAD to determine spontaneous cell death.

15. Label Samples with 7-AAD

15.1 Add 20 µL 21X 7-AAD to the experimental samples (at 400 µL) and mix or gently vortex.

15.2 Incubate 10 mins on ice protected from light and analyze as soon as possible (Section 16).

16. Flow Cytometry Analysis

16.1 Distinguish green target cells from unstained effector cells using FSC vs. SSC or CFSE (FL-1) vs. SSC.

16.2 Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to distinguish live green target cells from dead red and green target cells.

16.3 Create a gate on the green target cell population (R3).

16.4 Determine the number of red and green necrotic target cells (R2).

16.5 Calculate the percentage of cytotoxicity by dividing the number of red and green dead cells in the R2 region of R3 by the total number of green target cells in both the R1+R2 regions of R3 and multiplying by 100.

16.6 Calculate the percentage of cytotoxicity caused by the experimental treatment by subtracting the percentage of spontaneous cell death without the influence of effector cells from the samples (Control D).

Based on the number of green target cells in R3 cytotoxicity can be calculated as the percentage of the green target cells which are also stained red with 7-AAD in R2. This analysis reveals the population of necrotic target cells without interference from necrotic effector cells.

$$\text{Cytotoxicity (\%)} = \frac{\#\text{ Necrotic R2}}{\text{(R3 = \#Live R1 + \#Necrotic R2)}} \times 100$$

17. Notes

Technical Support

For all technical or commercial enquiries please go to:

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