Technical Tips for the Cell Cytotoxicity Assay using the DELFIA® Technology

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Introduction

Cytotoxicity assays are a fundamental part of the drug discovery process. One of the most common methods for detecting cell-mediated cytotoxicity is the conventional ^{51}Cr release assay in which cytotoxic cells are incubated with ^{51}Cr labeled tumor target cells, and the cytolytic activity is measured by detection of the ^{51}Cr released into the supernatant. As a non-radioactive alternative, we have developed the Europium TDA (EuTDA) cytotoxicity assay based on PerkinElmer's well known DELFIA technology. The EuTDA assay uses time-resolved fluorometry (TRF), which is the only non-radioactive method that offers sensitivity comparable to the ^{51}Cr release assay (Figure 1). There is no physical or chemical treatment of the cell membrane and the ligand is rapidly accumulated in the target cells, which facilitates excellent recovery of the loaded cells.

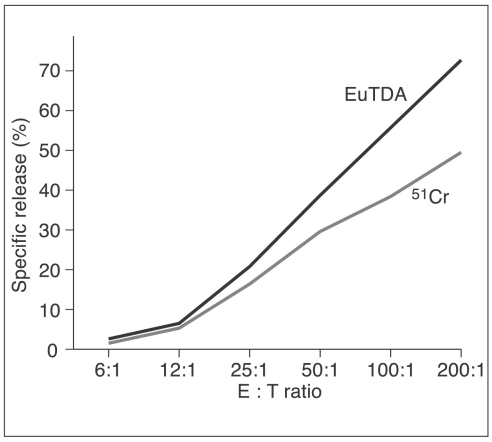

Figure 1. Comparison of the EuTDA assay with the ^{51}Cr release assay. The data is presented as % specific release from the target cells, lysed by the effector cells at different effector:target cell ratios.

The EuTDA assay is based on loading target cells with an acetoxymethyl ester of the fluorescence enhancing ligand (BATDA) (Figure 2). The ligand penetrates the cell membrane quickly. Within the cell the ester bonds are hydrolyzed to form a hydrophilic ligand (TDA) which no longer passes the membrane. When effector cells are placed in contact with the loaded target cells, the target cells are lysed and TDA is released. When the TDA is mixed with DELFIA Europium solution, the Europium and TDA form a highly fluorescent and stable chelate (EuTDA) (Figure 3). The measured fluorescence signal correlates directly with the amount of lysed cells.

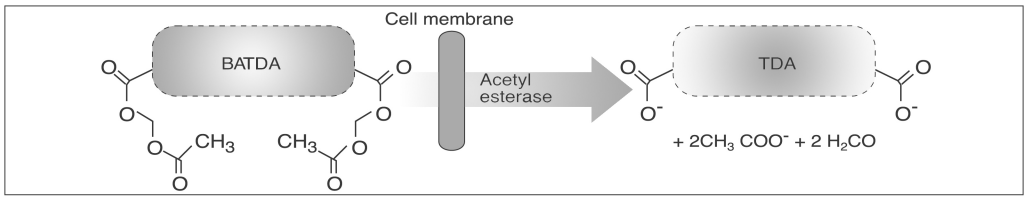

Figure 2. Principle of the cell labeling. After the BATDA ligand has penetrated the cell membrane it is hydrolyzed to TDA by the acetyl esterases in the cells.

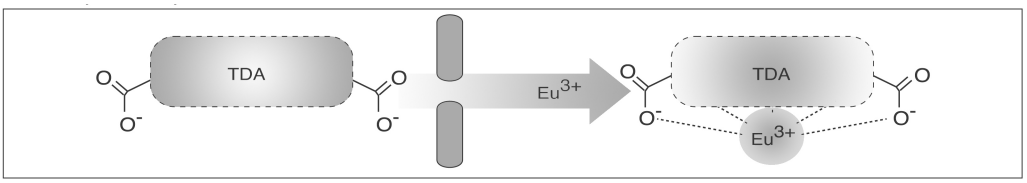

Figure 3. Detection of the released TDA. When effector cells are mixed with target cells, the target cells are lysed. After addition of Eu^{3+}-solution to the sample a fluorescent chelate is formed.

Here we provide a simple protocol for the EuTDA cell cytotoxicity assay, and demonstrate the robustness of the assay to a number of process improvements and simplifications. Firstly we will show that omitting the centrifugation step before transfer of sample does not compromise the assay performance, and secondly we will show that the assay can be performed in 96-well V-bottom plates and 384-well plates without significantly affecting the results. Finally, we will show that supernatant samples can be stored at -20°C prior to testing. We have also included a troubleshooting guide to optimize assays performed in alternative experimental conditions.

Materials and Methods

MATERIALS

All of the reagents used are available in the DELFIA EuTDA Cytotoxicity Reagents kit (PerkinElmer cat. no. AD0116) containing:

- DELFIA BATDA reagent (50 µL),
- DELFIA Lysis buffer (0.5 mL),
- DELFIA Europium solution (200 mL), and
- DELFIA microtitration plates

Alternatively, the reagents may be purchased separately:

DELFIA BATDA reagent, 50 µL (PerkinElmer cat. no. C136-100)
DELFIA Lysis buffer, 30 mL (PerkinElmer cat. no. 4005-0010)
DELFIA Europium solution, 200 mL (PerkinElmer cat. no. C135-100)
60 x 96-well microtitration plates (PerkinElmer cat. no. 1244-550)

CELL CULTURE AND PREPARATION

All experiments were performed using K562 cells as target cells and NK-92 (natural killer) cells or freshly isolated human peripheral blood lymphocytes (PBLs) as effector cells. All cells were grown at 37°C and 5% CO_2 in a humidified cell incubator. K562 cells were grown in DMEM medium supplemented with 10% FCS, 4 mM glutamine and 10 mM HEPES. NK cells were grown in MEM alpha medium supplemented with 12.5% FCS, 12.5% horse serum, 2 mM glutamine, 0.1 mM 2-betamercaptoethanol and 0.2 mM inositol in the presence of 10 – 100 U/mL recombinant interleukine 2 (IL-2). Human PBL cells were isolated by density gradient centrifugation and re-suspended in RPMI medium supplemented with 10% FCS. Prior to the assay, the PBL cells were grown overnight, for elimination of adherent cells (monocytes), in the presence of 10 – 100 U/mL recombinant IL-2.

ASSAY PROTOCOL

The K562 target cells were washed once with a balanced salt solution or with culture medium. The number of cells was adjusted to 1 x 10^6 /mL, and 5 mL of cells in culture medium was added to 5 µL of the fluorescence enhancing ligand (DELFIA BATDA reagent) in a 50 mL conical tube. The cells were loaded for 25 minutes in a humidified, 5% CO_2 atmosphere at 37°C. The cells were then washed three or four times with PBS supplemented with 20 mM HEPES and 2.5 mM probenecid by centrifugation at 1000 rpm for 4 minutes at room temperature. Probenecid is an inhibitor of the multidrug resistance (MDR) transporter, and was used to inhibit spontaneous release throughout the study. After the final wash the cell pellet was re-suspended in culture medium supplemented with 2.5 mM probenecid, and the cell density adjusted to 1 x 10^5 cells/mL.

The background, spontaneous release, and maximum release were measured in the same 96-well V-bottom plate (Greiner cat. no. 651180) used to incubate the target cells with the effector cells.

- The background measured the fluorescence from the culture medium without cells: an aliquot was removed from the loaded target cell suspension, centrifuged to pellet the cells, 50 µL of the supernatant was pipetted into the wells, and 50 µL of culture medium was added.
- The spontaneous release measured the fluorescence from the target cells without effector cells: loaded target cells (50 µL) were incubated with 50 µL of culture medium and were removed at the same time intervals as the assay samples.
- The maximum release measured the fluorescence of intentionally lysed target cells: loaded target cells (50 µL) were incubated with 50 µL of culture medium supplemented with 10 µL of Lysis buffer (DELFIA Lysis buffer).

For assays in 96-well V-bottom plates, 50 µL of loaded target cells were mixed with 50 µL of effector cells of varying cell concentration. For assays in 384-well plates, 25 µL of loaded target cells were mixed with 25 µL of effector cells. The target cells and effector cells can be incubated for between 15 minutes and 4 hours at 37°C. In the experiments presented here, the cells were incubated for the times shown in Table 1. The plate was centrifuged for 4 minutes at 1000 rpm and then 20 µL of the supernatant from the sample was transferred to a clear, flat bottom plate (DELFIA microtitration plate). Note that the centrifugation step can be omitted (see Figure 6). 200 µL of Europium solution (DELFIA Europium solution) was then added to the clear, flat bottom plate which was incubated for 15 minutes at room temperature on a shaker (1296-003/004 DELFIA Plateshake). The Europium signal was measured using the plate reader VICTOR^{3}™ Time-Resolved Fluorescence Model (PerkinElmer cat. no. 1420-012).

Experiment	Target Cells	Effector Cells	Incubation Time
Cell cytolysis	K562	PBL & NK	2 hours
Centrifugation	K562	NK	4 hours
384-well plate	K562	NK	4 hours
Storage at -20°C	K562	NK	4 hours

Table 1. Experimental conditions

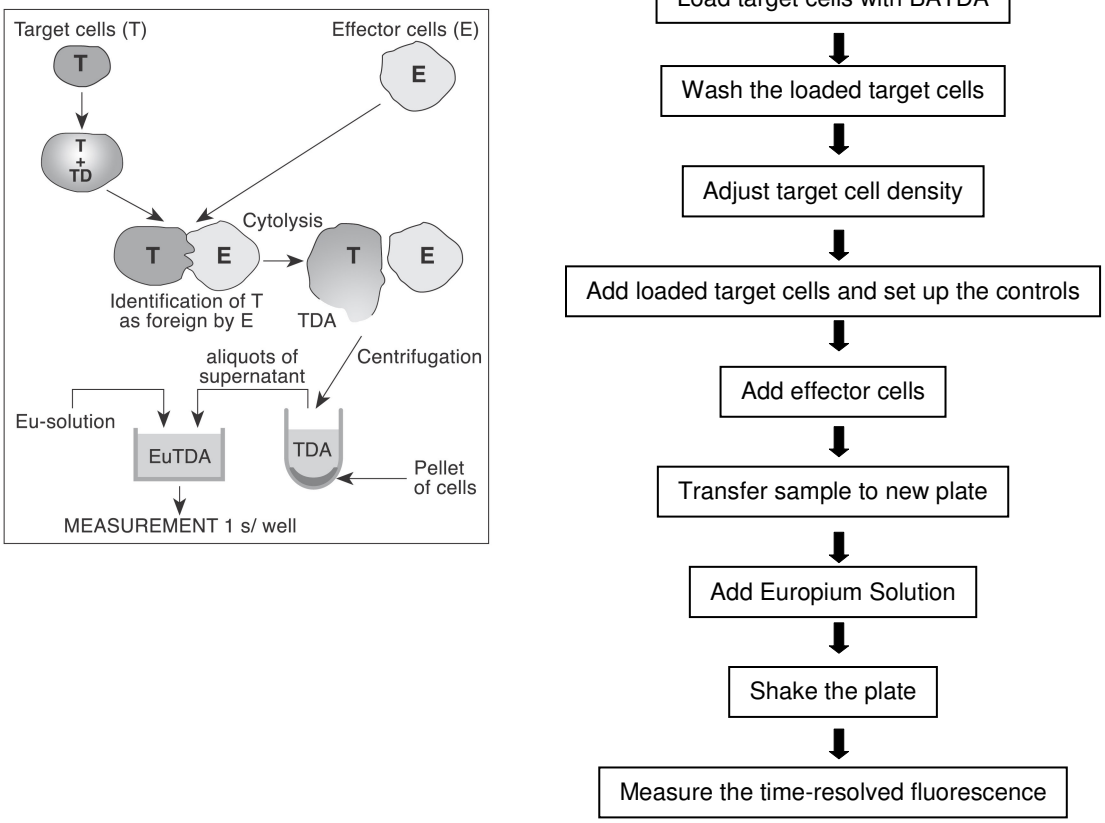

Figure 4. Principle and flow of the EuTDA assay.

When calculating the results, the background fluorescence must be subtracted from the spontaneous and maximum release values so that the relatively high background fluorescence does not bias the true specific release percentage. The formulas used in the calculations are given below:

$$\%\ \text{Specific release} = \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximum release (counts)} - \text{Spontaneous release (counts)}} \times 100$$

$$\%\ \text{Spontaneous release} = \frac{\text{Spontaneous release (counts)} - \text{background (counts)}}{\text{Maximum release (counts)} - \text{background (counts)}} \times 100$$

(Note that in rare cases the effector cells are more effective than the Lysis buffer in lysing the target cells, in which case the experimental release may be higher than the maximum release resulting in a specific release greater than 100%).

Results and Discussion

CYTOLYIS OF K562 TARGET CELLS INDUCED BY PBL AND NK EFFECTOR CELLS

To determine whether freshly isolated human PBL cells or cultured NK cells are more effective at causing cytolysis of K562 cells, the K562 target cells were incubated with both types of effector cells.

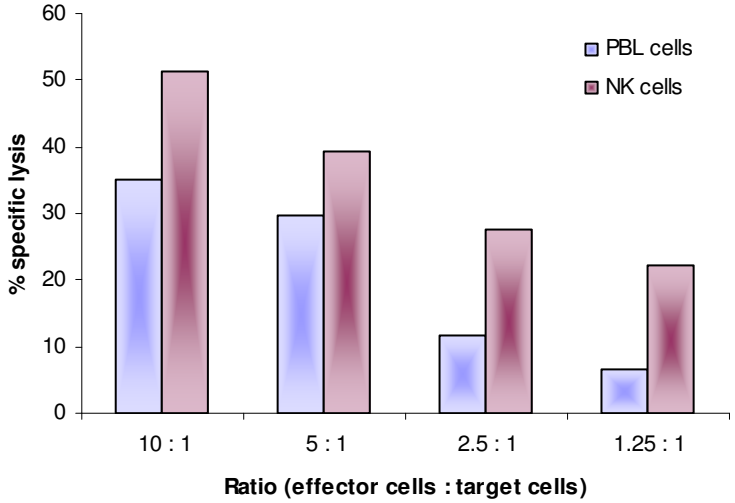

Figure 5. Comparison of cytolysis in K562 cells induced by NK-92 to that induced by human PBL cells.

Figure 5 shows that both effector cell lines induced cytolysis, but the NK cells were more prone to cause cytolysis of K562 cells than the PBL cells at all effector:target cell ratios.

EFFECT OF OMITTING THE CENTRIFUGATION STEP

Typically the plate is centrifuged before the supernatant sample is removed and mixed with Europium solution. However, because microtiter plate centrifuges are not available in all laboratories we investigated the effect of omitting this centrifugation step. Samples were collected from two 96-well V-bottom plates, where one plate was centrifuged before sample transfer and the other plate was not centrifuged before sample transfer.

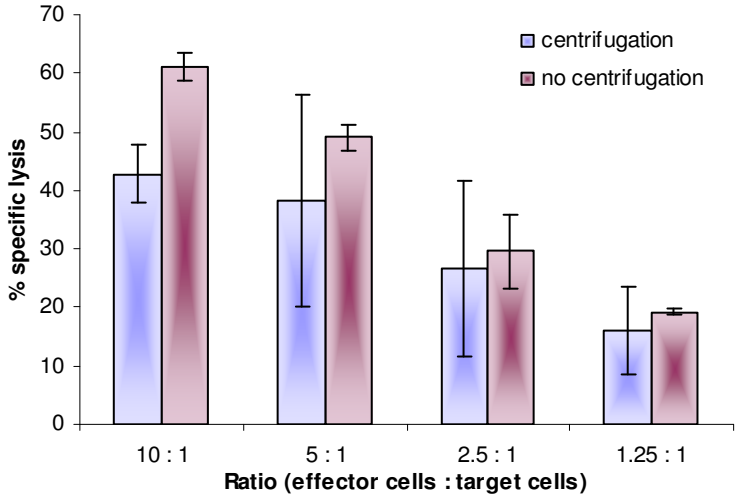

Figure 6. The impact of omitting the centrifugation step before sample transfer.

Figure 6 shows that the centrifugation step can be omitted from the assay protocol without reducing the % specific lysis. In fact the % specific lysis values are higher when the centrifugation step is omitted. This result may be specific to 96-well V-bottom plates. We postulate that the interaction between the cells at the bottom of the V-shaped wells makes it easier to remove the supernatant without accidentally transferring any cells. The results also show that the assay is more reproducible for samples that have not been centrifuged because the standard deviation is smaller than for samples that have been centrifuged. The standard deviation bars have been calculated by repeating the experiment three times.

EFFECT OF PERFORMING THE EUTDA ASSAY IN 384 WELL PLATES

The EuTDA assay was performed in a 384-well plate to determine if the assay can be scaled down without affecting the results.

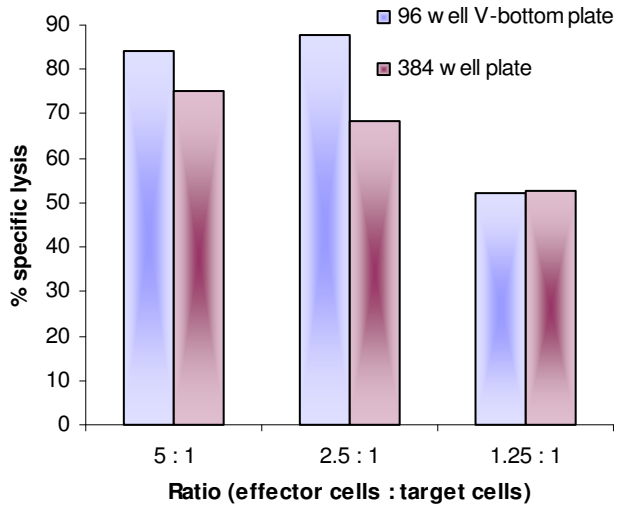

Figure 7. Comparison of the results obtained using 96-well and 384-well plates.

Figure 7 shows that the results do not differ significantly when the assay is performed in a 384-well plate compared to a 96-well plate. The advantage of using 384-well plates is that smaller cell volumes are required, so it is possible to generate more samples from the same cell suspension.

EFFECT OF FREEZING SAMPLES BEFORE ANALYSIS

In the normal assay protocol, after the target cells and effector cells have been incubated the supernatant is removed from the sample and immediately mixed with Europium solution. Figure 8 shows that the specific release from supernatant that has been stored at -20ºC is comparable to the specific release from fresh supernatant. This means that the entire assay does not need to be performed on the same day, and makes it possible to easily compare different lots of cells.

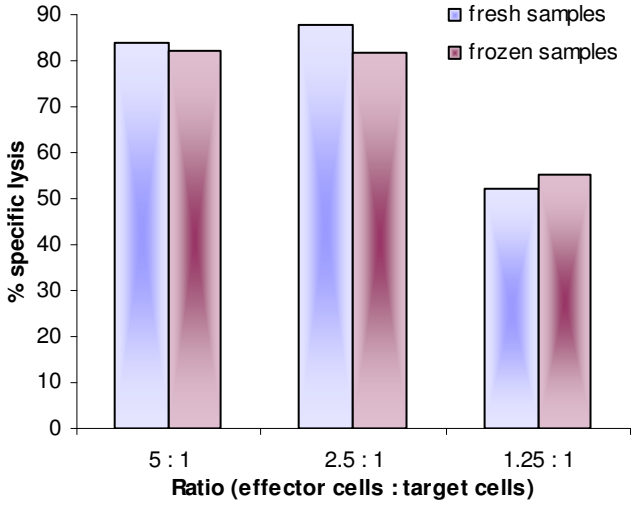

Figure 8. Comparison of fresh supernatant with supernatant that has been stored at – 20 °C.

Note that the supernatant was withdrawn from the sample before it was stored at -20ºC. We do not recommend freezing the cells because the freezing process may lyse the cells and obscure the assay results.

When the EuTDA assay is performed using different cell lines and different experimental conditions the protocol may have to be modified to optimize the assay. Table 2 shows a number of problems that may arise during assay development, and provides a selection of possible solutions to each problem.

Problem	**Solution**
Low recovery of loaded cells	• Check the composition and pH of the buffers and solutions. • Shorten the loading time. • Be careful when washing the cells. If necessary reduce the number of washes to three. • Use culture medium supplemented with serum throughout the assay. • Perform the cell loading under optimal culturing conditions.
High background	• Wash the loaded cells 5-6 times before use, carefully but quickly. Centrifuge for a maximum of 5 minutes at a speed suitable for live cells. • Avoid carryover from one wash to the following wash. • Check that your solutions are not contaminated with

	BATDA. • Check for culture medium interference.
High spontaneous release	• This test is suitable for short term assays (<4 h) - long incubation times increase the spontaneous release. Withdraw supernatant samples after only a few minutes of incubating target cells with effector cells, and monitor the release with time. • Note that this assay is typically faster than ^{51}Cr release. • Decrease the loading time. • Avoid leaving the cells waiting after loading and washing. • Try adding 1 - 10 mM probenecid or 1 – 10 µG/mL mitomycin C to the wash solution and to the medium used for diluting the cells after washing. Note: Probenecid is not soluble in the wash buffer or the media at these concentrations. A 200 x stock solution is first dissolved in 1 M NaOH. This is diluted 2-fold in the wash buffer (PBS supplemented with 20 mM HEPES). The 100 x probenecid stock solution is then diluted 100-fold in the wash buffer and the medium.

Table 2. Possible problems and suggestions for optimizing the EuTDA assay.

Conclusion

It has been shown that the DELFIA EuTDA cytotoxicity assay can be used to detect cytolysis of K562 target cells by NK cells or by human PBL cells. This non-radioactive assay displays comparable sensitivity to the traditional ^{51}Cr release assay, and can be readily scaled down to 384-well plate format which requires minimal sample volume. The assay is relatively insensitive to sample storage and freezing. To further increase the assay throughput and simplicity, centrifugation of the sample plates can be omitted.

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