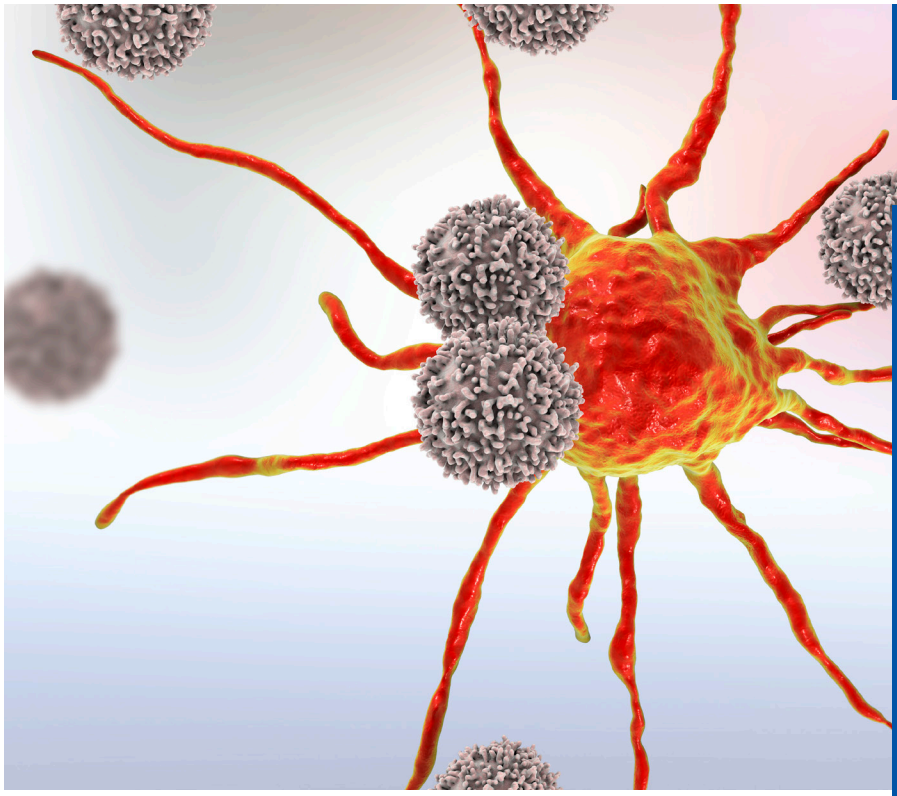

APPLICATION NOTE

DELFIA TRF

Author:

Adam Carlson

PerkinElmer, Inc.
Hopkinton, MA

Non-radioactive Detection of Antibody-Dependent Cellular Cytotoxicity Using DELFIA EuTDA Cytotoxicity Reagents

Introduction

Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism of cell-mediated immune response wherein an effector cell type of the immune system actively lyses a target cell opsonized with a therapeutic antibody. The availability of an *in vitro* assay to specifically measure cell death in target cells during ADCC is critical in the development and production of therapeutic antibodies. Current methods of labelling target cell populations include Calcein AM (fluorescent dye) or Chromium-51 (^{51}Cr; radioactive label). Additionally, general cytotoxicity assays have been described in the literature looking at cell components released into the supernatant such as lactate dehydrogenase (LDH); however these are not specific to the target cell population and measure cell death of both effector and target cell populations during the co-incubation time frame. The underlying assumption with a non-specific assay is that the majority of cell death is occurring in the target cells and contributions from the effector cells are minimal. DELFIA® time-resolved fluorescence (TRF) EuTDA Cytotoxicity Reagents facilitate the sensitive detection of ADCC using the same workflow as the Chromium-51 release assay but in a convenient-to-use, non-radioactive format.

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Acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL) are examples of cancer derived from a B-cell origin. One therapeutic approach to treating patients with these cancers is through binding of B-cell restricted surface antigens with monoclonal antibodies (mAb) that target cancer cells for elimination. The anti-CD20 mAb rituximab is effective against a variety of B-cell malignancies and research into its activity has shown its effectiveness is driven in part through mediation of ADCC.[1] Cells expressing tumor or pathogen-derived antigens on their surface are opsonized by specific therapeutic antibodies through the Fab region of the antibody while the Fcγ receptor (primarily FcγRIIIa/CD16) on immune effector cells bind the Fc region of the antibody to form a complex of effector and target cell. Once the complex is formed, the immune effector cells are activated and trigger a cascade of cell signaling resulting in the release of cytokines such as IFN-γ and TNF-α, and cytotoxic molecules perforin and granzymes which are responsible for cytolysis of the target cell.[2]

Immune effector cells describe a variety of cell types including macrophages, monocytes, granulocytes and natural killer (NK) cells, the latter of which represent 5 – 15% of the peripheral blood mononuclear cell (PBMC) population and play the biggest role in ADCC. NK cells are a subset of cytotoxic innate lymphoid cells which are able to kill virus-infected cells and tumor cells and help drive adaptive immunity by secretion of cytokines.[3] Some cell types, such as the monocytes, can act in an inhibitory manner to ADCC which might complicate co-culture experiments and reduce the measured effect when starting with a mixed population of cells found in PBMC samples. In this application note, purified NK cells were chosen to demonstrate the utility of the DELFIA EuTDA Cytotoxicity Reagents.

DELFIA EuTDA Cytotoxicity Reagents offer a time-resolved fluorescence assay format that can be used effectively to specifically label target cells with a ligand prior to performing ADCC co-culturing assays. No specially-engineered cells are required in this format. Figure 1 describes the assay principle which starts with loading the BATDA ligand, conversion of BATDA to TDA inside the cell, followed by TDA release into the supernatant, and finally the generation of the highly fluorescent lanthanide chelate EuTDA upon addition of the europium solution.

Figure 2 describes the ADCC process utilizing the DELFIA EuTDA Cytotoxicity Reagents wherein effector cells lyse the opsonized, labelled target cells and cause the release of the ligand into the supernatant where it can be quantified.

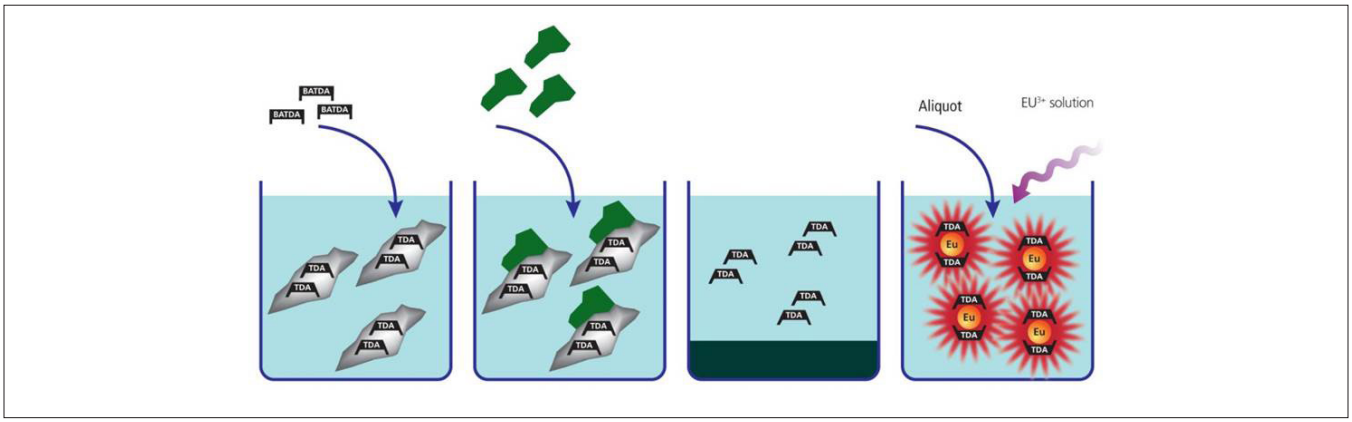

Figure 1. DELFIA EuTDA Cytotoxicity Assay Principle. Cells are loaded in a rapid and gentle method with a fluorescent enhancing ligand (BATDA) which crosses the cell membrane passively. Once inside the cell, the ligand is immediately hydrolyzed by cellular esterases to generate a hydrophilic molecule (TDA) that can no longer penetrate the cell membrane. Cytolysis releases TDA into the supernatant which forms a highly fluorescent lanthanide chelate when europium solution is added. Cytolysis levels are proportional to the amount of fluorescent signal produced by the final EuTDA product.

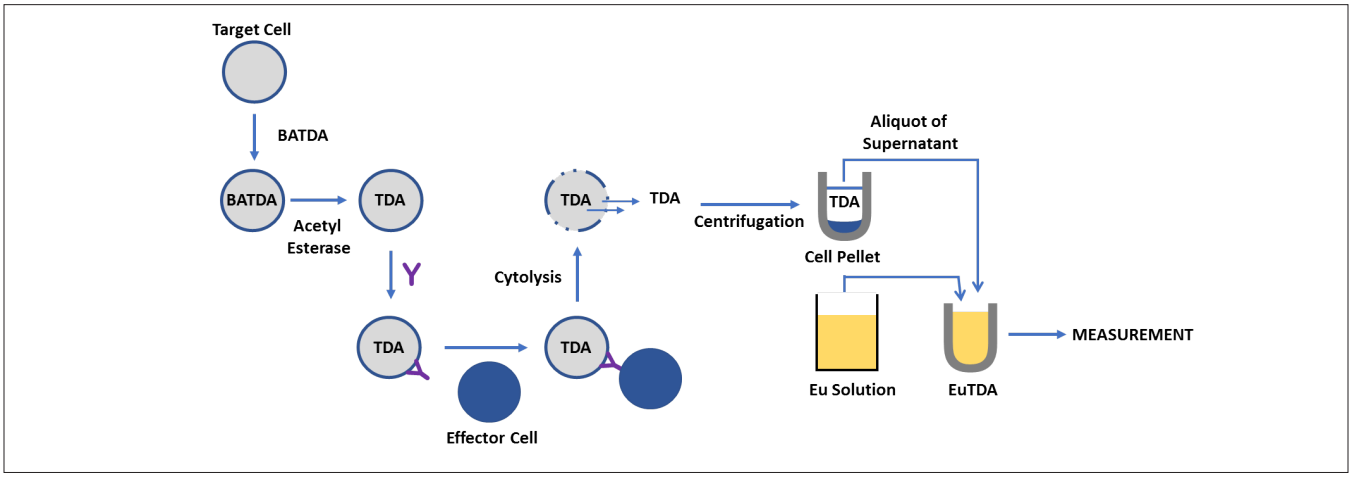

Figure 2. DELFIA EuTDA Cytotoxicity Assay for ADCC Determination. Target cells are first loaded with BATDA reagent then combined with antibody and co-cultured with chosen immune effector cell type. The antibody binds antigen on the target cell surface while the effector cells bind the Fc region of the antibody to form a complex with the target cells. Complex formation activates the immune effector cells which proceed to cytolyze the target cells releasing TDA into the supernatant. Supernatant is transferred to a fresh assay plate and europium solution added to form the active lanthanide (EuTDA). Signal is detected using DELFIA time-resolved fluorescence (TRF) settings on a compatible plate reader (excitation 340 nm and emission at 615 nm).

The DELFIA EuTDA Cytotoxicity Reagents provide a specific, non-radioactive based mechanism to label a cancer cell population and monitor cellular health in complex co-culture environments through release of the ligand into the supernatant. Samples can be taken from the supernatant for testing at multiple time points throughout the assay adding to the flexibility of the DELFIA EuTDA format.

Materials and Methods

Reagents

- DELFIA EuTDA Cytotoxicity Reagents (PerkinElmer, #AD0116)
- Raji cells (ATCC, #CCL-86)
- Natural Killer (NK) cells, CD56+/CD3- (Discovery Life Sciences)
- RPMI media (ATCC, #30-2001)
- Fetal bovine serum (FBS), heat inactivated (Thermo Fisher, #10082-147)
- Phosphate buffered saline (PBS), (Thermo Fisher, #10010-023)
- HEPES 1M solution (Thermo Fisher, #15630-080)
- Triton X-100 (Sigma Aldrich, #T8787)
- StorPlate™-96V (PerkinElmer, #6008299)
- CellCarrier™ Spheroid ULA 96-well Microplates (PerkinElmer, #6055330)
- Rituximab, anti-hCD20-hIgG1 (InvivoGen, #hcd20-mab1)
- Isotype control, anti-β-gal-hIgG1 (InvivoGen, #bgal-mab1)
- Non-fucosylated rituximab, anti-hCD20-hIgG1fut (InvivoGen, #hcd20-mab13)
- Falcon® 5 mL round-bottom polystyrene tubes (VWR, #60819-295)

ADCC Assay Protocol

Effector Cell Handling: Purified NK cells were negatively selected from a PBMC population and were characterized as CD56+/CD3- and ≥85% pure by the provider. Frozen NK cells (5 x 10^6 cells/vial) were removed from liquid nitrogen storage, thawed rapidly in the 37 °C water bath, washed one time in 10 mL cell culture media and prepared at appropriate plating density to match the effector-to-target cell ratio. 100 µL of NK cells at each density required, or media alone (control wells), was added to the assay plate.

Target Cell Loading: Raji cells which express CD20 on the cell surface were harvested from a T75 flask, washed one time with PBS and counted. Cell number was adjusted to 1 x 10^6 cells/mL in loading buffer (PBS + 10% FBS + 20 mM HEPES buffer) in a Falcon® round-bottom tube. Next, 3 µL of BATDA reagent was added to 2 mL of cells, mixed gently, and incubated for 15 min at 37 °C with 5% CO_2 in the cell culture incubator. After loading time was complete, cells were centrifuged and washed 3X with wash buffer (PBS + 20 mM HEPES buffer). At each wash step the cell pellet was carefully resuspended by gently flicking the tube before adding wash buffer. After the final wash, the cell pellet was resuspended in 1 mL of cell culture media and cell density counted again. Cells were adjusted to 2 x 10^5 cells/mL for plating in the assay (10,000 target cells/well in 50 µL plating volume).

Antibody Addition: A range of antibody dilutions was generated at 4X final desired concentration in cell culture media. 50 µL was added to each appropriate well, with no antibody (0 µg/mL) serving as a negative control.

Final assay volume in the 96-well CellCarrier Ultra low attachment (ULA) U-bottom shaped assay plate was 200 µL. The assay plate was centrifuged briefly after all components were present to promote contact between the cell types and help generate the ADCC complex. The U-bottom shape of the plate and low attachment surface also promoted settling of the effector and target cells together. Similarly, sterile V-bottom polystyrene assay plates could be used for the same effect of increasing cell to cell contact.

Data Collection and Analysis

Cell supernatant was carefully sampled from the assay plate so as not to disturb the cells. Collected supernatant was transferred to a fresh plate for centrifugation to remove any unwanted cell carryover. Next, 20 µL of cleared supernatant sample was mixed with 200 µL of europium solution in the provided DELFIA strip plate. The plate was incubated for 15 minutes at room temperature with shaking on a DELFIA Plateshake. DELFIA signal was measured on a PerkinElmer EnVision® multimode plate reader using default values for DELFIA TRF detection.

Formula Definitions:

- **Background (= Sample at Time Zero):** Aliquots of the loaded target cells were removed immediately after loading and plating density adjustment. Aliquots were centrifuged to pellet cells, and then 50 µL of supernatant was removed and pipetted into the assay plate well with 150 µL of cell culture media to reach the final 200 µL assay total volume.
- **Spontaneous Release (= Target Cells without Effector Cells Present, Sampled at Each Time Point):** Loaded target cells and antibody mix (100 µL combined) were incubated with 100 µL cell culture media instead of effector cells during the assay time frame. Supernatant was collected at specified time points.
- **Maximum Release (= Lysed Target Cells):** Loaded target cells (50 µL) were incubated with 150 µL of cell culture media supplemented with lysis buffer (10 µL per well).

Formula Calculations

%Specific Release

(Experimental Release – Spontaneous Release) / (Maximum Release – Spontaneous Release) x 100

%Spontaneous Release

(Spontaneous Release – Background) / (Maximum Release – Background) x 100

Results

ADCC Response is Effectively Measured by DELFIA TRF

Preliminary time-course experiments were conducted during target cell optimization by removing aliquots of supernatant at 0 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. Results indicated 2 hours provided a suitable time point for measuring target cell cytolysis due to the low spontaneous release (data not shown). For ADCC experiments, supernatant was collected 2 hours after the co-culture began and ADCC activity measured as described. Figure 3 shows the average DELFIA signal of the co-culture wells and the assay controls (background and maximum release) after 2 hours. The DELFIA signal increased with increasing numbers of NK effector cells present, and importantly, ADCC activity was only seen in the presence of anti-CD20 rituximab. Rituximab treatment alone did not cause cell death (no NK effector cells control), suggesting the effect is coming from the presence of the bound and activated NK cells in the co-culture.

Spontaneous release was calculated using the formula provided in the Data Analysis section. It is evident at this 2 hour time point that the Raji target cells were releasing the TDA reagent into the supernatant independent of ADCC activity. In Figure 3 the DELFIA signal increased from 20,000 counts (background) to approximately 40,000 counts in 2 hours using 0 µg/mL rituximab across each effector-to-target ratio. Figure 4 shows the calculated spontaneous release from the "No NK effector cell" wells, averaging approximately 15% spontaneous release. When optimizing BATDA loading for any cell line it is critical to find conditions where reagent loading is high and spontaneous release is low to generate a good assay window in the time frame sampled. Factors such as time and temperature can affect loading efficiency. If there is a high level of spontaneous release in a given target cell type, probenecid (Sigma, #P8761) or sulfinpyrazone (Sigma, #S9509) can be effective to reduce the background signal and could be included at 1-10 mM in the wash buffer so long as the concentration does not alter the pH of the wash buffer. Neither probenecid nor sulfinpyrazone was required for the Raji target cells.

Specific release was calculated as described in the Data Analysis section and is shown in Figure 5. After accounting for spontaneous release, cytolysis due to ADCC activity approaches 50% at higher effector-to-target cell ratios at 2 hours. There was a clear gain in specific release by adding more NK effector cells to the co-culture, which was most likely due to increased contact with the Raji target cells in the assay plate.

Differentiation of Antibody Potency in ADCC

Directed changes to an antibody can increase or decrease ADCC. Fucosylation and glycosylation status are two critical factors that affect the ADCC response. It has been reported that when rituximab is non-glycosylated it loses ADCC activity, and when it is non-fucosylated there is a marked increase in ADCC response.[4] Figure 6 shows a clear increase in ADCC activity with non-fucosylated rituximab relative to unaltered rituximab at two doses tested (0.25 or 1 µg/mL). This data suggests DELFIA EuTDA Cytotoxicity Reagents could be useful to assess the ADCC activity of modified antibodies or antibody variants.

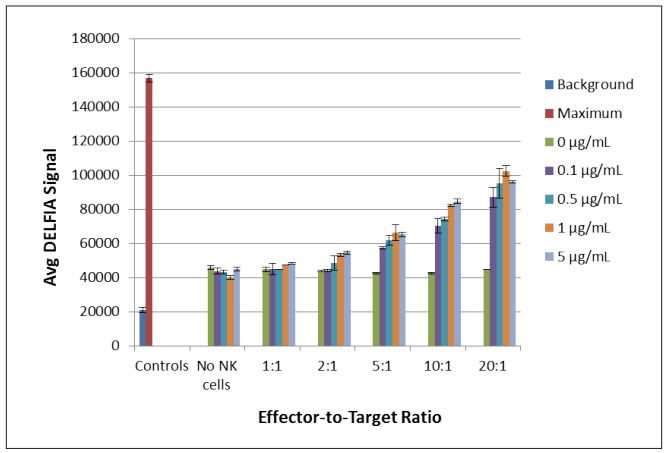

Figure 3. Average DELFIA Signal at 2 hours. Control wells (background and maximum release) represent the lower and upper limits of the DELFIA signal in the assay independent of the time point sampled. DELFIA signal increased with a corresponding increase of NK cells present (higher effector-to-target ratio). Cell death only occurred when combined with rituximab as evidenced by the 0 µg/mL antibody wells.

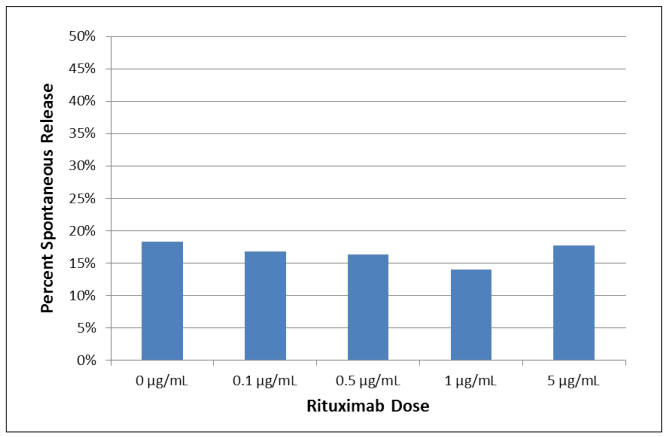

Figure 4. Spontaneous Release Values for Control Wells at 2 hours. Approximately 15% signal increase is due to spontaneous release from the Raji target cells. This is consistent across the dose range of rituximab and is an acceptable low amount of release for the DELFIA EuTDA Cytotoxicity assay to be run without the addition of probenecid or sulfinpyrazone.

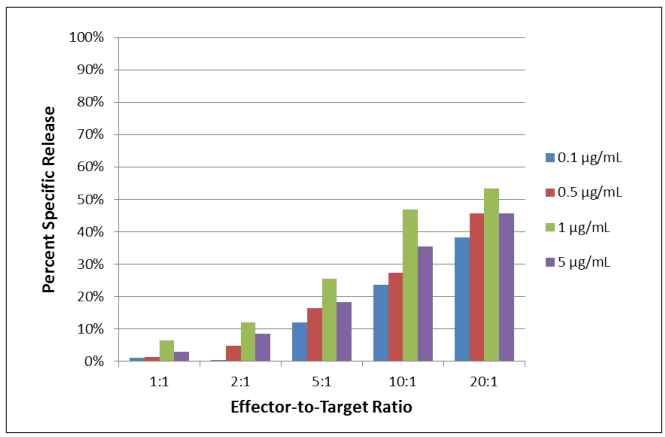

Figure 5. Percent Specific Release at 2 hours. Increased cytolysis is seen with increased effector-to-target cell ratio, as well as with increased rituximab concentration up to 1 µg/mL. The greatest ADCC response occurred with 1 µg/mL rituximab using a 20:1 effector-to-target cell ratio.

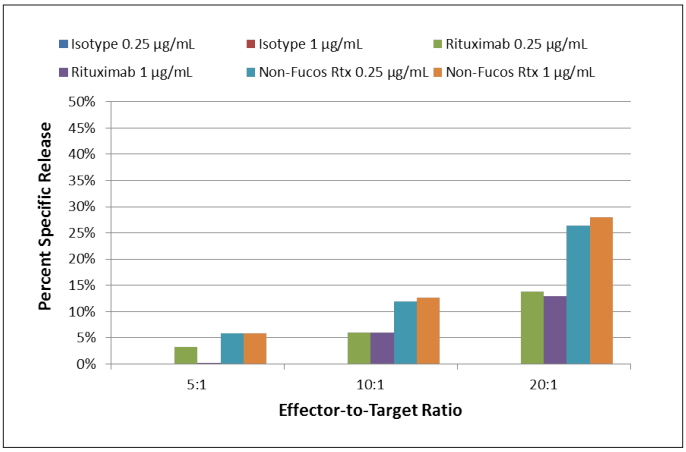

Figure 6. Antibody Differentiation. The non-fucosylated version of rituximab (Non-Fucos Rtx) is approximately 2-fold more potent when used at the same final concentrations as unaltered rituximab. This effect can be seen at each of the effector-to-target cell ratios tested. There was no cytolysis detected in the isotype control antibody wells.

Summary

After initial work to optimize BATDA reagent loading for the Raji cell type, the DELFIA EuTDA Cytotoxicity Reagents enabled measurement of target cell death in the complicated co-culture environment of ADCC using a non-radioactive alternative to Chromium-51 release assays. We were able to measure ADCC activity as early as 2 hours after setting up the co-culture experiment, suggesting that activation of the NK cells and ADCC occurs rapidly once the effector cells engage the opsonized target cells through the Fcγ receptor. Increasing the effector-to-target cell ratio resulted in more cytolysis and a corresponding increase in the DELFIA signal (Figure 3). This translated to almost 50% target cell death at the higher effector-to-target cell ratio (Percent Specific Release, Figure 5).

When using the DELFIA EuTDA Cytotoxicity Reagents for ADCC detection, including the proper control wells is critical to allow calculation of specific release caused by the activated effector cells and generating high quality, interpretable results. Once optimized for a given cell line, the DELFIA EuTDA Cytotoxicity Reagents provide a convenient, reliable, and non-radioactive alternative to more traditional Chromium-51 release assays.

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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