A simplified, gentle cell-labelling method for non-radioactive cytotoxicity assays.

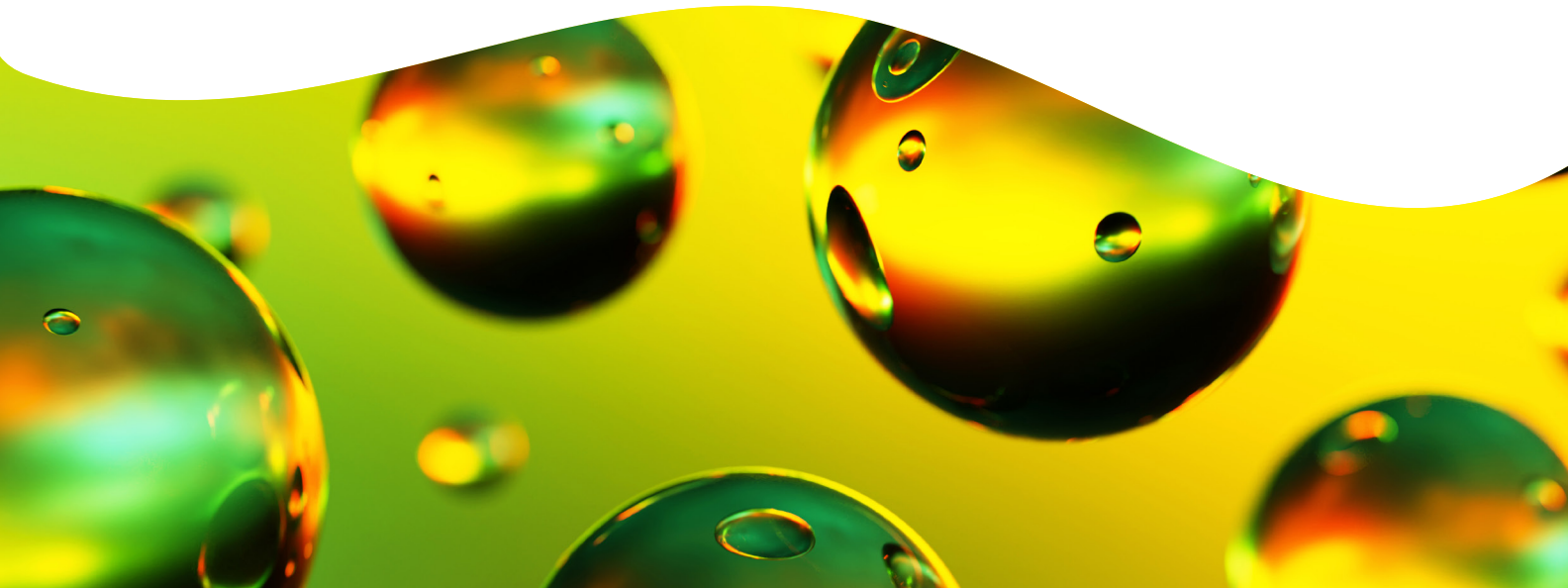
Benefits

- BATDA as a cell marker is a non-radioactive label
- Fast accumulation of label in cells
- Excellent recovery of labelled cells
- Fast release of label
- Fast measurement
- Stable fluorescent signal (5 hrs)

Time-resolved fluorescence offers a non-radioactive alternative to 51Cr with comparable sensitivity for cytotoxicity assays. Compared to the Eu-DTPA release concept, utilization of a fluorescent enhancing ligand is more suitable for fragile cell lines, making optimization of the labelling procedure easier. Cell membrane treatment is not required, and fast accumulation of the ligand in the target cells facilitates excellent recovery of the labeled cells.

Principle of the assay

This method is based on loading target cells with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA). The ligand penetrates the cell membrane quickly. Within the cell the ester bonds are hydrolysed to form a hydrophilic ligand (TDA) which no longer passes the membrane. After cytolysis the ligand is released and introduced to an Eu-solution. The Eu and the ligand form a highly fluorescent and stable chelate (EuTDA). The measured signal correlates directly with the amount of lysed cells.



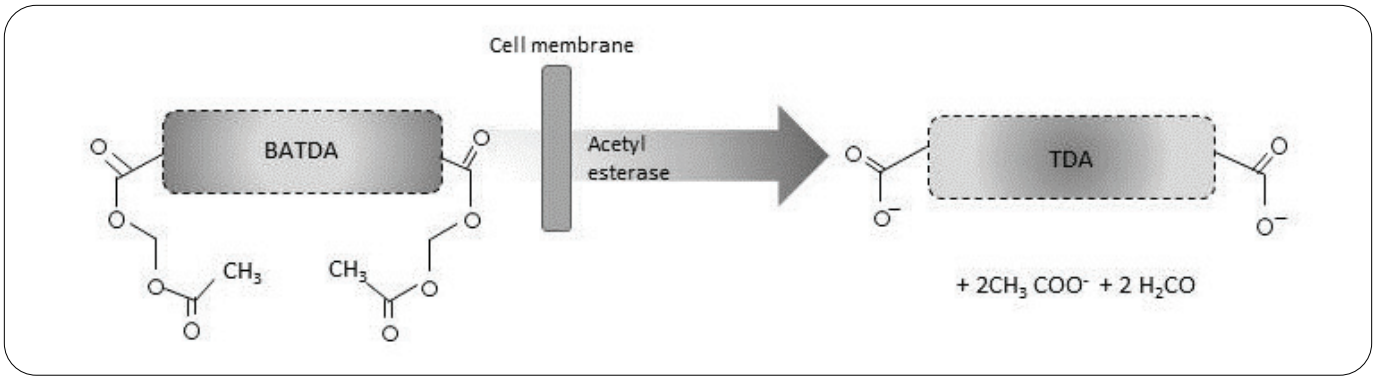


Figure 1. Principle of the cell labelling. After the BATDA ligand has penetrated the cell membrane it is hydrolysed to TDA by the acetyl esterases in the cells.

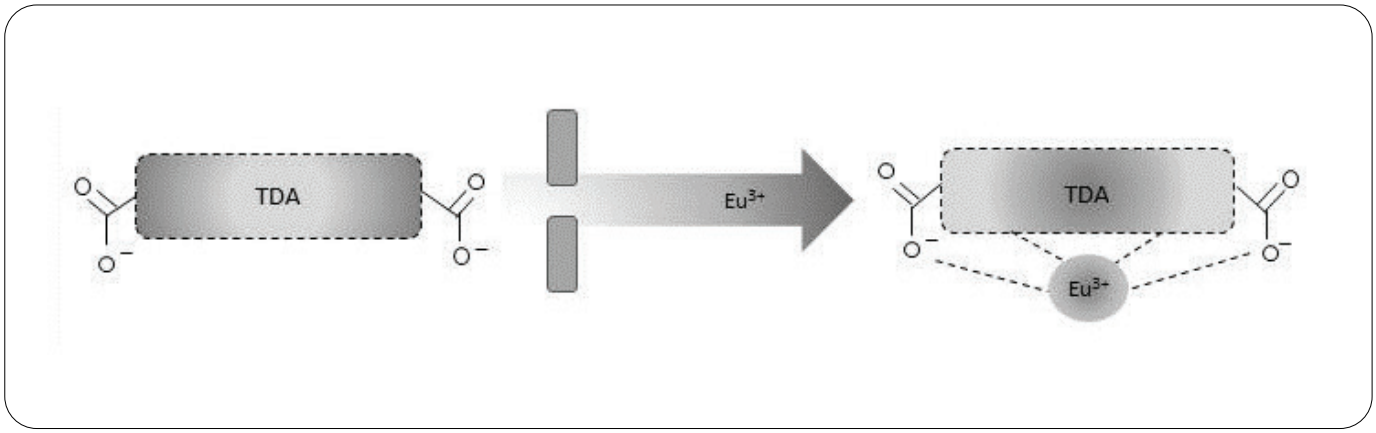


Figure 2. Detection of the released ligand. After addition of EU3+-solution to the sample a fluorescent chelate is formed.

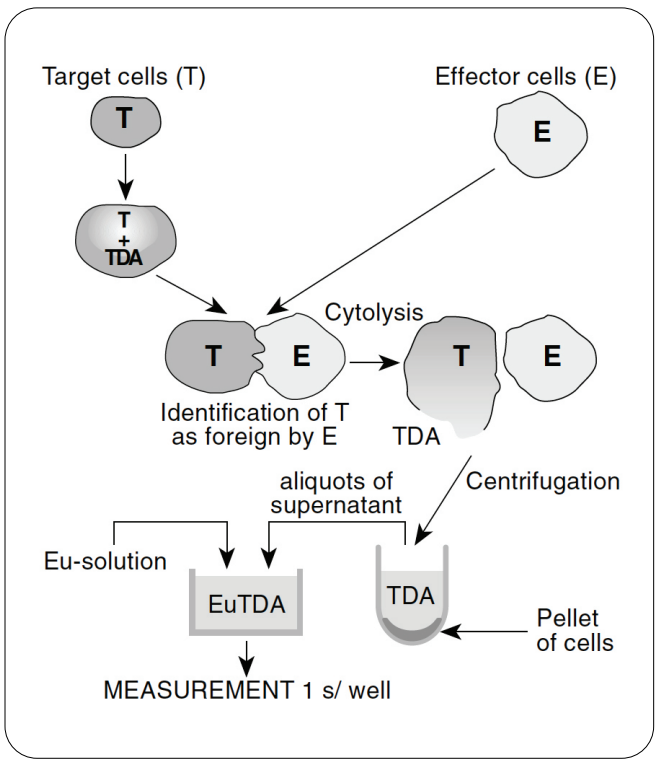


Figure 3. Principle of the release assay.

Labelling protocol (Guidelines)

Preparation of reagents:

1. Warm the lysis buffer in a water bath (37°C) just before use. Let the reagents reach room temperature before use. Check that the fluorescence enhancing ligand is thoroughly thawed before use.
2. Wash the cells once with a balanced salt solution or medium.
3. Adjust the number of cells to about 1 x 106 cells/mL. Add 2 - 4 mL of cells in culture medium to 5 µl of the fluorescence enhancing ligand. Incubate for 20 - 30 min at 37°C in a cell incubator.
4. Spin down the cells and resuspend in balanced salt solution.
5. Wash the cells 3 - 5 times. Resuspend the cell concentrate carefully. Avoid carryover from one wash step to the following.
6. After the final wash resuspend the pellet in culture medium and adjust to about 5 x 104 cells/mL.

Depending on the cell line the following parameters might be adjusted:

Labeling temperature:

4 - 37°C, use the temperature your cell line stands best. High temperature correlates with faster loading.

Labeling time:

5 - 30 min, usually a very sensitive cell line should not be labeled longer than 5 -10 min. Label until you get a maximum signal higher than 15000 - 20000, avoid loading too long.

Labeling concentrations:

Higher concentrations of BATDA results in higher signal until a plateau is reached.

Wash steps:

Try to wash the cells quickly yet suspend them very carefully. Add probenecid (1 - 2 mmol/l) (Sigma P8761) into the wash solution if necessary to lower the spontaneous release.

Guidelines for assay

1. 100 µl of loaded target cells (5000 cells) is pipetted to a round-bottom sterile plate.
2. 100 µl effector cells of varying cell concentration is added. An E:T from 6:1 to 100:1 is commonly used for NK cells.
3. Set up wells for detection of background, spontaneous release and maximum release. See instructions in following section.
4. Incubate two hours in a humidified 5 % CO2 atmosphere at 37°C.
5. Centrifuge for 5 min at 500 x g.
6. Transfer 20 µl of the supernatant to a flat bottom plate.
7. Add 200 µl of Eu-solution.
8. Shake at 250 rpm using a shaking radium of at least 3 mm.
9. Measure fluorescence in a time-resolved fluorometer within 5 hrs.

Note: Depending on the cell line the optimal amount of target cells per well is normally in the range of 5000 to 10000 cells. The ligand should be used only in short term assays and the incubation time should not exceed four hours.

Description of samples

Background:

An aliquot (taken immediately) from the ready diluted labelled target cell suspension is centrifuged. 100 µl of the supernatant is pipetted into the wells and 100 µl medium is added. 20 µl is transferred to the measuring plate and 200 µl of Eu solution is added. Shake the plate for 15 min and measure the fluorescence.

Spontaneous release:

Incubate the target cells (100 µl) with 100 µl of medium instead of effector cells. After centrifugation, transfer 20 µl of the supernatant to the flat bottomed plate and add 200 µl Eu solution. Shake for 15 min and measure.

Maximum release:

Incubate the target cells (100 µl) with 100 µl of medium supplemented with 10 µl of lysis buffer. After centrifugation, transfer 20 µl of the supernatant to the flat bottomed plate and add 200 µl Eu solution. Shake for 15 min and measure.

Calculation of results: *

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

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

In a natural killer cell assay the new EuTDA release assay shows good correlation with 51Cr-release assay (Figure 4).

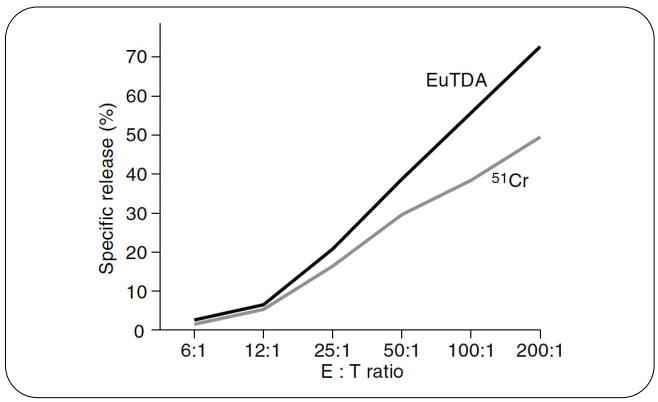


Figure 4. Comparison of the new assay to 51Cr-release assay.

The specific release is faster than in a ^{51}Cr-release assay. The spontaneous release from *K562, Molt-4* and *Jurkat* cells is typically around 10 % in a two hour assay. After labelling, the cell recovery is very high, close to 90%, for the mentioned cell lines. Also *Wein 133, P815, EBV transformed B cells* and *CD4 positive CEM.NKr cells* have been used as target cells. See figures 5 and 6 for DELFIA® Cytotoxicity assay results when CHO and P815 cells are used.

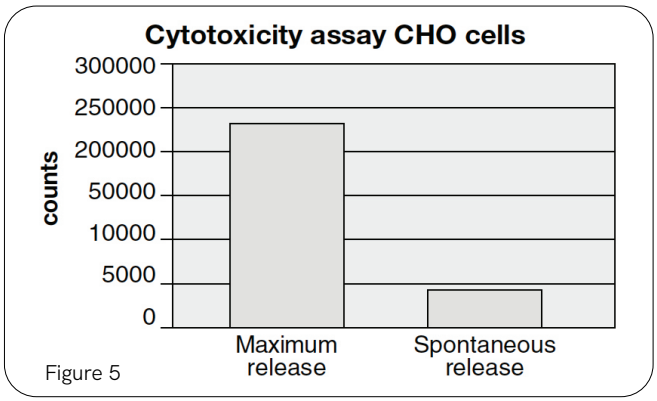


Figure 5

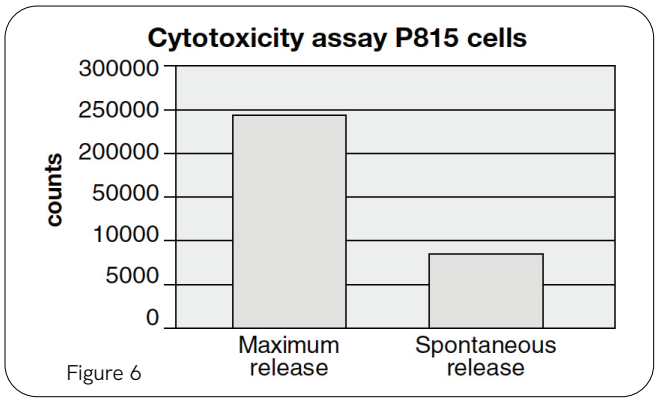


Figure 6

Figure 5 and 6. Cells, 1000000/mL were incubated for 15 min at 37°C with 5 µl DELFIA BATDA reagent. The cells were washed four times in PBS and cell number was adjusted to 100000/mL. 200 µl (20000 cells) was incubated with 20 µl lysis buffer five min. for maximum release. 20 µl was transferred to a flat bottom plate and 200 µl of the DELFIA Europium Solution was added. The plate was incubated on shaker for 15 min before measuring.

* Make sure that background fluorescence is subtracted from the spontaneous and maximal release counts. This is necessary to get the true specific release percentage, otherwise biased by the relatively high background fluorescence.

Table 1. Technical tips for Eutda-Release test.

Problem	Possible reason	How to avoid the problem?
Low recovery of labelled cells	- Poor conditions	- Check the composition and pH of the buffers and solutions used - Shorten the labelling time - Be careful when washing the cells. If necessary reduce the number of washings to three - Use culture medium supplemented with serum throughout the assay - Perform the cell labelling under optimal culturing conditions
Low label incorporation Poor sensitivity	- Cell line difficult to label - Cells are in poor condition	- Increase the label concentration and or the labelling temperature - Try to use Pluronic® F-127, 0.02 % (w/w)(Calbiochem 540025) during loading - Optimize loading temperature - Check the pH of loading buffer
Low maximum release	- Quench of signal	- SDS quenches fluorescence, use lysis buffer from the kit or Triton X-100
High background Poor sensitivity	- Inadequate washing - Solutions or pipettes	- Wash the labelled cells 5-6 times before use, carefully but quickly - Try adding up to 2 mM probenecid to wash solution - Avoid carryover from one wash to the following - Check that your solutions are not contaminated with ligand

Table 1. Technical tips for Eutda-Release test. (continued)

Problem	Possible reason	How to avoid the problem?
High spontaneous release	- Dependent on cell line	- This test is suitable for short term assay (<four h), long incubation times increase spontaneous release. - Note that this assay is typically faster than 51Cr release - Decrease loading time - Introduce up to 2 mM probenecid or sulfinpyrazole (Sigma S9509) into the cell culture medium and wash solution. - Supplement the culture medium with 50 µm 2-mercaptoethanol
	- Dependent on cell line	- Make sure that cells are in log phase of their growth when being labelled - Remember to measure the background and subtract that to get real spontaneous release - Try conditioning cells after labelling: Incubate 30 min. in a conditioned incubator, wash once, count and dilute for use
Poor precision	- Contamination	- Use a dedicated pipette for the Eu solution - Wash carefully
	- Equipment and technique	- Check your pipetting technique - Balance the centrifuge

Reagents available separately

AD0116	DELFIA EuTDA Cytotoxicity Assay Reagents
C136-100	BATDA Fluorescence enhancing ligand, 0.5 mL
4005-0010	Lysis buffer, digitonin 0.3 mg/mL, 30 mL
C135-100	Eu-solution, 200 mL
AAAND-0001	96-well microtitration plates (60)
C136-100	DELFIA BATDA reagent, 50 µl
4005-0010	DELFIA Lysis buffer, 0.5 mL

Store the reagents between + 2 - 8°C.

Instrument

The signals were read using the VICTOR™ Multilabel Plate Reader.

Reagents required but not included in the product:

- Tissue culture plates (round-bottom).
- Balanced salt solution for washing of the ligand.
- Loaded cells, e.g. PBS or cell culture medium.
- Cell culture medium, e.g. RPMI 164 medium.

Acknowledgement

For testing of the reagents we would like to thank Dr William Biddison at SRA Technologies Inc. and Dr. Mireille Viguier at Cochin University Hospital, Paris.

References

1. Blomberg K, Hautala R, Lövgren J, Lindqvist C, Åkerman K. Time-resolved fluorometric assay for natural killer activity using targets labelled with a fluorescence enhancing ligand. J Immunol Methods 1996;193:199-206.

2. Cafaro A, Caputo A, Fracasso C, Maggiorella MT, Goletti D, Baroncelli S, Pace M, Sernicola L, Koanga-Mogtomo ML, Betti M, Borsetti A, Belli R, Akerblom L, Corrias F, Butto S, Heeney J, Verani P, Titti F, Ensoli B. Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. Nature Med 1999;5:643-50.

3. Ding Yuan-Ding, Smith KJ, Garboczi DN, Utz U, Biddison WE, Wiley DC. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. Immunity 1998; 8: 403-11.

4. Hurttia H, Saarinen K, Leino L. Increased adhesion of peripheral blood neutrophils from patients with localized juvenile periodontitis. J Periodontal Res 1998; 33: 292-7.

5. Leino L, Saarinen K, Kivisto K, Koulu L, Jansen CT, Punnonen K. Systemic suppression of human peripheral blood phagocytic leukocytes after whole-body UVB irradiation. J Leukoc Biol 1999;65:573-82.

6. Lengagne R, Gnatjic S, David A-L, Lacabanne V, Guillet J-G, Viguier M. Determination of T cellmediated cytotoxicity by Time-resolved fluorometry using europium-ligand labelled target cells. Wallac Word Wide 1998;Summer:4-9.

7. Saarinen K, Kivistö K, Blomberg K, Punnonen K, Leino L. Time-resolved fluorometric assay for leukocyte adhesion using a fluorescence enhancing ligand. J Immunol Methods 2000; 236: 19-26.

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