Print

Cell Proliferation Kit II (XTT)

Colorimetric assay (XTT based) for the non-radioactive quantification of cell proliferation and viability

Cat. No. 11 465 015 001

Version 18
Content version: March 2016

1 Kit (for 2,500 tests)

Store at −15 to −25°C

1. Product overview

Kit contents

Vial	Label	Contents
1	XTT labeling reagent	• 5× 25 ml XTT (sodium 3´-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent, in RPMI 1640, without phenol red • 1 mg/ml, filtered through 0.2 µm pore size membrane
2	Electron-coupling reagent	• 5× 0.5 ml PMS (N-methyl dibenzopyrazine methyl sulfate) • 0.383 mg/ml (1.25 mM), in phosphate buffered saline (PBS), filtered through 0.2 µm pore size membrane

Introduction

The determination of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Such an example is based on the capability of the cells to incorporate a radioactively labeled substance ([^{3}H]- thymidine), or to release a radioisotope such as [^{51}Cr] after cell lysis. Alternatively, the incorporation of 5-bromo-2´-deoxyuridine (BrdU)* in place of thymidine is monitored as a parameter for DNA synthesis and cellular proliferation in immunohisto- and cytochemistry, in a cell ELISA and FACS analysis (kits and reagents for these applications are available from Roche Diagnostics).

Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts MTT and XTT are especially useful for assaying the quantification of viable cells.

Both, MTT and XTT work by being to a formazan dye only by metabolic active cells (fig. 1; for UV absorbance spectrum, see fig. 2).

Assay principle

The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells (fig. 1) (5). Therefore, this conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer (ELISA reader). This ensures a high degree of accurracy, enables on-line computer processing of the data (data collection, calculation and report generation) and, thereby, allows the rapid and convenient handling of a high number of samples.

Cells, grown in a 96 well tissue culture plate, are incubated with the yellow XTT solution (final concentration 0.3 mg/ml) for 4–24 h (see fig. 3 and assay procedure). After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader. An increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed, as monitored by the absorbance (see fig. 3).

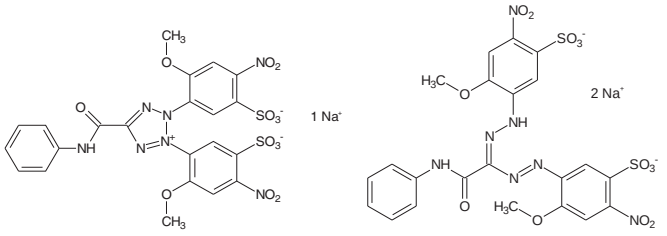

Fig. 1: Metabolization of XTT to a water soluble formazan salt by viable cells.

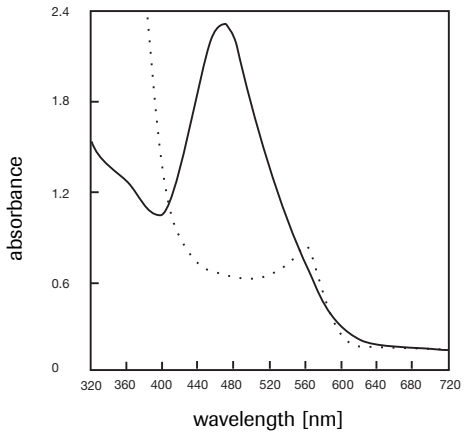

Fig. 2: Comparison of UV-spectra of XTT labeling mixture (dotted line) ant the formazan dye, formed by mitochondrial dehydrogenase activity.

Background information

The non-radioactive, colorimetric assay system using XTT was first described by Scudiero, P.A. *et al.* (1, 2) and improved in subsequent years by several other investigators (3, 4). The assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes. It is used for the measurement of cell proliferation in response to growth factors, cytokines and nutrients (see fig. 4). It is also used for the measurement of cytotoxicity, like the quantification of tumor necrosis factor -α or -β effects (see fig. 5) or the assessment of cytotoxic or growth inhibiting agents such as inhibitory antibodies (see fig. 6).

The XTT-assay is also used as a non-radioactive alternative for the [^{51}Cr]-release cytotoxicity assay (6). Here, the assay is as sensitive as the radioactive assay, but shows a significantly lower inter- and intra-test variability and a lower background, especially after long term incubation (6).

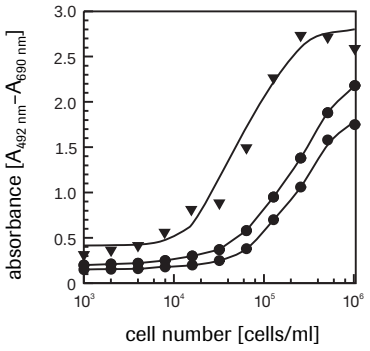

Fig. 3: Effect of different numbers of cells on color formation (example given, using Ag8 cells) after 2 h (●), 6 h (○) and 20 h (▼) incubation with XTT labeling mixture as described below (section 4).

Application

The XTT assay was originally described for the measurement of drug sensitivity of tumor cell lines (2). The XTT assay is also used for the quantitative determination of cellular proliferation and activation *e.g.*, in response to growth factors and cytokines such as IL-2 and IL-6. It is also used for the quantification of antiproliferative or cytotoxic effects *e.g.,* mediated by tumor necrosis factor α- or β-and for the measurement of virus induced cytophatic effects (3). A non-radioactive alternative for the [^{51}Cr] release cytotoxicity assay using XTT is described (6).

Storage/ stability

The components of the kit are stable at −15 to −25°C, protected from light until the expiration date printed on the label.

Thaw reagents immediately before use. It is recommended to prepare appropriate aliquots [5 ml XTT labeling reagent and 0.1 ml electron coupling reagent are required for the performance of the assay with one microplate (96 wells)] and to avoid repeated thawing and freezing.

Note: Precipitates will form during shipment or storage at −15 to −25°C, in which case the container should be warmed to 37°C and mixed to obtain a clear solution.

Advantages

Compared to radioactive isotope techniques, the Cell Proliferation Kit II (XTT) shows the following advantages:

Benefits	Features
safer	no radioactive isotopes are used
accurate	the absorbance revealed, strongly correlates to the cell number, (see fig. 3)
sensitive	low cell numbers are detected (see fig. 3)
fast	the use of multiwell-ELISA readers allows for processing a large number of samples
easy	no washing steps and no additional reagents are required

2. Protocols and required materials

2.1 Before you begin

Preparation of solutions

Thaw XTT labeling reagent and electron-coupling reagent, respectively in a waterbath at 37°C. Mix each vial thoroughly to obtain a clear solution.

XTT labeling mixture

To perform a cell proliferation assay (XTT) with one microplate (96 wells) mix 5 ml XTT labeling reagent with 0.1 ml electron coupling reagent.

Note: To obtain reliable results thaw and mix XTT labeling reagent and electron coupling reagent immediately before use.

Working instruction

Cells are grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl culture medium per well, according to the media needs of the cells in a humidified atmosphere (*e.g.,* 37°C, 6.5% CO_2).

The incubation period of the cell cultures depends on the particular experimental approach and on the cell line, used for the assay. For most experimental setups, the incubation of cells for 24 to 96 h is appropriate.

After the incubation period, add to each well 50 µl of the XTT labeling mixture, prepared as described above (final XTT concentration 0.3 mg/ml).

Incubate the microplate for 4 to 24 h in a humidified atmosphere (*e.g.,* 37°C, 6.5% CO_2).

Notes: The incubation time varies with the individual experimental setup (*e.g.,* cell type and cell concentration, used). Therefore, we recommend to measure the absorption as described at different time points after addition of XTT labeling mixture (*e.g.,* 4, 6, 8, 12, and 18 h) using one and the same microplate to determine the optimal incubation period for the particular experimental setup.

Protocol overview

If for the initial incubation of the cells a larger volume of culture medium is required, increase the amount of XTT labeling mixture correspondingly (*e.g.,* 75 µl XTT labeling mixture, when cells are cultured in 150 µl culture medium).

Step	Procedure	Vol/well	Time	Temp. (°C)
	Perform tissue culture using 96 welll microplates (tissue culture plates	100 µl	24 - 96 h	37°C
1	Thaw XTT labeling reagent and electron coupling reagent and thoroughly mix each vial to obtain a clear solution.	–	–	37°C
2	Prepare XTT labeling mixture by mixing XTT labeling reagent and electron coupling reagent .	50 µl 1 µl	–	37°C
3	Add XTT labeling mix-ture and incubate in a humidified atmosphere	50 µl	4 – 24 h	37°C
	Measure absorbance using an ELISA reader at 450–500 nm with a reference wavelength at 650 nm .	–	–	–

2.2 Cell growth assay procedures

Introduction

To determine the activity of human interleukin-6 (hIL-6) on 7TD1 cells (mouse-mouse hybridoma) (see fig. 4).

Additional reagents required

- Culture medium, *e.g.,* DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrat, 50 µM 2-mercaptoethanol, HT-media supplement (1×), containing 0.1 mM hypoxanthine, and 16 µM thymidine.
  Note: If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin* or gentamicin.
- Interleukin-6, human (hIL-6) (200,000 U/ml, 2 µg/ml), sterile.
- Cell Proliferation Kit II (XTT).

Protocol

Please refer to the following table.

Step	Action
1	Seed 7TD1 cells at a concentration of 4× 10^3 cells/well in 100 µl culture medium containing various amounts of IL-6 [final concentration *e.g.,* 0.1–100 U/ml (0.001 – 1 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
2	Incubate cell cultures for 4 days at 37°C and 6.5% CO_2.
3	Add 50 µl XTT labeling mixture per well and incubate for 4 h at 37°C and 6.5% CO_2.
4	Measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 450 and 500 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm.

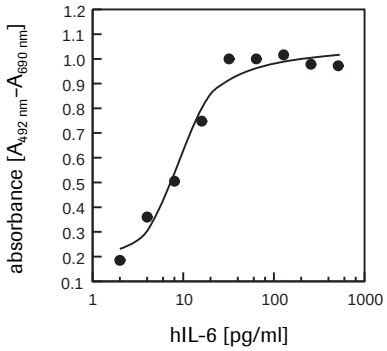

Fig. 4: Proliferation of 7TD1 cells (mouse-mouse hybridoma) in response to recombinant human interleukin-6 (hIL-6) using the procedure described.

2.3 Cytotoxicity assay procedure

Introduction

To determine the cytotoxic effect of human tumor necrosis factor-α (hTNF-α) on WEHI-164 cells (mouse fibrosarcoma) (see fig. 5).

Additional reagents required

- Culture medium, *e.g.,* RPMI 1640 containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, and actinomycin C1 (actinomycin D), 1 µg/ml. If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin* or gentamicin.
- Tumor necrosis-factor-α, human (hTNF-α) (10 µg/ml), sterile.
- Cell Proliferation Kit II (XTT).

Protocol

Please refer to the following table.

Step	Action
1	Preincubate WEHI-164 cells at a concentration of 1× 10^6 cells/ml in culture medium with actinomycin C1, 1 mg/ml for 3 h at 37°C and 6.5% CO_2.
2	Seed cells at a concentration of 5× 10^4 cells/well in 100 µl culture medium containing actinomycin C1 (1 mg/ml) and various amounts of hTNF-α (final concentration *e.g.*, 0.001–0.5 ng/ml) into microplates (tissue culture grade, 96 wells, flat bottom).
3	Incubate cell cultures for 24 h at 37°C and 6.5% CO_2.
4	Add 50 µl XTT labeling mixture and incubate for 18 h at 37°C and 6.5% CO_2.
5	Measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 450 and 500 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm

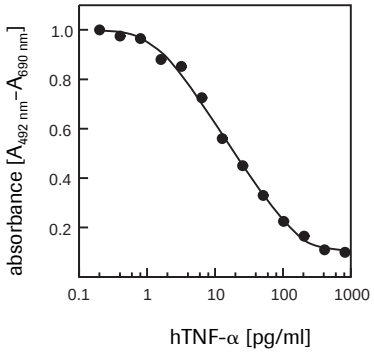

Fig. 5: Determination of the cytotoxic activity of recombinant human TNF-α (hTNF-α) on WEHI-164 cells (mouse fibrosarcoma) using the procedure described.

2.4 Assay procedure for the analysis of neutralizing monoclonal antibodies to growth factors or cytokines

Introduction

To determine the inhibitory activity of a murine, monoclonal antibody to human interleukin-2 (anti-hIL-2) on hIL-2 activity on CTLL-2 cells (IL-2 dependent mouse T-cell line) (see fig. 6).

Additional reagents required

- Culture medium, *e.g.,* RPMI 1640 containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids (NEAA) (1×), 10 mM Hepes*, and 50 µM 2-mercaptoethanol.
  Note: If an antibiotic is to be used, additionally supplement media with penicillin/streptomycin* or gentamicin.
- Interleukin-2, human (hIL-2) (*e.g.,* 10,000 U/ml, 5 µg/ml)*
- anti-hIL-2
- Cell Proliferation Kit II (XTT).

Protocol

Please refer to the following table.

Note: If for the initial incubation of the cells a larger volume of culture medium is required, increase the amount of XTT labeling mixture correspondingly (*e.g.*, 75 µl XTT labeling mixture, when cells are cultured in 150 µl culture medium).

Step	Action
1	Preincubate 50 µl of culture medium for approx. 30 min at 15–25°C containing hIL-2 (4 U/ml, 2 ng/ml) and various amounts of anti-hIL-2 [final concentration *e.g.,* 0.01–40 µg/ml)] in microplates (tissue culture grade, 96 wells, flat bottom).
2	Harvest sensitive cells, *e.g.,* CTLL-2 cells and wash them three times by centrifugation in culture medium without IL-2.
3	Add CTLL-2 cells at a concentration of 4× 10^3 cells/well in 50 µl culture medium to the preincubated mixture of IL-2 and anti-IL-2 and incubate for 48 h.
4	Add 50 µl XTT labeling mixture and incubate for 4 h at 37°C and 6.5% CO_2.
5	Measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 450 and 500 nm according to the filters available for the ELISA reader, used. The reference wavelength should be more than 650 nm

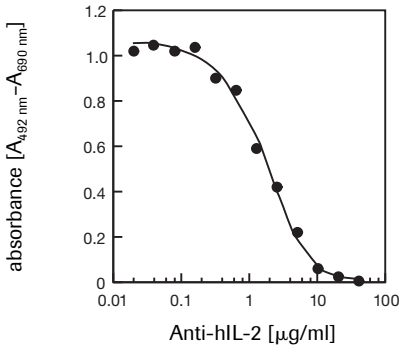

Fig. 6: Inhibition of recombinant human interleukin-2 (hIL-2) (4 U/ml; 2 ng/ml) activity on CTLL-2 cells (mouse T-cell line) by anti-hIL-2 (clone B-G5) using the procedure described.

3. Appendix

3.1 References

1 Paull, K. D. et al. (1988) *J. Heterocycl. Chem.* **25**, 911–914.
2 Scudiero, P. A. et al. (1988) *Cancer Res.* **48**, 4827–4833.
3 Weislow, O. S. et al. (1989) *J. Natl. Cancer Inst.* **81**, 577–586.
4 Roehm, N. W. et al. (1991) *J. Immunol. Methods* **142**, 257–265.
5 Gerlier, D. & Thomasset, N. (1986) *J. Immunol. Methods* **94**, 57–63.
6 Jost, L. M., Kirkwood, J. M. & Whiteside, T. L. (1992) *J. Immunol. Methods* **147**, 153–165.

3.2 Related products

Parameter	Detection by	Products	Cat. No.
BrdU labeling of proliferating cells	*In situ* assay	BrdU Labeling and Detection Kit I	11 296 736 001
		BrdU Labeling and Detection Kit II	11 299 964 001
		BrdU Labeling and Detection Kit III	11 444 611 001
		In Situ Cell Proliferation Kit, FLUOS	11 810 740 001
	ELISA	Cell Proliferation ELISA, BrdU (colorimetric)	11 647 229 001
		Cell Proliferation ELISA, BrdU (chemiluminescent)	11 669 915 001
	Single reagents for *in situ* assays and ELISA applications	Anti-BrdU formalin grade	11 170 376 001
		Anti-BrdU -Fluorescein, formalin grade	11 202 693 001
		Anti-BrdU -Peroxidase, Fab fragments, formalin grade	11 585 860 001
Measurement of metabolic activity	Quantification in microplate	Cell Proliferation Kit I (MTT)	11 465 007 001
		Cell Proliferation Reagent WST-1	11 644 807 001

* *available from Roche Diagnostics*

Changes to Previous Version Editorial Changes

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