For life science research only.
Not for use in diagnostic procedures.

Cell Proliferation Kit II (XTT)

Version: 19
Content Version: January 2021

Colorimetric assay (XTT based) for the nonradioactive quantification of cell proliferation and viability

Cat. No. 11 465 015 001 1 kit
2,500 tests

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	red	Cell Proliferation Kit II (XTT), XTT labeling reagent	▪ 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.	5 bottles, 25 ml each
2	red	Cell Proliferation Kit II (XTT), Electron coupling reagent	▪ 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.	5 bottes, 0.5 ml each

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	red	XTT labeling reagent	Store at −15 to −25°C. ⚠ ***Keep protected from light.*** ⚠ ***Avoid repeated freezing and thawing. If precipitates form during shipment or storage, warm the container to +37°C and mix thoroughly to obtain a clear solution.*** Thaw reagents immediately before use. Prepare appropriate aliquots required for the performance of the assay with one 96-well microplate, for example, 5 ml XTT labeling reagent and 0.1 ml Electron coupling reagent.
2	red	Electron coupling reagent	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- +37°C incubator
- Humidified chamber
- Centrifuge with rotor for microplates (for suspension cells only)
- ELISA reader for microplates.
  - *The wavelength to measure the absorbance of the formazan product is between 450 and 500 nm; the reference wavelength should be >650 nm.*
- Multichannel pipettes (10 µl, 50 µl, and 100 µl)
- Sterile pipette tips
- Flat-bottomed 96-well microplates, tissue-culture grade

For the measurement of the proliferation of 7TD1 cells after IL-6 stimulation

- Culture medium, such as DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM L-glutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine-monohydrate, 50 µM 2-mercaptoethanol, 1x HT media supplement, containing 0.1 mM hypoxanthine and 16 µM thymidine
  If an antibiotic will be used, supplement media with Penicillin-Streptomycin or gentamicin.*
- Interleukin-6, human (hIL-6)* (200,000 U/ml, 2 µg/ml), sterile filtered

For the measurement of the cytotoxic effect of human tumor necrosis factor-α (hTNF-α) on WEHI-164 cells

- Culture medium, such as RPMI 1640 containing 10% heat inactivated FCS, 2 mM L-glutamine and 1 µg/ml actinomycin C1 (actinomycin D)
  If an antibiotic will be used, supplement media with Penicillin-Streptomycin or gentamicin.*
- Tumor Necrosis Factor-α, human (hTNF-α)* (10 µg/ml), sterile filtered

For the analysis of neutralizing monoclonal antibodies to growth factors or cytokines

- Culture medium, such as RPMI 1640 containing 10% heat inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids (NEAA), 10 mM HEPES, and 50 µM 2-mercaptoethanol
  If an antibiotic will be used, supplement media with Penicillin-Streptomycin or gentamicin.*
- Interleukin-2, human (hIL-2)*, for example, 10,000 U/ml, 5 µg/ml, sterile filtered
- Anti-hIL-2

1.4. Application

The Cell Proliferation Kit II (XTT) can be used in a variety of applications:

- Originally used for the measurement of drug sensitivity of tumor cell lines.
- Designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes.
- Measurement of cell proliferation and activation in response to growth factors, cytokines (IL-2, IL-6), and nutrients (Fig. 1).
- Useful for the measurement of cytotoxicity. Examples include the quantification of tumor necrosis factor-α or -β effects (Fig. 2) or the assessment of cytotoxic or growth inhibiting agents, such as inhibitory antibodies (Fig. 3).
- For the replacement of the radioactive [^{51}Cr]-release cytotoxicity assay, protocols using XTT have been developed. The XTT assay is as sensitive as the radioactive method, but shows a significantly lower background, especially after long-term incubation.

2. How to Use this Product

2.1. Protocols

Assay protocol

An overview of the steps is shown in the following table:

Step	Description	Volume/well [µl]	Time [Hours]	Temperature [°C]
1	Perform tissue culture using 96-well, tissue culture grade, flat-bottom microplates.	100	24 – 96	+37
2	Thaw XTT labeling reagent and Electron coupling reagent and thoroughly mix each bottle to obtain a clear solution. ⚠ ***To obtain reliable results, thaw and mix XTT labeling reagent and Electron coupling reagent immediately before use.***	–	–	+37
3	Prepare XTT labeling mixture by mixing XTT labeling reagent, and Electron coupling reagent.	50 1	–	+37
4	Add XTT labeling mixture and incubate in a humidified atmosphere.	50	4 – 24	+37
5	Evaluate microplate using an ELISA reader at 450 to 500 nm with a reference wavelength of 650 nm.			

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

1. Grow cells 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, such as +37°C, 5 to 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, incubate cells for 24 to 96 hours.

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

3. Incubate the microplate for 4 to 24 hours in a humidified atmosphere, such as +37°C, 5 to 6.5% CO_2.

   The incubation time varies with the individual experimental setup, for example, cell type and cell concentration used. Therefore, measure the absorption as described at different time points after addition of XTT labeling mixture, for example, 4, 6, 8, 12, and 18 hours, using one and the same microplate to determine the optimal incubation period for the particular experimental setup.

Measurement of the proliferation of 7TD1 cells after IL-6 stimulation

For the determination of human Interleukin-6 (hIL-6) activity on 7TD1 cells, see Figure 1.

1. Seed 7TD1 cells (mouse-mouse hybridoma) at a concentration of 4 × 10^3 cells/well in 100 µl culture medium containing various amounts of IL-6 (final concentration approximately 0.1 to 100 U/ml (0.001 to 1 ng/ml) into microplates (tissue-culture grade, 96 wells, flat bottom).

2. Incubate cell cultures for 4 days at +37°C and 5 to 6.5% CO_2.

3. After the incubation period, add 50 µl of the XTT labeling mixture to each well.

4. Incubate the microplate for 4 hours in a humidified atmosphere, such as +37°C, 5 to 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. Use a reference wavelength >650 nm.

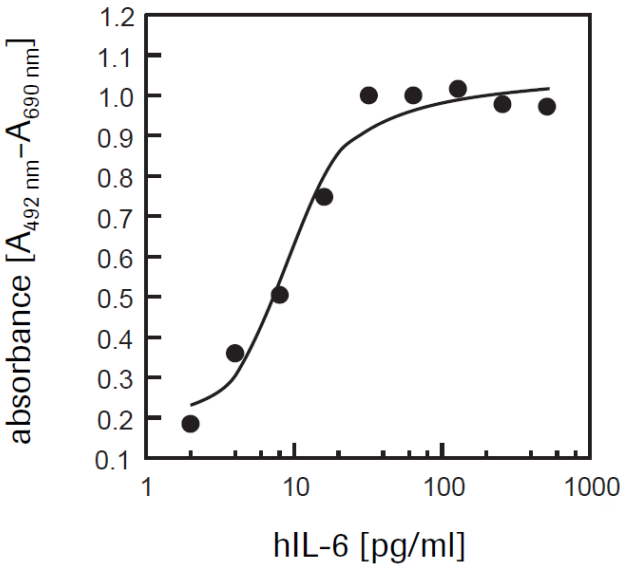

Fig. 1: Proliferation of 7TD1 cells (mouse-mouse hybridoma) in response to recombinant human interleukin-6 (hIL-6) using the described protocol.

Measurement of the cytotoxic effect of human tumor necrosis factor-α (hTNF-α) on WEHI-164 cells

For the determination of the cytotoxic effect of hTNF-α on WEHI-164 cells, see Figure 2.

1. Preincubate WEHI-164 cells at a concentration of 1 × 10^6 cells/ml in culture medium with 1 mg/ml actinomycin C1 for 3 hours at +37°C and 5 to 6.5% CO_2.

2. Seed cells at a concentration of 5 × 10^4 cells/well in 100 µl culture medium containing 1 mg/ml actinomycin C1 and various amounts of hTNF-α (final concentration approximately 0.001 to 0.5 ng/ml) into microplates (tissue-culture grade, 96 wells, flat bottom).

3. Incubate cell cultures for 24 hours at +37°C and 5 to 6.5% CO_2.

4. After the incubation period, add 50 µl of the XTT labeling mixture to each well.

5. Incubate the microplate for 18 hours in a humidified atmosphere, such as +37°C, 5 to 6.5% CO_2.

6. 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. Use a reference wavelength >650 nm.

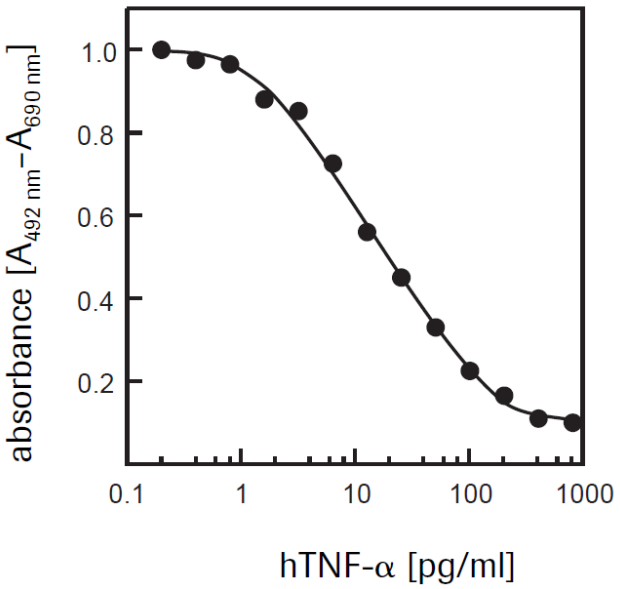

Fig. 2: Determination of the cytotoxic activity of recombinant human TNF-α on WEHI-164 cells (mouse fibrosarcoma) using the described protocol.

Analysis of neutralizing monoclonal antibodies to growth factors or cytokines

For the determination of 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) (Fig. 3).

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

1. Preincubate 50 µl of culture medium containing hIL-2 (4 U/ml, 2 ng/ml) and various amounts of anti-hIL-2 (final concentration approximately 0.01 to 40 µg/ml) in microplates (tissue-culture grade, 96 wells, flat bottom) for approximately 30 minutes at +15 to +25°C.

2. Harvest sensitive cells, such as 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 hours.

4. After the incubation period, add 50 µl of the XTT labeling mixture to each well.

5. Incubate the microplate for 4 hours in a humidified atmosphere, such as +37°C, 5 to 6.5% CO_2.

6. 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. Use a reference wavelength >650 nm.

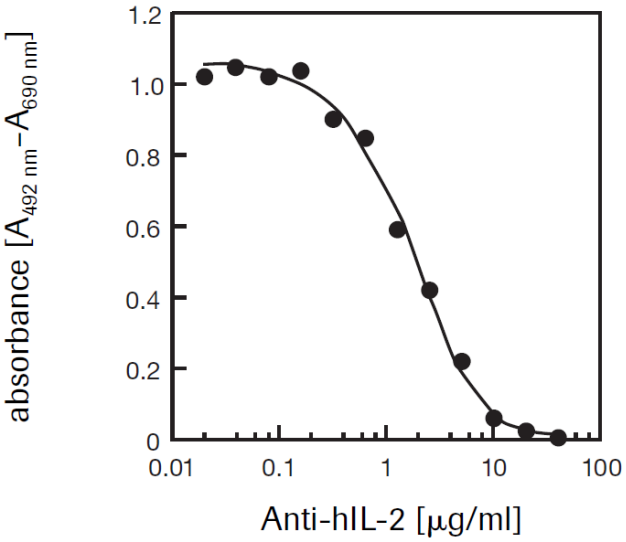

Fig. 3: Inhibition of recombinant human interleukin-2 (hIL-2) (4 U/ml; 2 ng/ml) (●), activity on CTLL-2 cells (mouse T-cell line) by anti-hIL-2 (clone B-G5) using the described protocol.

2.2. Parameters

Accuracy

The absorbance revealed strongly correlates to the cell number (Fig. 5).

Sensitivity

Low cell numbers are detected (Fig. 5).

3. Additional Information on this Product

3.1. Test Principle

The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells (Fig. 4). 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 accuracy, enables online computer processing of the data (data collection, calculation, and report generation) and, thereby, enables the rapid and convenient handling of a high number of samples.

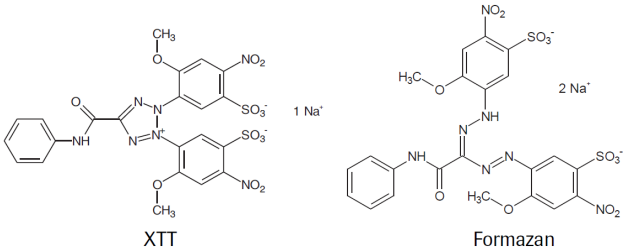

Fig. 4: Metabolization of XTT to a water-soluble formazan salt by viable cells.

Overview of the basic steps

1. Cells grown in a 96-well tissue-culture plate are incubated with the yellow XTT solution (final concentration 0.3 mg/ml) for 4 to 24 hours, see Figure 5 and section, **Assay protocol**.

2. After this incubation period, an orange formazan solution is formed.

3. The solubilized formazan product is spectrophotometrically quantified using an ELISA reader.
   – An increase in the 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 (Fig. 5).

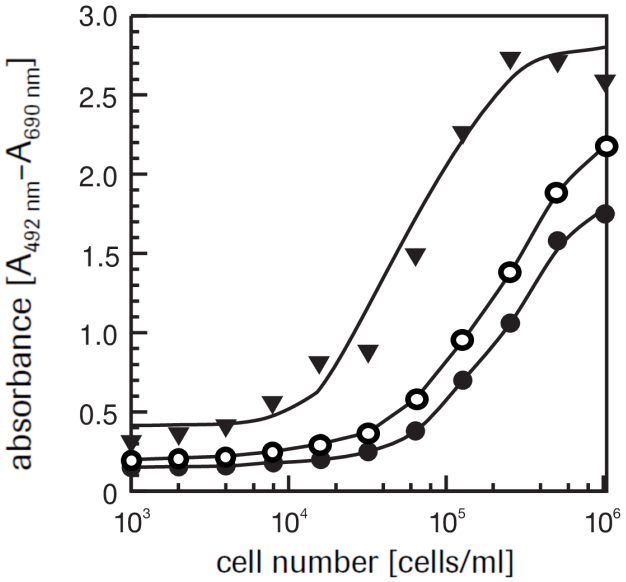

Fig. 5: Effect of different numbers of cells on color formation (example given using Ag8 cells) after 2 hours (●), 6 hours (○), and 20 hours (▼) incubation with XTT labeling mixture, see section, **Principle**.

Background information

The determination of cellular proliferation, viability, and activation are key areas in a wide variety of cellular 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, such as [^{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 immunohistocytochemistry, in a cell ELISA, and FACS analysis. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts, such as MTT, XTT, and WST-1 are especially useful for assaying the quantification of viable cells, because they are cleaved to form a formazan dye (Fig. 4; for UV absorbance spectrum, see Figure 6) only by metabolically active cells.

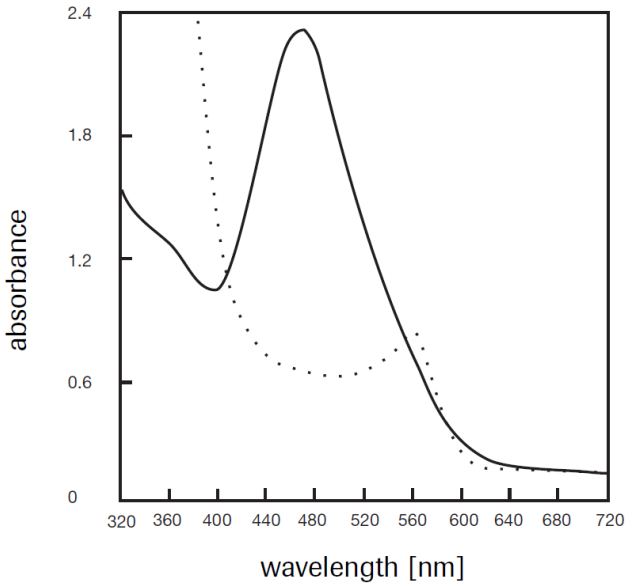

Fig. 6: Comparison of UV-spectra of XTT labeling mixture (dotted line) and the formazan dye, formed by mitochondrial dehydrogenase activity.

4. Supplementary Information

4.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

4.2. Changes to previous version

Layout changes.
Editorial changes.

4.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Tumor Necrosis Factor-α, human (hTNF-α)	1,000,000 U, 10 µg, 1 ml	11 371 843 001
Interleukin-6, human (hIL-6)	200,000 U, 2 µg, 1 ml	11 138 600 001
Interleukin-2, human (hIL-2)	10,000 U, 5 µg, 50 ml	10 799 068 001
	10,000 U, 5 µg, 1 ml	11 011 456 001
	50,000 U, 25 µg, 5 ml	11 147 528 001
Penicillin-Streptomycin	for 20 ml, 500x	11 074 440 001

4.4. Trademarks

All product names and trademarks are the property of their respective owners.

4.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

4.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

4.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

4.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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