Table of contents

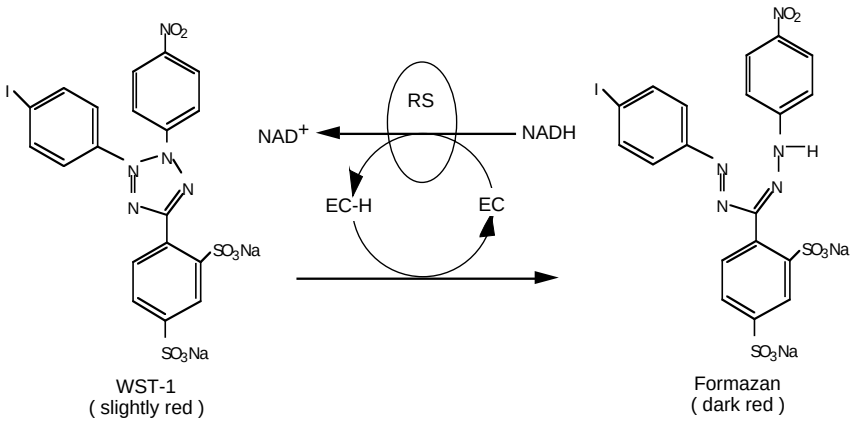
1. Description

In the life science it is very important to measure the cell proliferation or the cell viability.
Some standard methods were developed for satisfying the need of sensitive, accurate, speedy, and simple method. In these methods DNA synthesis is detected by the measurement of RI-labeled nucleoside uptaken into DNA, as DNA synthesis is accompanied with the cell proliferation or the cell viability.
Recently new measuring method has emerged for the detection of cell proliferation or cell viability. In this new method the number of viable cells are measured by the detection of cleaved tetrazolium salts that added into a medium. In this new method wash and collection of cells are not required, and all procedure, from the culture in small scale to the data analysis with ELISA reader, can be carried out in the same microtiter plate.
The PreMix WST-1 enables to measure the cell proliferation and cell viability with colorimetric assay, and bases on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells. This product takes the place of RI-labeled nucleoside, and provides non-RI method for the analysis of cell proliferation or cell viability.

2.Principle

Recently various kinds of tetrazolium salts (e.g. MTT[2,3,4], XTT[5,6,7], MTS[8], and so on) are available to measure cell proliferation or cell viability. These teterazolium salts are cleaved to formazan dye by the succinate-tetrazolium reductase (EC 1.3.99.1)[9], which exists in mitochondrial respiratory chain and is active only in viable cells. Total activity of this mitochondrial dehydrogenase in a sample rises with the increase of viable cells. As the increase of enzymes' activity leads an increase of the production of formazan dye, the quantity of formazan dye is related directly with the number of metabolically active cells in the medium. The formazan dye formed by metabolically active cell can be quantitated by measuring its absorbance by ELISA reader, which enables to measure cell proliferation activity and viability. The absorbance of formazan dye solution is in direct proportion to the number of viable cells. (Fig. 1)
This product is also designed for non-radioactive and spectrophotometric quantification of cell growth and viability in proliferation and chemosensitivity assay.
It can be used for

- The measurement of cell proliferation which is sensitive to growth factors, cytokines, mitogens, and nutrients.
- The analysis of cytotoxic and cytostatic compounds such as anticancer drugs, etc.
- The evaluation of physiological mediator and antibodies which inhibit cell growth.



WST-1
(slightly red)

Formazan
(dark red)

Fig.1 Cleavage of the tetrazolium salt (WST-1) to fromazan.
(EC = electron coupling reagent, RS = mitochondrial succinate-tetrazolium-reductase system)

3.Features

1. *Safety*: No radioactive isotopes are required
   No volatile organic solvent is required for solubilization.
2. *Accuracy*: Absorbance strongly correlates to the number of viable cells.
3. *Sensitivity*: More sensitive than using MTT.
   Sensitive as using XTT.
4. *Ease of use*: • Supplied as a ready-to-use, sterile solution.
   • Short reaction time.
   • The entire assay is performed in one microtiter plate.
   • No need for washing, harvesting or solubilization
   Processing a large number of samples is possible by using a multiwell-ELISA reader.
   No isotope disposal and radiation safety paperwork.
5. *Flexibility*: Plates can be read and returned several times to the incubator for further color development.

4.Kit component

(For 2,500 tests)
25 ml × 1 bottle of PreMix WST-1
The PreMix WST-1 is a ready-to-use solution containing WST-1 and an electron coupling reagent, diluted in phosphate buffered saline, sterile. It is recommended to add 10 μl / well PreMix WST-1 to the cells already cultured in 100 μl / well (1:10 final dilution). If the cells are cultured in 200 μl / well, add 20 μl / well PreMix WST-1. Using the 100 μl / well cell culture volume, one bottle will contain sufficient volume to perform 2,500 tests (25 microtiter plates).

5.Storage

- Shipped at -20 °C
- Stored at -20 °C (protected from light)

If the precipitate is observed when dissolving this reagent, redissolve any precipitate by warming at 37°C for several minutes with shaking gently.
Once thawed, it can be stored at 4°C, protected from light, for several weeks.
For longer storage, it is recommended to store in aliquots at -20°C.
Do not repeat freeze-thaw cycle.

6. Protocol

6-1. Reagent and instrument required other than this kit

- Incubator (37 °C)
- Centrifuge
- Microtiter plate (ELISA) reader with a filter for the use of a wavelength between 420-480 nm (if a reference wavelength is to be substracted, a filter for above 600 nm is recommended.).
- Microscope
- Hemacytometer
- Multichannel pipettor (10, 50, 100 μl)
- Sterile pipette tips
- 96-well microtiter plates (tissue culture grade, flat bottom)

6-2. Protocol

1. Culture cells in microtiter plates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 μl/well culture medium in a humidified atmosphere (e.g. 37 °C. 5 % CO_2).
   *The incubation period and cell density of the culture depend on the particular experiment conditions and on the cell line used. For most experimental setups, a cell concentration between 0.1 and 5 $\times 10^4$ / well and an incubation time of 24 to 96 hrs is appropriate.
2. After the incubation period, add 10 μl / well PreMix WST-1.
3. Incubate the cells for 0.5 to 4 hrs in a humidified atmosphere (e.g. 37 °C 5 % CO_2).

4. Measure the absorbance of the samples against a background control (see 7-3) as blank using a microtiter plate (ELISA) reader. The wave length for measuring the absorbance of the formazan product is between 420-480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader. The reference wavelength should be more than 600 nm.
   Note:
   The appropriate incubation time after the addition of PreMix WST-1 depends on the individual experimental setup (e.g. cell type and cell concentration used). Therefore, it is recommended to measure the absorption repeatedly at different points in time after the addition of PreMix WST-1 (e.g. 0.5, 1, 2, and 4 hrs) in a preliminary experiment.
   • If high sensitivity is required, incubate the cells in the presence of PreMix WST-1 for longer periods of time.

6-3. Background control (blank)

Add the same volume of culture medium and PreMix WST-1 as used in the experiment into one well (e.g. 100 μl culture medium plus 10 μl PreMix WST-1). Use this background control (absorbance of culture medium plus PreMix WST-1 in the absence of cells) as a blank position for the ELISA reader.
Slight spontaneous absorbance occurs if PreMix WST-1 is added to culture medium in the absence of cells. This background absorbance depends on the culture medium, the incubation time and exposure to light. Typical background absorbance after 2 hrs is between 0.1-0.2 absorbance units.

7.Application

7-1. Cell proliferation assay (IL-2)

Determination of the activity of human interleukin-2 (IL-2) on the mouse T cell line CTLL-2
Reagents:
- Culture medium, e.g.
  RPMI 1640 containing 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM Na-pyruvate, 1× non-essential amino acids, and 50 μM 2-mercaptoethanol.
  Optionally, add penicillin / streptomycin or gentamicin
- Human IL-2 (10,000 U / ml; 5 μg / ml), sterile
- PreMix WST-1

Procedure:
1. Seed CTLL-2 cells at a concentration of 4×10^3 cells / well in 100 μl culture medium containing various amounts of IL-2 (final concentration e.g. 0.005-25 ng / ml) into microtiter plates (tissue culture grade, 96 wells, flat bottom).
2. Incubate cells for 48 h at 37 °C and 5 % CO_2
3. Add 10 μl/well PreMix WST-1 and incubate for 4 hrs at 37 °C and 5 % CO_2
4. Measure the absorbance as described in the protocol (6-2).

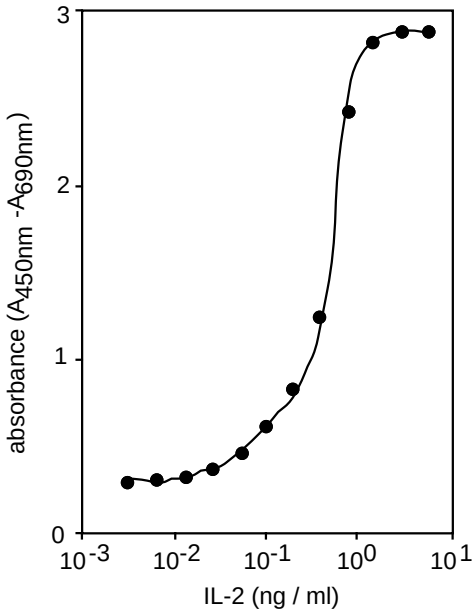


Fig.2 Meaurement of cell proliferation of CTLL-2 cell under existence of Human IL-2.

7-2. Cytotoxicity assay (TNF-α)

Determination of the cytotoxic effect of human tumor necrosis factor-α (TNF-α) on the mouse fibrosarcoma cell line WEHI-164.

Reagents:

- Culture medium, e.g.
  RPMI 1640 containing 10 % heat-inactivated FCS, 2 mM L-glutamine and actinomycin C1, (actinomycin D) , 1 μg / ml
  Optionally, add penicillin/streptomycin or gentamicin.
- human TNF-α (10 mg / ml), sterile.
- PreMix WST-1.

Procedure:

1. Preincubate WEHI-164 cells at a concentration of 1×10^6 cells / ml in culture medium with actinomycin C1, 1 μg / ml for 3 h at 37 °C and 5 % CO_2.
2. Seed cells at a concentration of 5×10^4 cells / well in 100 μl culture medium containing actinomycin C1, (1 μg / ml) and various amounts of TNF-α (final concentration e.g. 0.001-0.5 ng / ml) into microtiter plates (tissue culture grade. 96 wells, flat bottom).
3. Incubate cell cultures for 24 hrs at 37 °C and 5 % CO_2.
4. Add 10 μl / well PreMix WST-1 and incubate for 4 hrs at 37 °C and 5 % CO_2.
5. Measure the absorbance as described in the protocol (6-2).

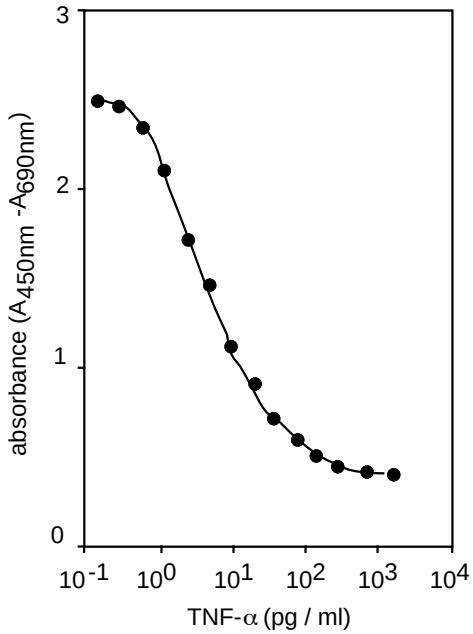


Fig.3 Measurement of cytotoxic activity of human TNF-α to WEHI-164 cells.

8. Q & A

Q-1 What does the PreMix mean ?

A-1 With conventional kit employing WST-1, electron coupling reagent and WST-1 have to be mixed before the mesurement. This mixture could be stored only for 3 days at 4 °C or for one month at - 20 °C. Takara's PreMix WST-1 is a ready-to-use solution in which all reagents are mixed. No need to mix before use. Futhermore, the PreMix can be stored for at least one year at - 20 °C.
Even if once thawed, it can be available for at least 2 weeks if stored at 4 °C. This kit is appropriate for customers who do not use this kit so frequently. PreMix WST-1 allows more sensitive detection than conventional kits had to be mixed before the mesurement.

Q-2 In measurement of cell proliferation, various tetrazolium salts (MTT, XTT, MTS, and so on) are used. What kind of difference does PreMix WST-1 possess from them ?

A-2 Principle of the measurement is the same, but formazan dyes derived from each tetrazolium salt are completely different in water solubility. In case of MTT, insoluble crystal of formazan dye is formed, and it is needed to solve the crystal with surfactants or organic reagents. In case of XTT and MTS, soluble formazan dye is formed, but its solubility is lower than that of WST-1.
So WST-1 is the most sensitive and possesses the widest range compared with other tetrazolium salts.

Q-3 What kind of differences are there between WST-1 and AlamarBlue™, water-soluble reagent for cell proliferation ?

A-3 Cell proliferation can be detected in absorbance and fluorescence with AlamarBlue™. In case that the same sensitity as WST-1 is desired using AlamarBlue™, cell proliferation has to be measured in fluorescence.

Q-4 Do components of medium or additives affect measurement ?

A-4 Theoretically, reducing substances may affect the value of absorbace. In case that reducing reagents are added, cells should be washed with PBS once before measuring.

Q-5 How long does it take to preculture the cells before adding Premix WST-1 ?

A-5 The time for adding PreMix WST-1 depends on the cell types, the cell phase (growth phase, static phase, and so on), and the objectives of experiment. Usually overnight (16 hrs) is enough for preculture. All experiment were done after 2-3 hr preculture for taking our data.

Q-6 Is the measurement affected by the lactate dehydrogenase (LDH) derived from dead cells ?

A-6 The activity of LDH is often measured to detect the activity of cell injury. However, as the LDH's activity of dead cells is much lower than dehydrogenases' activity of viable cells, the LDH activity does not affect results of measurements.

Q-7 What wavelength is used for the measurement of frormazan dye ?

A-7 The wavelenght between 420 nm and 480 nm is appropriate. The Maximum absorbance is at 440 nm. The absorbance decrease when the wavelength is out of that range.

9. Quick reference of protocol

Step	Procedure	Volume (μl well)	Time
1.	Perform culture in 96-well microtiter plate.	100 μl / well 0.1 - 5.0 x 10^4 cells / well	24 - 96 hrs
2.	Add PreMix WST-1 and incubate at 37°C in a humidified atmosphere.	10 μl / well	0.5 - 4 hrs
3.	Measure absorbance using an ELISA reader at 420 nm, with a reference wavelength > 650 nm.		

10. References

1) Cook, J. A. & Mitchell, J. B. (1989) *Anal. Biochem* **179**, 1-7.
2) Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55-63.
3) Carmichael, J. et al. (1987) *Cancer Res.* **47**, 936-942.
4) Vistica, D. T. et al. (1991) *Cancer Res.* **51**, 2515-2520.
5) Scudiero, D. A. et al. (1988) *Cancer Res.* **48**, 4827-4833.
6) Weislow, O. S. et al. (1989) *J. Natl. Cancer. Inst.* **81**, 577-586.
7) Roehm, N. W. et al. (1991) *J. Immunol. Methods* **142**, 257-265.
8) Cory, A. H. et al. (1991) *Cancer Commun.* **3**, 207-212.
9) Slater, T. F., Sawyer, B. & Sträult, U. (1963) *Biochim. Biophys. Acta* **77**, 383-393.

11. Note

This product is for research use only. Not for use in diagnostic procedures for clinical purposes. For in vitro use only