Technical Bulletin

CellTiter 96® AQueous One Solution Cell Proliferation Assay

INSTRUCTIONS FOR USE OF PRODUCTS G3580, G3581 AND G3582.

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CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay

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1. Description

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay[a] is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQ_{ueous} One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[a]] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. This convenient "One Solution" format is an improvement over the first version of the CellTiter 96® AQ_{ueous} Assay, where phenazine methosulfate (PMS) is used as the electron coupling reagent, and the PMS Solution and MTS Solution are supplied separately.

The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium (Figure 1, 1). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (2). Assays are performed by adding a small amount of the CellTiter 96® AQ_{ueous} One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490nm with a 96-well plate reader (3,4).

Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA
Toll Free in USA 800-356-9526 · Phone 608-274-4330 · Fax 608-277-2516 · **www.promega.com**
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1. Description (continued)

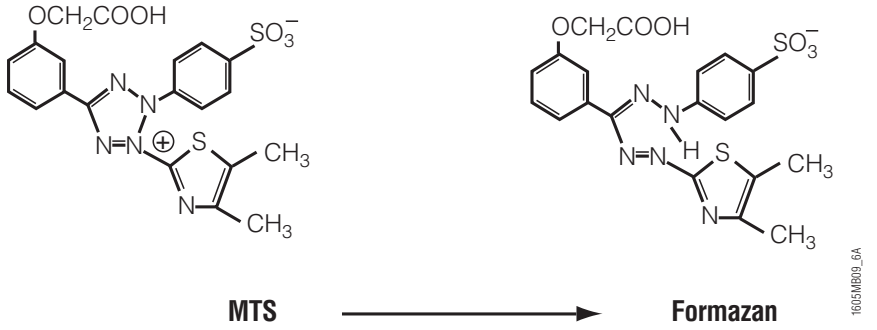

Figure 1. Structures of MTS tetrazolium and its formazan product.

The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture (Figure 2). Because the MTS formazan product is soluble in tissue culture medium, the CellTiter 96® AQ_{ueous} One Solution Assay requires fewer steps than procedures that use tetrazolium compounds such as MTT or INT (5,6). The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570nm (7).

If you currently use a [^{3}H]thymidine incorporation assay, addition of the CellTiter 96® AQ_{ueous} One Solution Reagent can be substituted for the pulse of [^{3}H]thymidine at the time point in the assay when the pulse of radioactive thymidine is usually added. Bioassay data comparing [^{3}H]thymidine incorporation to the MTS-based CellTiter 96® AQ_{ueous} Assay and the original MTT-based CellTiter 96® Assay demonstrate that tetrazolium reagents can be substituted for [^{3}H]thymidine incorporation (4,7).

Advantages of the CellTiter 96® AQ_{ueous} One Solution Assay include:

- **Easy-to-Use:** Add the CellTiter 96® AQ_{ueous} One Solution Reagent to cells, incubate and read absorbance.
- **Convenient:** Supplied as a single solution, filter-sterilized and ready for adding to assay plates.
- **Fast:** Perform the assay in a 96-well plate with no washing or cell harvesting. Also eliminates solubilization steps normally required for MTT assays.
- **Non-Radioactive:** Requires no scintillation cocktail or radioactive waste disposal.

- **Flexible:** Plates can be read and returned to incubator for further color development.
- **Safe:** Requires no volatile organic solvent to solubilize the formazanproduct (unlike MTT).

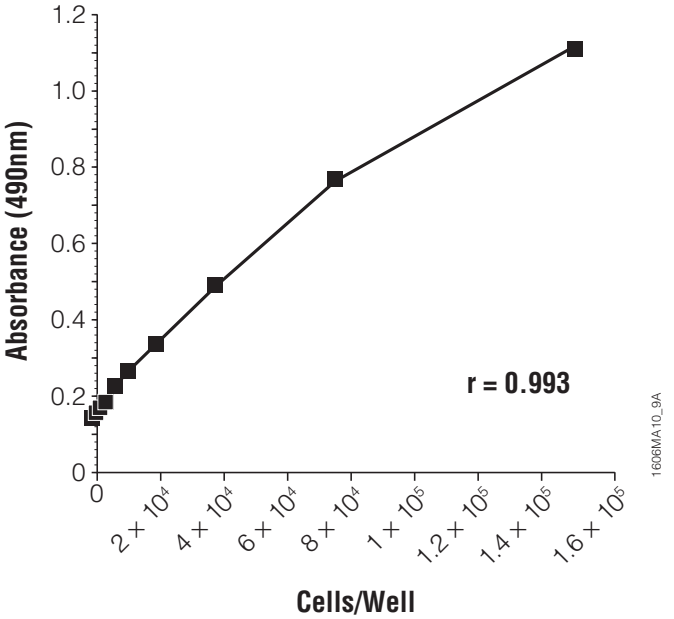

Figure 2. Effect of cell number on absorbance at 490nm measured using the CellTiter 96® AQ_{ueous} One Solution Assay. Various numbers of B9 hybridoma cells were added to the wells of a 96-well plate in RPMI containing 50µM 2-mercaptoethanol and supplemented with 5% FBS and 2ng/ml IL-6. The medium was allowed to equilibrate for 1 hour, then 20µl/well of CellTiter 96® AQ_{ueous} One Solution Reagent was added. After 1 hour at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490nm was recorded using an ELISA plate reader. Each point represents the mean ± SD of 4 replicates. The correlation coefficient of the line was 0.993, indicating a linear response between cell number and absorbance at 490nm. The background absorbance shown at zero cells/well was not subtracted from these data.

2. Product Components and Storage Conditions

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	200 assays	G3582

Includes:

- 4ml CellTiter 96® AQ_{ueous} One Solution Reagent

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	1,000 assays	G3580

Includes:

- 20ml CellTiter 96® AQ_{ueous} One Solution Reagent

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	5,000 assays	G3581

Includes:

- 100ml CellTiter 96® AQ_{ueous} One Solution Reagent

Storage Conditions: For long-term storage, store the CellTiter 96® AQ_{ueous} One Solution Reagent at –20°C, protected from light. See the expiration date on the Product Information Label. For frequent use, solutions may be stored at 4°C, protected from light, for up to 6 weeks.

Note: The performance of CellTiter 96® AQ_{ueous} One Solution Reagent after 10 freeze-thaw cycles was demonstrated to be equal to that of freshly prepared solution.

Safety: To the best of our knowledge, the chemical, physical and toxicological properties of this product have not been thoroughly investigated; therefore, we recommend the use of gloves, lab coats and eye protection when working with these or any chemicals.

Light-Sensitivity: The CellTiter 96® AQ_{ueous} One Solution Reagent is light-sensitive and is supplied in an amber container. Discoloration may occur if solutions are exposed to light outside of the container for several hours. This discoloration may cause slightly higher background 490nm absorbance readings, but it should not affect the performance of the CellTiter 96® AQ_{ueous} One Solution Assay.

3. Protocols

Materials to Be Supplied by the User

- 96-well plates suitable for tissue culture
- repeating pipettes, digital pipettes or multichannel pipettes
- 96-well plate reader

3.A. General Protocol

1. Thaw the CellTiter 96® AQ_{ueous} One Solution Reagent. It should take approximately 90 minutes at room temperature, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.

2. Pipet 20µl of CellTiter 96® AQ_{ueous} One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium.

   Note: We recommend repeating pipettes, digital pipettes or multichannel pipettes for convenient delivery of uniform volumes of CellTiter 96® AQ_{ueous} One Solution Reagent to the 96-well plate.

3. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO_2 atmosphere.

   Note: To measure the amount of soluble formazan produced by cellular reduction of MTS, proceed immediately to Step 4. Alternatively, to measure the absorbance later, add 25µl of 10% SDS to each well to stop the reaction. Store SDS-treated plates protected from light in a humidified chamber at room temperature for up to 18 hours. Proceed to Step 4.

4. Record the absorbance at 490nm using a 96-well plate reader.

3.B. Example of a Protocol for Bioassay of IL-6 Using B9 Cells

1. Maintain stock cultures of B9 cells in RPMI 1640 medium containing 5% FBS, 50µM 2-mercaptoethanol (2-ME) supplemented with 5ng/ml human recombinant IL-6 . Subculture the stock cultures of cells to 2×10^4 cells/ml, and refeed with human recombinant IL-6 every 3 days or when a density of 2×10^5 cells/ml is reached.

   Note: B9 cells used for the bioassay should be from stock cultures 2 days after the last subculture (feeding with IL-6).

2. Add 50µl/well of IL-6 samples or standards to be measured, diluted in RPMI 1640 medium containing 5% FBS and 50µM 2-ME. Start the titration of the IL-6 standard at 4ng/ml in column 12, and perform serial twofold dilutions across the plate to column 2 (to 4pg/ml). (After the cell suspension is added in Step 5 below, the final concentration of the titrated standard will be 2ng/ml in column 12 to 2pg/ml in column 2.) Use column 1 for the negative control: RPMI 1640 medium (and supplements) without IL-6. Equilibrate the plate at 37°C in a humidified, 5% CO_2 atmosphere while harvesting the cells for assay.

3.B. Example of a Protocol for Bioassay of IL-6 Using B9 Cells (continued)

3. Wash the B9 cells twice in RPMI 1640 containing 5% FBS and 50µM 2-ME by centrifugation at 300 × *g* for 5 minutes.
4. Determine cell number and viability (by trypan blue exclusion), and resuspend the cells to a final concentration of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FBS and 50µM 2-ME.
5. Dispense 50µl of the cell suspension (5,000 cells) into all wells of the plate prepared in Step 2. The total volume in each well should be 100µl.
6. Incubate the plate at 37°C for 48–72 hours in a humidified, 5% CO_2 atmosphere.
7. Add 20µl per well of CellTiter 96® AQ_{ueous} One Solution Reagent.
8. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO_2 atmosphere.

   Note: To measure the amount of soluble formazan produced by cellular reduction of MTS, proceed immediately to Step 9. Alternatively, to measure the absorbance at a later time, add 25µl of 10% SDS to each well to stop the reaction. Store SDS-treated plates protected from light in a humidified chamber at room temperature for up to 18 hours. Proceed to Step 9.
9. Record the absorbance at 490nm using a 96-well plate reader.
10. Plot the corrected absorbance at 490nm (Y axis) versus concentration of growth factor (X axis). Determine the X-axis value corresponding to one-half the difference between the maximum (plateau) and minimum (no growth factor control) absorbance values; this is the ED_{50} value (ED_{50} = the concentration of growth factor necessary to give one-half the maximum response.)

4. General Considerations

4.A. Background Absorbance

A small amount of spontaneous 490nm absorbance occurs in culture medium incubated with CellTiter 96® AQ_{ueous} One Solution Reagent. The type of culture medium used, type of serum, pH and length of exposure to light are variables that may contribute to the background 490nm absorbance. Background absorbance is typically 0.2–0.3 absorbance units after 4 hours of culture. Background absorbance may result from chemical interference of certain compounds with tetrazolium reduction reactions. Strong reducing substances, including ascorbic acid, or sulfhydryl-containing compounds, such as glutathione, coenzyme A and dithiothreitol, can reduce tetrazolium salts nonenzymatically and lead to increased background absorbance values. Culture medium at elevated pH or extended exposure to direct light also may cause an accelerated spontaneous reduction of tetrazolium salts and result in increased background absorbance values. If phenol red containing medium is

used, an immediate change in color may indicate a shift in pH caused by the test compounds. Specific chemical interference of test compounds can be confirmed by measuring absorbance values from control wells containing medium without cells at various concentrations of test compound.

Background 490nm absorbance may be corrected as follows: Prepare a triplicate set of control wells (without cells) containing the same volumes of culture medium and CellTiter 96® AQ_{ueous} One Solution Reagent as in the experimental wells. Subtract the average 490nm absorbance from the "no cell" control wells from all other absorbance values to yield corrected absorbances.

4.B. Optional Wavelengths to Record Data

Figure 3 shows an absorbance spectrum of the formazan product resulting from reduction of MTS. We recommend recording data at the absorbance peak of 490nm; however, if your 96-well plate reader does not have a 490nm filter, data can be recorded at wavelengths of 450–540nm. Absorbance may be recorded at other wavelengths if necessary, but loss in sensitivity will result. A reference wavelength of 630–700nm may be used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance.

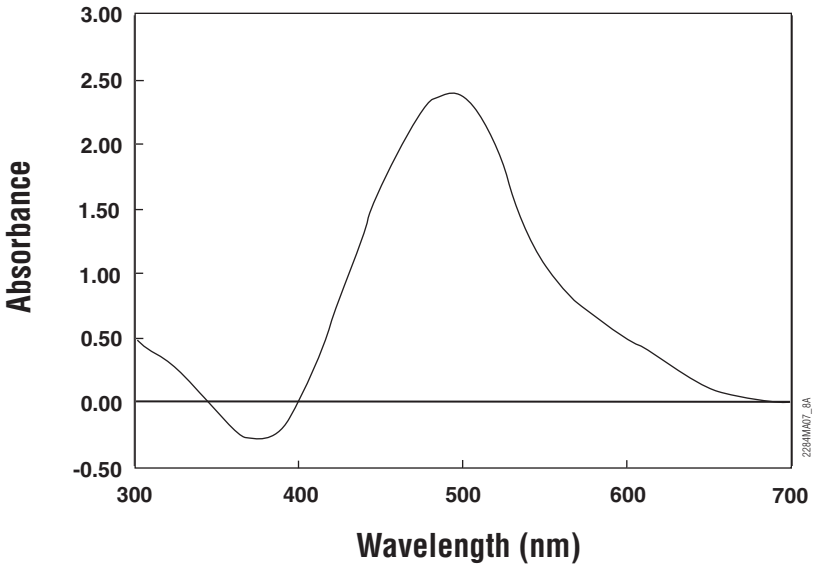

Figure 3. Absorbance spectrum of MTS/formazan. The absorbance spectrum of the formazan product resulting from reduction of the MTS tetrazolium compound shows an absorbance maximum at 490nm. The negative absorbance values (382nm) correspond to the disappearance of MTS as it is converted into formazan.

4.C. Lymphocyte Assays

Lymphocytes may produce less formazan than other cell types (8). To achieve significant absorbance changes with lymphocytes, increase the number of cells to approximately 2.5–10 × 10^4 cells/well and incubate the plate with CellTiter 96® AQ_{ueous} One Solution Reagent for the entire 4-hour period.

4.D. Reagent Optimization

The concentrations of tetrazolium and electron transfer reagents have been optimized for general use with a wide variety of cell lines cultured in 96-well plates containing 100µl of medium. If different volumes of culture medium are used, adjust the volume to maintain a ratio of 20µl CellTiter 96® AQ_{ueous} One Solution Reagent per 100µl culture medium. This reagent:medium ratio results in a final concentration of 317µg/ml MTS in the assay wells. Minor variations in the optimum concentrations of tetrazolium and electron transfer reagents occur with different cell lines; however, assay sensitivity is seldom compromised using the formulation in the CellTiter 96® AQ_{ueous} One Solution Reagent. If reagent optimization is critical to your assay procedure, we recommend using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Cat.# G5421, G5430, G5440) or the CellTiter 96® AQ_{ueous} MTS Reagent Powder products (Cat.# G1111, G1112) that supply the chemicals separately.

4.E. Cell Number Optimization

Cell proliferation assays require cells to grow over a period of time. Therefore, choose an initial number of cells per well that produces an assay signal near the low end of the linear range of the assay. This helps to ensure that the signal measured at the end of the assay will not exceed the linear range of the assay. This cell number can be determined by performing a cell titration as shown in Figure 2.

Different cell types have different levels of metabolic activity. Factors that affect the metabolic activity of cells may affect the relationship between cell number and absorbance. Anchorage-dependent cells that undergo contact inhibition may show a change in metabolic activity per cell at high densities, resulting in a nonlinear relationship between cell number and absorbance. Factors that affect the cytoplasmic volume or physiology of the cells will affect metabolic activity.

For most tumor cells, hybridomas and fibroblast cell lines, 5,000 cells per well is recommended to initiate proliferation studies, although fewer than 1,000 cells can usually be detected. The known exception to this is blood lymphocytes, which generally require 25,000–250,000 cells per well to obtain a sufficient absorbance reading.

5. References

1. Barltrop, J.A. *et al.* (1991) 5-(3-carboxymethoxyphenyl)-2-(4,5-dimenthylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. *Bioorg. Med. Chem. Lett.* **1**, 611-4.

2. Berridge, M.V. and Tan, A.S. (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch. Biochem. Biophys.* **303**, 474–82.

3. Cory, A.H. *et al.* (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **3**, 207–12.

4. Riss, T.L. and Moravec, R.A. (1992) Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. *Mol. Biol. Cell (Suppl.)* **3**, 184a.

5. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63.

6. Bernabei, P.A. *et al.* (1989) In vitro chemosensitivity testing of leukemic cells: Development of a semiautomated colorimetric assay. *Hematol. Oncol.* **7**, 243–53.

7. *CellTiter 96® Non-Radioactive Cell Proliferation Assay Technical Bulletin* #TB112, Promega Corporation.

8. Chen, C.-H., Campbell, P.A. and Newman, L.S. (1990) MTT colorimetric assay detects mitogen responses of spleen but not blood lymphocytes. *Int. Arch. Allergy Appl. Immunol.* **93**, 249–55

6. Related Products

MTS/MTT-Based Cell Viability Assay Systems

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay	1,000 assays	G5421
	5,000 assays	G5430
	50,000 assays	G5440
CellTiter 96® AQ_{ueous} MTS Reagent Powder*	250mg	G1112
	1g	G1111
CellTiter 96® Non-Radioactive Cell Proliferation Assay	1,000 assays	G4000
	5,000 assays	G4100

*PMS is not supplied with MTS Reagent Powder and must be obtained separately.

6. Related Products (continued)

Luminescent-Based Cell Viability Assay System

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573

Resazurin-Based Cell Viability Assay System

Product	Size	Cat.#
CellTiter-Blue® Cell Viability Assay	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082

Fluorescent-Based Cell Viability Assay

Product	Size	Cat.#
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
	5 × 10ml	G6081
	2 × 50ml	G6082

Cytotoxicity Assay Systems (LDH)

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
	1,000–4,000 assays	G7891
CytoTox 96® Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
	5 × 10ml	G9291
	2 × 50ml	G9292

Apoptosis Assay Systems

Product	Size	Cat.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791
Caspase-Glo® 2 Assay	10ml	G0940
	50ml	G0941
Caspase-Glo® 6 Assay	10ml	G0970
	50ml	G0971
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
	100ml	G8212
CaspACE™ Assay System, Colorimetric	50 assays	G7351
	100 assays	G7220
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
DeadEnd™ Colorimetric TUNEL System	40 reactions	G7130
	20 reactions	G7360

Apoptosis Reagents

Product	Size	Cat.#
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
	125µl	G7462
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-Cytochrome c mAb	100µg	G7421
Anti-pS^{473} Akt pAb	40µl	G7441
Anti-PARP p85 Fragment pAb	50µl	G7341
Caspase Inhibitor Z-VAD-FMK	125µl	G7232
	50µl	G7231
Caspase Inhibitor, Ac-DEVD-CHO	100µl	G5961

6. Related Products (continued)

Viability and Cytotoxicity Assay

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
	5 × 10ml	G9201
(live/dead cell protease activity determination)	2 × 50ml	G9202
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
	5 × 10ml	G9261
(dead cell protease activity determination)	2 × 50ml	G9262
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
	5 × 10ml	G9271
(live/dead cell protease activity determination)	2 × 50ml	G9272

[a]The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.