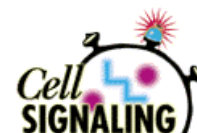


# Improved Non-Radioactive Assay to Measure Cellular Proliferation or Toxicity: The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Cell Proliferation Assay



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*Tetrazolium compounds are an increasingly popular, non-radioactive alternative for cellular proliferation and cytotoxicity assays. Promega's CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay features the tetrazolium compound MTS\*, a more stable and soluble version of previous tetrazolium compounds. MTS can be directly substituted for a radioisotope in assays to determine cellular proliferation or death.*

*\*The MTS tetrazolium compound is the subject of U.S. Patent No. 5,185,450 assigned to the University of South Florida, which is licensed exclusively to Promega Corporation.*

## Introduction

There are several assay methods available to measure the number of viable cells in proliferation or cytotoxicity assays. The most reliable method is treatment of the sample with a vital dye such as trypan blue followed by counting of viable cells using a hemocytometer and microscope. This is a direct, unequivocal measure of the number of viable cells remaining at the end of an assay; however, it is not practical for a large number of samples because it is tedious and time-consuming. The most commonly used method for assessing cellular proliferation is measurement of [<sup>3</sup>H]thymidine incorporation into DNA. This radioactive method has been extensively reported in scientific literature. However, the assay has several disadvantages, the most obvious being the use of radioactive isotopes. In addition, the assay is expensive and prone to artifacts because incorporation of [<sup>3</sup>H]thymidine measures DNA synthesis which is often not directly related to the number of viable cells present. One such artifact occurs if an improper labeling interval is used, especially for cell populations that have been quiescent and are subsequently stimulated to enter the S phase of the cell cycle after treatment with a "progression" growth factor. If the interval of [<sup>3</sup>H]thymidine labeling is too short and the S phase of the cell cycle is partially or completely missed, inaccurate results will be obtained. This problem can usually be corrected by increasing the labeling period to 24 hours, but this results in a longer assay. In addition, there is a large and growing body of literature describing artifacts from [<sup>3</sup>H]thymidine incorporation assays caused by mycoplasma contamination of either the indicator cell line or the factor being tested (1-4).

## Advances in tetrazolium compound development

Tetrazolium reagents have become popular as convenient, non-radioactive alternatives for determining numbers of viable cells in proliferation and cytotoxicity assays. Since the first reported use of MTT for measuring cellular proliferation (5) there have been several advances made to the original technique. Methods for solubilizing the formazan crystals of the MTT tetrazolium and forming a stable colored product were optimized for the original CellTiter 96<sup>®</sup> Assay System. A second major advance was made by designing and synthesizing the XTT tetrazolium reagent (6) which, when administered to cells along with a secondary electron transfer reagent such as menadione or phenazine methosulfate (PMS), results in the formation of an aqueous, soluble formazan product (7). This eliminated the need for a solubilization step in the procedure. However, the XTT tetrazolium compound has the disadvantages of limited solubility and stability in solution, requiring preparation of fresh solutions prior to each assay.

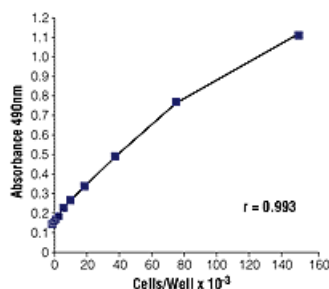
A further advance was made by the invention of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (8). The MTS tetrazolium compound (Owen's reagent) is much more soluble than XTT. In addition, MTS solutions remain stable for several months allowing use of the same solution for several assays. The MTS tetrazolium compound is used in combination with micromolar quantities of an electron transfer reagent resulting in the formation of an aqueous, soluble formazan compound when bioreduced by viable cells in culture (9). Thus, results can be recorded directly from the assay plate without further processing; there is no need to solubilize the formazan product before recording the absorbance, as is required for the use of the MTT compound.

## MTS reagent in CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System

The MTS tetrazolium compound is the basis of all of the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Assays. The first version of the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Assay was supplied with separate solutions of MTS and PMS which were combined prior to use (10). Product improvements have resulted in a new, improved formulation, with all reagents in "One Solution", which is stable for several months (11). The CellTiter 96<sup>®</sup>

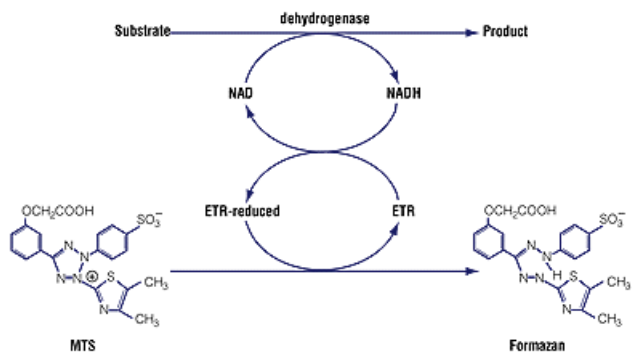
AQ<sub>ueous</sub> One Solution Cell Proliferation Assay is an improved colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent is composed of the MTS tetrazolium compound and phenazine ethosulfate (PES) as the electron coupling reagent. The concentrations of MTS and electron transfer reagent have been optimized based on results from a variety of anchorage-dependent and independent (suspension) cell types, including human lymphocytes.

The cellular conversion of tetrazolium compounds to the ultraviolet-absorbing formazan product has been demonstrated to be directly proportional to the number of viable cells for most commonly used assay procedures (10-13; [Figure 1](#)). It has been reported recently that there may not be a direct requirement for mitochondria or succinate dehydrogenase to accomplish the reduction of tetrazolium compounds in proliferation assays (14). Cellular metabolism resulting in the formation of reducing equivalents such as NADH or NADPH is thought to be responsible for the conversion of the tetrazolium compounds to the colored formazan products ([Figure 2](#)).



**Figure 1. Effect of cell number on absorbance at 490nm measured using the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution**

**Assay.** Various numbers of B9 hybridoma cells were added to the wells of a 96 well plate in RPMI containing 50 $\mu$ M 2-mercaptoethanol and supplemented with 5% FBS and 2ng/ml IL-6. The medium was allowed to equilibrate for 1 hour, then 20 $\mu$ l/well of CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent was added. After 1 hour at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, the absorbance at 490nm was recorded using an ELISA plate reader. Each point represents the mean  $\pm$  SD of 4 replicates. The correlation coefficient of the line was 0.993, indicating that there was a linear response between cell number and absorbance at 490nm. The background absorbance shown at zero cells/well was not subtracted from these data.



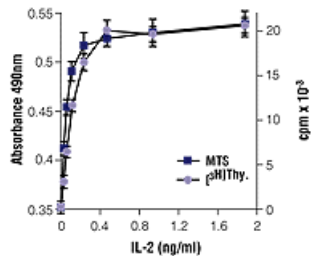
**Figure 2. Schematic diagram showing cellular metabolism resulting in the conversion of MTS to formazan.** The primary means of reducing MTS to its formazan product by metabolically active cells is thought to occur as a result of the action of dehydrogenase enzymes generating reducing equivalents such as NADH or NADPH. NADH can transfer its electrons to an electron transfer reagent (ETR) such as PMS or PES resulting in reduction of these compounds. The reduced ETRs, in turn, can directly interact with and reduce the MTS tetrazolium compound producing the deeply colored formazan product.

## MTS can replace [<sup>3</sup>H]thymidine proliferation and/or cytotoxicity assays

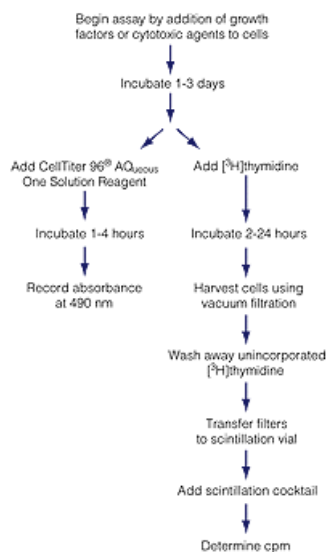
The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System can be directly substituted in many applications where proliferation is measured using [<sup>3</sup>H]thymidine incorporation. A comparison of results obtained using the two assay procedures is shown in [Figure 3](#) demonstrating that the critical parameter of ED<sub>50</sub> (the quantity of factor resulting in half the maximal response) is similar in both assays.

The most significant protocol change needed for laboratories to convert from [<sup>3</sup>H]thymidine incorporation assays to the CellTiter 96<sup>®</sup>

AQ<sub>ueous</sub> One Solution Assay System is a reduction in the number of steps in the protocol. A flow diagram comparing the two procedures and the number of steps involved in each procedure, is shown in [Figure 4](#). Both proliferation assays are set up with cells and factors to be tested in 96 well plates using the same initial steps. Instead of adding the [<sup>3</sup>H]thymidine pulse, the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent can be added to the plate. The culture plate is returned to the incubator, usually for a 1- to 4-hour period, then the 490nm absorbance is recorded using a 96 well plate reader. The processing time and labor required for [<sup>3</sup>H]thymidine incorporation assays is much greater because the procedure requires washing steps to remove unincorporated [<sup>3</sup>H]thymidine from cells, harvesting by vacuum filtration onto a glass fiber mat and subsequent transfer of glass fiber filter disks to scintillation vials for determining cpm. Typically, the time required to determine cpm in 96 samples is much greater than the time to record absorbance from one 96 well plate.



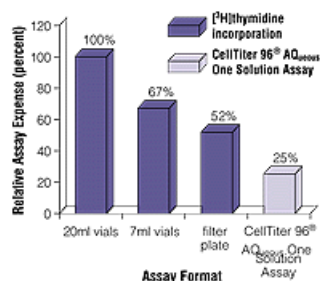
**Figure 3. Comparison between MTS and [<sup>3</sup>H]thymidine incorporation assays.** CTLL-2 cells were harvested from stock cultures supplemented with IL-2 and washed twice by centrifugation. Identical assay plates were prepared containing various concentrations of Human Recombinant IL-2 (Cat.# G5521). CTLL-2 cells were dispensed into 96 well assay plates ( $5 \times 10^3$  cells/well) and cultured for 20 hours in the presence of IL-2. After 1 day in culture, the parallel samples received either 0.2 $\mu$ Ci of [<sup>3</sup>H]thymidine or 20 $\mu$ l of CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent, then the assay plates were incubated for an additional 4 hour period. The 490nm absorbance was recorded directly from the CellTiter 96<sup>®</sup> Assay plate. The [<sup>3</sup>H]thymidine incorporation assay plate was harvested onto glass fiber filter mats using a Cambridge PhD. Cell Harvester. The filter mats were washed, transferred to scintillation vials, scintillation cocktail added and cpm determined. The Y-axis scales have been adjusted to show the similar shapes of the curves indicating that the CellTiter 96<sup>®</sup> One Solution Assay technology provides similar results for determining ED<sub>50</sub> in proliferation assays.



**Figure 4. Flow diagram comparing steps required for [<sup>3</sup>H]thymidine incorporation versus the CellTiter 96<sup>®</sup> One Solution assays.** The initial steps for setting up both assays are identical and the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent can be added at the step when [<sup>3</sup>H]thymidine would normally be added. The greatest difference between the two procedures is in the number of additional steps in the [<sup>3</sup>H]thymidine incorporation assay before obtaining data.

## Cost of [<sup>3</sup>H]thymidine versus CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System

The cost of the reagents required to perform a [<sup>3</sup>H]thymidine incorporation assay is generally much greater than for the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System. Figure 5 demonstrates the difference in total assay costs for the two assay types. For example, the cost of 96 samples assayed using [<sup>3</sup>H]thymidine in standard 20ml vials is approximately 4X the cost of assaying 96 samples with the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System. A 96 sample assay using [<sup>3</sup>H]thymidine in a 96 well plate is 2X the cost of 96 samples in the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System. The most significant contributing factor to [<sup>3</sup>H]thymidine incorporation assay costs is the cost of the radioisotope and subsequent disposal of solid, radioactive waste. The disposal of one 55 gallon drum of solid waste currently costs approximately \$1,500 - \$1,700 (U.S. dollars). Contaminated materials requiring radioactive disposal include gloves, pipette tips, cell harvesting mats, absorbent paper used to cover bench tops and 96 well plates. Other costs associated with [<sup>3</sup>H]thymidine incorporation assays include the extra labor involved in performing the cell harvesting and washing steps and the administrative responsibilities of logging radioisotope use and tracking its disposal. In addition, the use of radioactivity is meeting increased opposition by environmental groups, triggered in part by incidents of contamination caused by scientists working with these compounds.



**Figure 5. Relative assay expenses were compared using three [<sup>3</sup>H]thymidine incorporation assay formats and the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Assay System.** The most expensive format, [<sup>3</sup>H]thymidine incorporation using 20ml vials, was equated to 100 percent and the other assay formats were expressed as percentages relative to the 20ml vial [<sup>3</sup>H]thymidine incorporation assay cost. Costs used for this calculation were for the components necessary to perform 96 assays with each format and included: scintillation vials, scintillation cocktail, filter bottom plates, glass fiber harvesting filters, [<sup>3</sup>H]thymidine, disposal of solid radioactive waste and the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Reagent.

## Summary

The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Cell Proliferation Assay offers a convenient, less expensive, non-radioactive alternative to using [<sup>3</sup>H]thymidine incorporation assays. The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Assay System provides comparable results and requires less time and fewer sample manipulations by laboratory workers than conventional [<sup>3</sup>H]thymidine incorporation assays. The advantages of the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> One Solution Cell Proliferation Assay over [<sup>3</sup>H]thymidine incorporation include: i) convenience of a single reagent solution, filter sterilized and ready for use; ii) the efficiency of a 96 well plate assay, with no washing, cell harvesting or sample transfer; iii) the safety of non-radioactive reagents and elimination of volatile organic solvents required for preparation of previous tetrazolium compounds (like MTT); iv) the flexibility of plate reading at several levels of color development instead of at a fixed time; and v) stability of the solution for several months when it is stored frozen.

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Also visit the Technical Resources section of Promega's Web site (<http://www.promega.com>) for a bibliography of CellTiter 96<sup>®</sup> Assay applications.

### Ordering Information

Product	Size	Cat.#
CellTiter 96 <sup>®</sup> AQ <sub>ueous</sub> One Solution Cell Proliferation Assay System	1,000 assays	G3580
CellTiter 96 <sup>®</sup> AQ <sub>ueous</sub> One Solution Cell Proliferation Assay System	5,000 assays	G3581
CellTiter 96 <sup>®</sup> AQ <sub>ueous</sub> Non-Radioactive Cell Proliferation Assay System	1,000 assays	G5421
CellTiter 96 <sup>®</sup> AQ <sub>ueous</sub> Non-Radioactive Cell Proliferation Assay System	5,000 assays	G5430

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