Technical Bulletin

CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay

INSTRUCTIONS FOR USE OF PRODUCTS G5421, G5430, G5440, G1111 AND G1112.

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CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay

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1. Description

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay[a] is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. The CellTiter 96® AQ_{ueous} Assay is composed of solutions of 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 methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium (1). The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates without additional processing (2,3).

The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells (Figure 1). The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture (Figure 2).

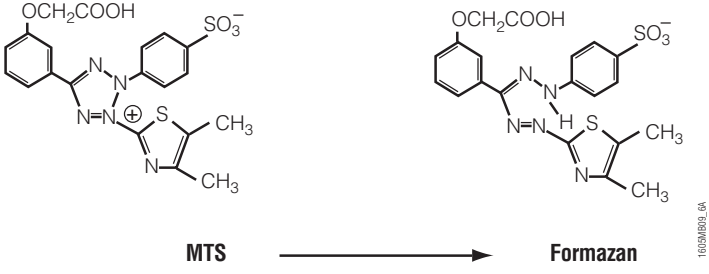

Figure 1. Structures of MTS tetrazolium salt and its formazan product.

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

- **Easy to Use:** Combine MTS and PMS solutions, add combined reagent to cells, incubate and read.
- **Fast:** Assay is performed in a 96-well plate with no washing or cell harvesting. Solubilization steps eliminated because the MTS formazan product is soluble in tissue culture medium.
- **Safe:** Requires no volatile organic solvent to solubilize the formazan product (unlike MTT).
- **Convenient:** Supplied as ready-to-use, stable, frozen sterile solutions (unlike XTT).
- **Flexible:** Plates can be read and returned to incubator for further color development (unlike MTT).
- **Non-Radioactive:** Requires no scintillation cocktail or radioactive waste disposal.

XTT, commonly used in cell proliferation assays, is a tetrazolium compound similar to MTS, which is bioreduced into an aqueous soluble formazan product (4). Unlike MTS, XTT has limited solubility and is not stable in solution. Procedures using XTT require daily preparation of fresh solutions using prewarmed (37°C) or hot (60°C) culture medium to produce a 1mg/ml solution (5,6). In contrast, the components of the CellTiter 96® AQ_{ueous} Assay are supplied as filter-sterilized solutions in physiological buffered saline ready for use.

Because the MTS formazan product is soluble in tissue culture medium, the CellTiter 96® AQ_{ueous} Assay requires fewer steps than procedures that use tetrazolium compounds such as MTT (supplied in Promega's original CellTiter 96® Assay) or INT (7,8). 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 (9).

If you currently use a [^{3}H]thymidine incorporation assay, addition of the combined MTS/PMS solution can be substituted for [^{3}H]thymidine at the time point in the assay when the pulse of radioactive thymidine is usually added. Data from proliferation bioassays comparing the CellTiter 96® AQ_{ueous} Assay and [^{3}H]thymidine incorporation show similar results (see Figure 3, Section 3.C). This is in agreement with similar radioactivity incorporation studies performed using Promega's original CellTiter 96® Assay (Cat.# G4000; 8).

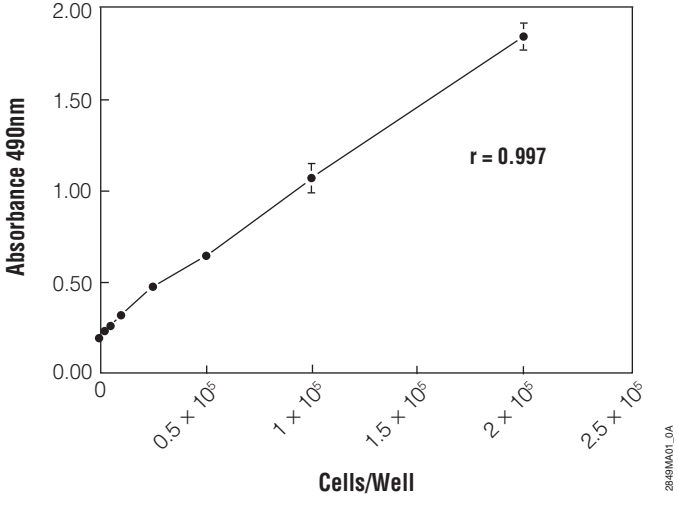

Figure 2. Effect of cell number on absorbance at 490nm measured using the CellTiter 96® AQ_{ueous} Assay. Various numbers of K562 (human chronic myelogenous leukemia) cells in RPMI supplemented with 5% fetal bovine serum (FBS) were added to the wells of a 96-well plate. The medium was allowed to equilibrate for 1 hour, then 20µl/well of combined MTS/PMS solution were 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 0.997, 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} Non-Radioactive Cell Proliferation Assay	1,000 assays	G5421

Includes:

- 20ml MTS Solution
- 1ml PMS Solution

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay	5,000 assays	G5430

Includes:

- 100ml MTS Solution
- 5ml PMS Solution
-

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay	50,000 assays	G5440

Includes:

- 1,000ml MTS Solution (10 × 100ml)
- 50ml PMS Solution (10 × 5ml)

Storage Conditions: For long-term storage, the MTS and PMS Solutions should be frozen at –20°C and 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 six weeks.

Note: The performance of these solutions processed through 10 freeze-thaw cycles has been demonstrated to be equal to that of freshly prepared solutions.

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} MTS Reagent Powder[(a)]	250mg	G1112
	1g	G1111

Note that PMS is not supplied with this product and must be obtained separately.

Storage Conditions: Store MTS Reagent Powder desiccated at 4°C protected from light. See the expiration date on the Product Information Label.

PMS Solution is included with products G5421, G5430 and G5440. PMS must be supplied by the user for products G1111 and G1112.

3. Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 5.)

- Dulbecco's phosphate buffered saline (DPBS)
- phenazine methosulfate (PMS; e.g., Sigma Cat.# P9625)

Section 3.A describes the preparation of MTS Solution from powder. If you have purchased the CellTiter 96® AQ_{ueous} Assay, proceed directly to Section 3.B.

3.A. Preparation of MTS Solution from CellTiter 96® AQ_{ueous} MTS Reagent Powder

The following protocol is recommended for the preparation of 21ml of MTS Solution (sufficient for ten 96-well plates).

1. Select a light-protected container or wrap a container with foil.
2. Add 21ml of DPBS to the container.
3. Weigh out 42mg of MTS Reagent Powder and add to DPBS.
4. Mix at moderate speed on a magnetic stir plate for 15 minutes or until the MTS is completely dissolved.
5. Measure the pH of the MTS Solution. The optimum pH is between pH 6.0–6.5. If the solution is above pH 6.5, adjust to pH 6.5 with 1N HCl.
6. Filter-sterilize the MTS Solution through a 0.2µm filter into a sterile, light-protected container.
7. Store the MTS Solution at –20°C protected from light.

3.B. General Protocol for Use of CellTiter 96® AQ_{ueous} Assay Reagents

The following recommendations are for the preparation of reagents sufficient for one 96-well plate containing cells cultured in a 100µl volume.

1. Thaw the MTS Solution and the PMS Solution. It should take approximately 90 minutes at room temperature or 10 minutes in a 37°C water bath to completely thaw the 20ml size of MTS Solution.

   Note: For convenience, the first time the product is thawed, the entire contents of the 1ml tube of PMS Solution can be transferred to the 20ml bottle of MTS Solution. This mixture should be stored at –20°C between uses. If storing PMS and MTS Solutions at 4°C, do not combine these solutions until immediately before addition to the assay plate.

2. Remove 2.0ml of MTS Solution from the amber reagent bottle using aseptic technique and transfer to a test tube.
3. Add 100µl of PMS Solution to the 2.0ml of MTS Solution immediately before addition to the culture plate containing cells.

3.B. General Protocol for Use of CellTiter 96® AQ_{ueous} Assay Reagents (continued)

4. Gently swirl the tube to ensure complete mixing of the combined MTS/PMS solution.

5. Pipet 20µl of the combined MTS/PMS solution into each well of the 96 well assay plate containing 100µl of cells in culture medium.

   Note: We recommend repeating pipettes, electronic digital pipettes or multichannel pipettes for addition of the combined MTS/PMS solution to wells of the 96-well plate. It may be convenient to dilute the combined MTS/PMS solution into a sufficient volume of culture medium to allow for the extra nonrecoverable volume required when using reagent reservoirs and multichannel pipettes.

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

   Note: To measure the amount of soluble formazan produced by cellular reduction of the MTS, proceed immediately to Step 7. 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 7.

7. Record the absorbance at 490nm using an ELISA plate reader.

   Note: The recommended concentrations of MTS Solution and PMS Solution have been optimized for a wide variety of cell lines cultured in 96 well plates containing 100µl of medium per well. If different volumes of medium are used, adjust the volume of the combined MTS/PMS solution to maintain a ratio of 20µl combined MTS/PMS solution per 100µl culture medium. This results in final concentrations in the assay of 333µg/ml MTS and 25µM PMS. If sensitivity in your assay (adequate 490nm absorbance readings) is not a limiting factor, 20µl of the combined MTS/PMS solution may be adequate for use with volumes as large as 200µl/well, but this will reduce the MTS and PMS to suboptimal concentrations. At suboptimal concentrations of MTS and at high cell numbers (50,000–200,000 K562 cells/well), we have observed that the absorbance at 490nm is not linear with cell number.

3.C. Example of a Protocol for Bioassay of IL-6 Using B9 Cells and the CellTiter 96® AQueous Assay

1. Maintain stock cultures of B9 cells in RPMI 1640 medium containing 5% FBS, 50µM 2-mercaptoethanol (2-ME), and 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 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 2ng/ml in column 12 and perform serial twofold dilutions across the plate to column 2 (to 2pg/ml). 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. 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 suspend 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 for 48–72 hours at 37°C in a humidified, 5% CO_2 atmosphere.

7. Add 20µl per well of MTS/PMS solution (see Section 3.A).

8. Incubate the plate for 1–4 hours at 37°C in a humidified, 5% CO_2 atmosphere. To measure the amount of soluble formazan produced by cellular reduction of the MTS, proceed immediately to Step 9.

   Note: 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 an ELISA plate reader.

10. Plot the corrected absorbance at 490nm (Y axis) versus concentration of growth factor (X axis), and determine the ED_{50} value by determining the X-axis value corresponding to one-half the difference between the maximum (plateau) and minimum (no growth factor control) absorbance values. ED_{50} = the concentration of growth factor necessary to give one-half the maximal response. In the example shown in Figure 3, the ED_{50} = 1.5pg/ml of IL-6.

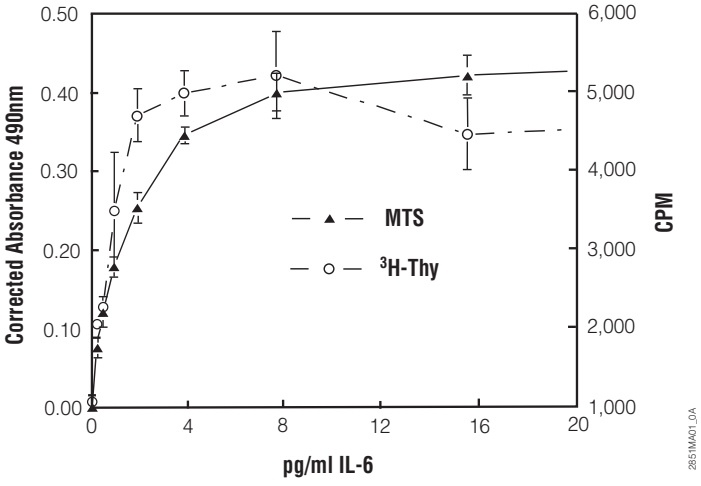

Figure 3. Proliferation of B9 cells in response to various concentrations of IL-6 measured using the CellTiter 96® AQ_{ueous} Assay and [^{3}H]-thymidine incorporation assays.

4. General Considerations

4.A. Light Sensitivity

The PMS and MTS Solutions and MTS Reagent Powder are light sensitive and supplied in amber containers. Slight discoloration may occur if solutions are stored at 4°C for several days to weeks. This discoloration may cause higher background 490nm absorbance readings, but it should not affect the performance of the CellTiter 96® AQ_{ueous} Assay.

4.B. Safety

MTS: Because the toxicological properties of MTS have not been thoroughly investigated, caution should be used. Other tetrazolium compounds are generally classified as irritants.

PMS: PMS is classified as a carcinogen; however, according to 29 CFR (U.S. Code of Federal Regulations) 1910.1200, a mixture that contains less than 0.1% by weight or volume of a carcinogenic, hazardous component is not considered hazardous unless there is evidence to the contrary. Suitable precautions should be taken in the use and disposal of this product.

4.C. Background Absorbance

A slight amount of spontaneous 490nm absorbance occurs in culture medium incubated with combined MTS/PMS solution. 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.

If background 490nm absorbance is significant using your experimental conditions, correct for it as follows. Prepare a triplicate set of control wells (without cells) containing the same volumes of culture medium and combined MTS/PMS solution as in the experimental wells. Subtract the average 490nm absorbance from these "no cell" control wells from all other absorbance values to yield corrected absorbances.

4.D. Optional Wavelengths to Record Data

Figure 4 shows an absorbance spectrum of MTS/formazan after bioreduction by K562 cells. The spectrum indicates an absorbance peak at 490nm. We recommend recording data at 490nm; however, if the ELISA reader available does not have a 490nm filter, data can be recorded at wavelengths anywhere between 450–540nm. Absorbance may be recorded at other wavelengths if necessary, but loss of sensitivity will result. For example, data recorded at 570nm results in absorbance readings of approximately one-third of the readings obtained at 490nm. A reference wavelength between 630–700nm may be used. Use of a reference wavelength eliminates background contributed by cell debris, fingerprints and other nonspecific absorbance.

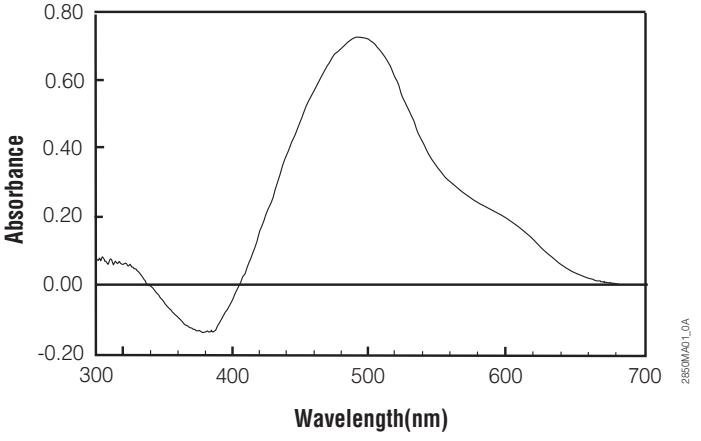

Figure 4. Absorbance spectrum of MTS/formazan after bioreduction by K562 cells. The K562 cells were cultured in RPMI 1640 supplemented with 10% FBS. The blank used to generate this absorbance spectrum was culture medium containing MTS that was not bioreduced by cells. The negative absorbance values (382nm) correspond to the disappearance of MTS as it is converted into formazan.

4.E. Lymphocyte Assays

Lymphocytes may produce less formazan than other cell types (10). 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 the MTS/PMS solution for the entire 4-hour period.

4.F. Cell Number Optimization

Cell proliferation assays require cells to grow over a period of time. Therefore, at the beginning of the culture period, choose a 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 should be empirically determined for each cell line 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. Composition of Buffers and Solutions

DPBS

0.2g	KCl
8.0g	NaCl
0.2g	KH_2PO_4
1.15g	Na_2HPO_4
100mg	$MgCl_2 \cdot 6H_2O$
133mg	$CaCl_2 \cdot 2H_2O$

Add room temperature, deionized water to the KCl, NaCl, KH_2PO_4 and Na_2HPO_4 to a 1 liter final volume. Adjust pH to 7.35 using 1N HCl or 1N NaOH. Add the $MgCl_2 \cdot 6H_2O$; mix thoroughly; then add the $CaCl_2 \cdot 2H_2O$ and mix thoroughly.

Phenazine methosulfate (PMS)

0.92mg/ml PMS in DPBS

Filter-sterilize through a 0.2µm filter into a sterile, light-protected container. Store at –20°C.

6. Related Products

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

6. Related Products (continued)

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
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
DeadEnd™ Colorimetric TUNEL System	20 reactions	G7360
	40 reactions	G7130
CaspACE™ Assay System, Colorimetric	50 assays	G7351
	100 assays	G7220

Apoptosis Reagents

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

Product	Size	Cat.#
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581
CellTiter 96® Non-Radioactive Cell Proliferation Assay	1,000 assays	G4000
	5,000 assays	G4100
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter-Blue® Cell Viability Assay	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
	5 × 10ml	G6081
	2 × 50ml	G6082
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
	5 × 10ml	G9291
	2 × 50ml	G9292
CytoTox 96® Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890

7. References

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[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.

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