



Technical Bulletin

CellTiter 96® Non-Radioactive Cell Proliferation Assay

INSTRUCTIONS FOR USE OF PRODUCTS G4000 AND G4100.



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CellTiter 96® Non-Radioactive Cell Proliferation Assay

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1. Description

Proliferation assays are widely used in cell biology for the study of growth factors, cytokines, nutrients and cytotoxic agents. There are several ways to determine the number of cells in a proliferation assay. Cell number can be determined: 1) directly, by counting using a microscope or an electronic particle counter, or 2) indirectly, by measuring incorporation of radioactive precursors, by the use of chromogenic dyes to quantitate total protein, or by measuring metabolic activity of cellular enzymes.

The CellTiter 96® Non-Radioactive Cell Proliferation Assay is a collection of qualified reagents that provide a rapid and convenient method of determining viable cell number in proliferation, cytotoxicity (1,2), cell attachment (3,4), chemotaxis (5), and apoptosis (6) assays. The CellTiter 96® Assay is based on the cellular conversion of a tetrazolium salt into a formazan product that is easily detected using a 96-well plate reader. The original form of this assay was

described by Mosmann (7). In subsequent years, several investigators (8–11) have worked to make improvements. Technical problems addressed include: 1) serum protein precipitation caused by the addition of organic solvent, 2) interference of phenol red, 3) incomplete solubilization of formazan crystals resulting in a lowered sensitivity, and 4) stability of the colored product. In addition, direct comparisons between ^3H thymidine incorporation and tetrazolium conversion have demonstrated less than a 5% difference between the two assays for determination of growth factor content of several samples (8). Promega has contributed to this body of work and has done extensive optimization of the component formulations and conditions used to create the CellTiter 96® Non-Radioactive Cell Proliferation Assay for eukaryotic cell proliferation, cell attachment (3,4), and apoptosis (6) studies. Tetrazolium assay technology also has been used to measure eukaryotic cytotoxicity (1,2) and chemotaxis (5) as well as to measure yeast (12–15) and bacteria (16) cell numbers.

The CellTiter 96® Assay procedure is outlined in Figure 1. The assay is performed by the addition of a premixed optimized Dye Solution to culture wells of a 96-well plate usually containing various concentrations of growth factor or test substance. During a 4-hour incubation, living cells convert the tetrazolium component of the Dye Solution into a formazan product. The addition of Dye Solution can be substituted for a pulse of ^3H thymidine at the time point in the assay when this pulse is normally performed. The Solubilization Solution/Stop Mix is then added to the culture wells to solubilize the formazan product, and the absorbance at 570nm is recorded using a 96-well plate reader. The 570nm absorbance reading is directly proportional to the number of cells normally used in proliferation assays. Although the absorbance maximum for the formazan product is 570nm and pure solutions appear blue, the color at the end of the assay may not be blue and depends on the quantity of formazan present relative to other components (including serum, acidified phenol red and unreduced MTT) in the culture medium.

Figure 2 shows the linear relationship ($r = 0.99$, 0 to 200,000 cells/well) between the number of cells and color formation and demonstrates that as few as 1,000 cells/well can be detected using a 96-well plate reader. Note that the incubation time can be reduced with high cell numbers. The ability to convert the tetrazolium salt in the Dye Solution into the formazan product varies among different cell types depending on their metabolic capacity. Most eukaryotic cells in culture, including mammalian, plant and yeast cell types (15), reduce the tetrazolium salt sufficiently to perform CellTiter 96® assays accurately at low cell numbers. The known exception to this is blood lymphocytes (17); for these cells, it is often necessary to increase the cell number to $1\text{--}5 \times 10^5/\text{ml}$ to obtain a significant 570nm absorbance reading (17–20).

Cell Number Optimization

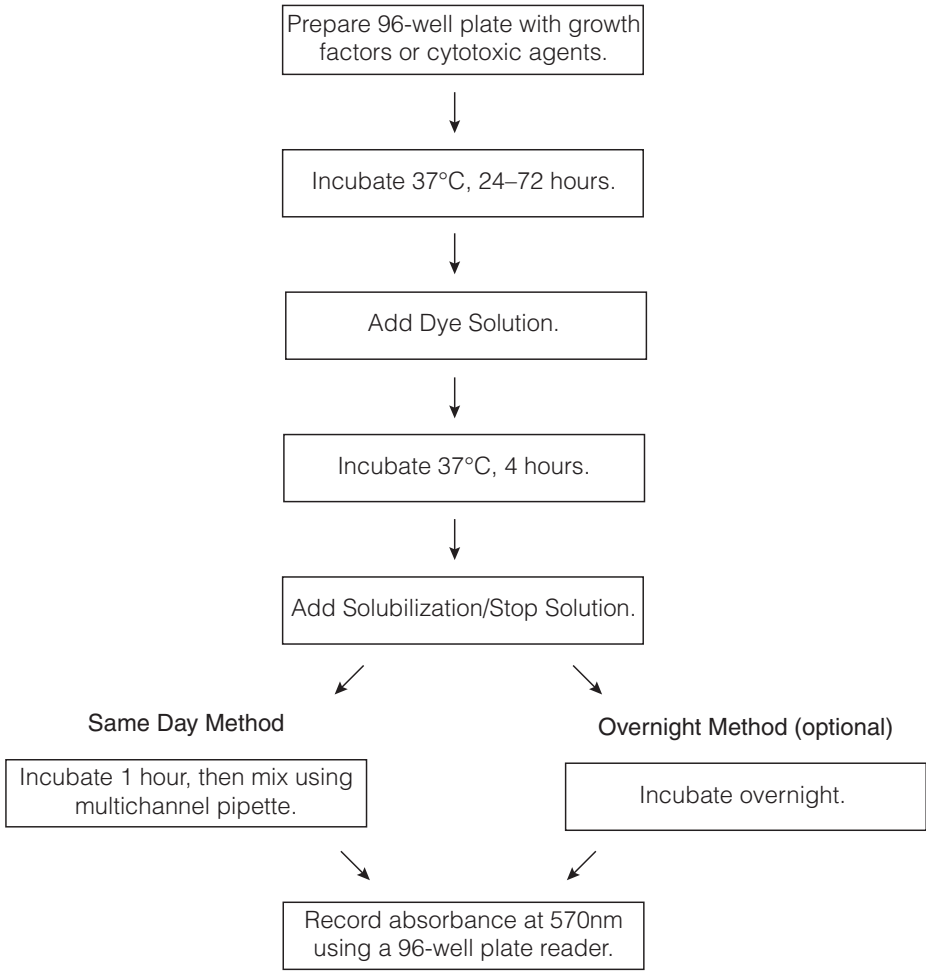
Because cell proliferation assays require cells to grow over a period of time, choose a number of cells per well that produces an assay signal near the low end of the linear range of the assay. This helps to ensure that the signal measured at the end of the assay will not exceed the linear range of the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

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 at high densities 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 usually can 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.

Advantages

The CellTiter 96® Assay has several advantages over conventional cell number or ³[H]thymidine incorporation assays. Because the CellTiter 96® Assay is non-radioactive, the required documentation of isotope use and the costs of scintillation cocktail as well as the subsequent disposal costs of spent isotope, cocktail, vials and wash solutions are eliminated. There is a minimum amount of labor involved in doing the CellTiter 96® Assay. The assay is done entirely in a 96-well plate with no steps that require washing the cells or the removal of solution from the wells. The assay can be used for both anchorage-dependent or suspension cells with no change in the protocol. The assay plates are read using a 96-well plate reader, making it easy to computerize data collection, calculations and report generation. A comparison of results obtained with the CellTiter 96® Assay and ³[H]thymidine incorporation assay is shown in Figure 3. There is no significant difference between the bioactivity values obtained with these two assays.



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Figure 1. CellTiter 96® Assay flow chart.

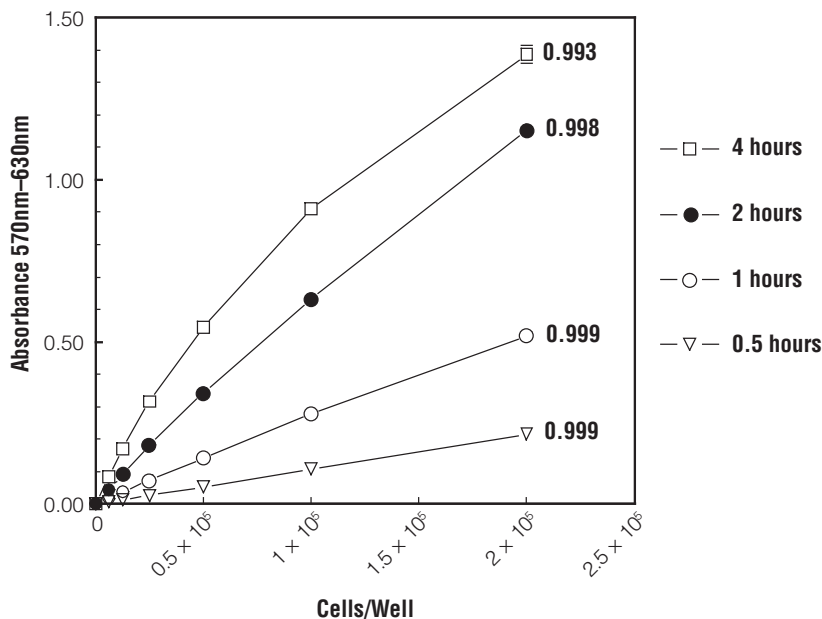


Figure 2. Effect of B9 hybridoma cell number on color formation.

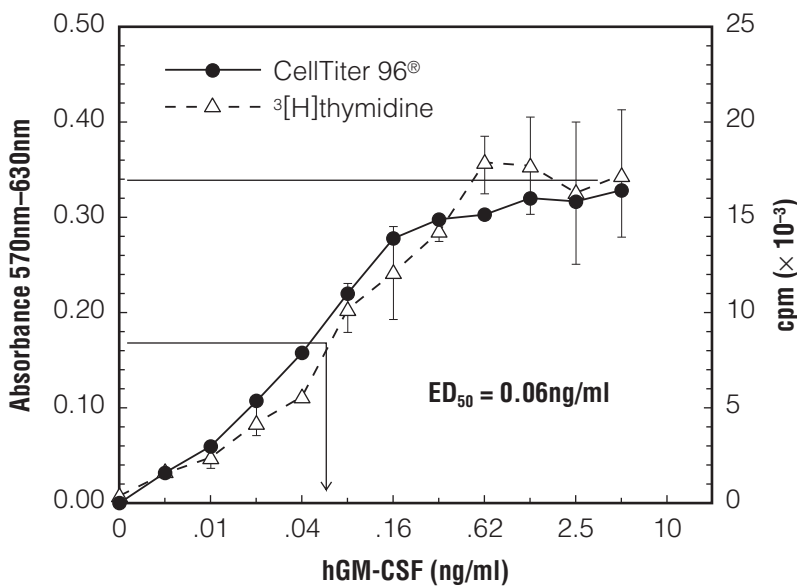


Figure 3. CellTiter 96® and ³[H]thymidine assays of hGM-CSF using TF-1 cells (21). A blank absorbance value of 0.065 was subtracted from all CellTiter 96® values.

2. Product Components and Storage Conditions

Product	Size	Cat.#
CellTiter 96® Non-Radioactive Cell Proliferation Assay	1,000 assays	G4000

Includes:

- 100ml Solubilization Solution/Stop Mix
- 15ml Dye Solution

Product	Size	Cat.#
CellTiter 96® Non-Radioactive Cell Proliferation Assay	5,000 assays	G4100

Includes:

- 500ml Solubilization Solution/Stop Mix
- 75ml Dye Solution

Packaging: Cat.# G4000 and G4100 are each composed of two packages. G4000 consists of Part# G4001 and G4002. G4100 consists of Part# G4101 and G4102. The G4001 and G4101 component packages contain the Solubilization Solution/Stop Mix. The G4002 and G4102 component packages contain the Dye Solution.

Solubilization Solution/Stop Mix: Store at room temperature.

Note: If a precipitate forms during shipment or storage, warm the container to 37°C and mix.

Dye Solution: Dispense under sterile conditions. Store at -20°C. Avoid multiple freeze-thaw cycles or exposure to frequent temperature changes. These fluctuations can greatly alter product stability. For daily/weekly use, the Dye Solution can be stored at 2-4°C in an amber bottle and protected from light.

Note: A precipitate may form in the Dye Solution but will not affect the performance of the system.

 **Protect the Dye Solution from light.**

 **Dye Solution is an irritant. Avoid contact with skin or eyes.**

3. Standard CellTiter 96® Assay Protocol for 96-Well Plates

Materials to Be Supplied by the User

- sterile 96-well cell culture plates
- 96-well plate reader
- latex or plastic gloves

3.A. Preparation of Assay Plates

1. Add 50µl of culture medium containing various concentrations of growth factor samples or standards to each well of a 96-well plate.

For example, to prepare a standard assay of a growth factor, add 50µl of culture medium (without growth factor) to all wells. Then prepare a working dilution of growth factor that is 4 times the highest final concentration to be assayed. Add 50µl of this working dilution to a triplicate set of wells in column 12. Using a multichannel pipette, perform 50µl twofold serial dilutions across the 96-well plate from column 12 to column 2 (leaving column 1 as the negative control to be used as the blank).

2. Equilibrate the plate at 37°C in a humidified, 5% CO₂ atmosphere while preparing the cells to be used for the assay.

3.B. Harvesting Cells Used for Assay

1. Cells used for bioassay are typically from stock cultures; however, the culture conditions used to grow cells can affect results. We recommend that culture conditions be taken into consideration when analyzing results of proliferation bioassays. Record the following culture conditions: passage number, medium composition, cell density and time in culture since last medium change.
2. Wash the cells twice by centrifugation in assay medium that is free of the growth factor(s) to be tested.
3. Determine cell number and trypan blue viability, and suspend the cells to a final concentration of 1×10^5 /ml in assay medium.
4. Dispense 50µl of the cell suspension (5,000 cells) into all wells of the pre-equilibrated 96-well plate (Section III.A, Step 2). The total volume in the plate should now be 100µl/well.

Note: For lymphocytes, the number may have to be increased to 50,000–100,000 cells/well.

5. Incubate the plate at 37°C for 48–72 hours in a humidified, 5% CO₂ atmosphere.

3.C. Color Development and Recording of Data

1. Add 15µl of the Dye Solution to each well.

! Dye Solution is an irritant. **Avoid contact with skin or eyes.**

2. Incubate the plate at 37°C for up to 4 hours in a humidified, 5% CO₂ atmosphere.
3. After incubation, add 100µl of the Solubilization Solution/Stop Mix to each well.

Note: It is not necessary to keep the plate sterile after this stage. The colored formazan product is stable at 4°C, and absorbance can be recorded several days later. To avoid evaporation upon storage, place the plate(s) in a humid atmosphere (a sealed box with a small container of water or moist paper towel will work).

! **Caution:** Solubilization Solution/Stop Mix contains an organic solvent. We recommend that the Solubilization Solution/Stop Mix be dispensed in a fume hood and, upon completion of the assay, the contents of the plate be disposed of into organic waste.

4. **SAME DAY METHOD:** One hour after addition of the Solubilization Solution/Stop Mix, the contents of the wells may be mixed to get a uniformly colored solution. Mixing can be done using a multichannel pipette. However, care should be taken to avoid bubble formation. Bubbles on the surface may interfere with the accurate recording of absorbance values.

OPTIONAL OVERNIGHT METHOD: Allow the plate to stand overnight in a sealed container with a humidified atmosphere at room temperature to completely solubilize the formazan crystals. Incubation at 37°C will accelerate solubilization of crystals and is recommended for assays with high cell numbers and a large quantity of formazan formation.

5. Record the absorbance at 570nm wavelength using a 96-well plate reader. The use of a reference wavelength will reduce background contributed by cell debris, fingerprints and other nonspecific absorbance. Any reference wavelength between 630–750nm may be used; greater sensitivity is obtained by using a reference wavelength of 650nm or above. If your plate reader does not have dual wavelengths, the reference wavelength may be eliminated with only a minimum effect on accuracy among replicate samples. If the plate reader is equipped with a shaking device to mix the contents of the wells, it is recommended that the plate be shaken prior to reading to ensure a uniformly colored solution.

Note: The absorbance maximum for formazan in the Solubilization Solution/Stop Mix is 570nm, but the absorbance can be read between 550–600nm (see Figure 4).

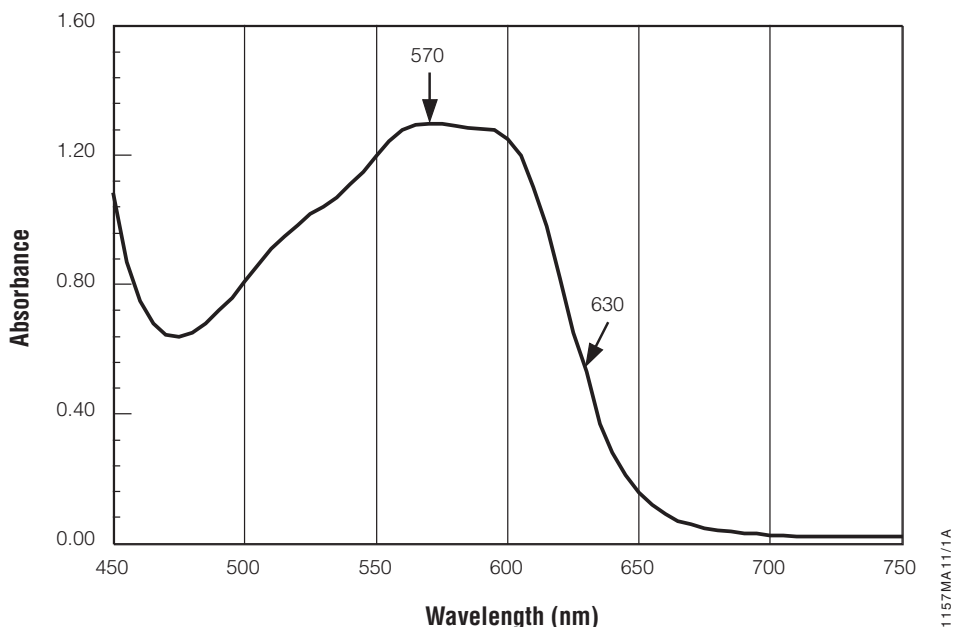


Figure 4. Absorbance spectrum of formazan in Solubilization Solution/Stop Mix.

3.D. Background Absorbance

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 or 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. In addition, the type of culture medium used and the source of serum may have slight influences on background absorbance values.

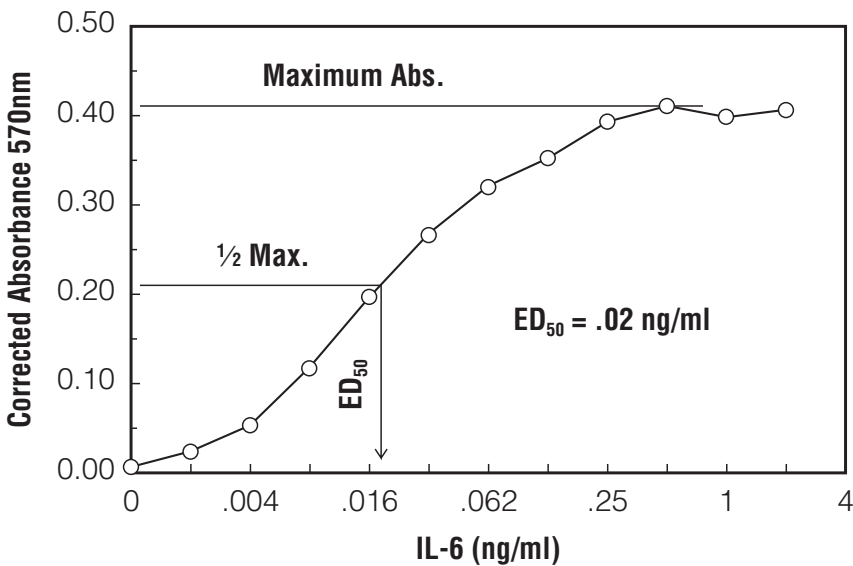
To test for the effects of chemical interference, prepare a set of control wells without cells containing the same volumes of culture medium, the vehicle used to deliver test compound, and tetrazolium reagent. Incubate in parallel with test samples, and subtract the average 570nm absorbance of the no-cell control wells from all experimental wells to yield corrected absorbance.

If specific chemical interference of test compounds is suspected, absorbance values from control wells containing medium without cells at various concentrations of test compound should confirm whether or not chemical interference is occurring. If phenol red-containing medium is used, an immediate change in color may indicate a shift in pH caused by the test compounds.

3.E. Calculations

1. The average of the absorbance values in column 1 (negative control) may be used as a blank value and subtracted from all absorbance values to yield Corrected Absorbance Values.
2. Plot Corrected Absorbance Values 570nm (Y axis) versus concentration of growth factor (X axis, log scale). Determine the ED_{50} value by locating the X-axis value corresponding to one-half the maximum (plateau) absorbance value (see Figure 5).

Note: ED_{50} = one unit of bioactivity or the concentration of growth factor necessary to give half the maximal response.



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Figure 5. Bioassay of IL-6.

4. Example of CellTiter 96® Cytotoxicity Assay Protocol

The following protocol illustrates the use of the CellTiter 96® Non-Radioactive Cell Proliferation Assay System to examine TNF- α cytotoxicity using murine L929 cells.

4.A. Preparation of Assay Plates and Harvesting Cells

1. Grow murine L929 cells (ATCC CCL 1) in Ham's F12 and Dulbecco's modified Eagle's media (1:1, v/v ratio) containing 1.2g/L sodium bicarbonate, 15mM HEPES (pH 7.35) and supplemented with 10% (v/v) horse serum.
2. Harvest the cells in log phase growth using trypsin:EDTA. Determine cell number and trypan blue viability, and suspend the cells to a final concentration of 2.22×10^5 cells/ml in the above medium.
3. Dispense 90 μ l of the cell suspension (containing 2×10^4 cells) into each well of a 96-well tissue culture plate.
4. Incubate overnight (18–24 hours) at 37°C in a humidified, 5% CO₂ atmosphere.
5. Prepare twofold serial dilutions of TNF- α in the above medium containing 10 μ g/ml actinomycin D. Add 10 μ l per well of these dilutions to yield a final concentration of 1 μ g/ml actinomycin D in all wells. The TNF- α concentrations in assay wells should range from 0.78–1.5pg/ml. The positive control should be 200ng/ml TNF- α in 1 μ g/ml actinomycin D. The negative control should not contain TNF- α .
6. Incubate the plate at 37°C for 20 hours in a humidified, 5% CO₂ atmosphere.

4.B. Color Development and Recording of Data

1. Add 15 μ l of the Dye Solution to each well.

 Dye Solution is an irritant. **Avoid contact with skin or eyes.**

2. Incubate the plate at 37°C for 4 hours in a humidified, 5% CO₂ atmosphere.
3. After 4 hours, add 100 μ l of the Solubilization Solution/Stop Mix to each well.

Note: It is not necessary to keep the plate sterile after this stage. The colored formazan product is stable, and absorbance can be recorded several days later. To avoid evaporation upon storage, place the plate(s) in a humid atmosphere (a sealed box with a small container of water or moist paper towel will work).

 **CAUTION:** Solubilization Solution/Stop Mix contains an organic solvent. We recommend that the Solubilization Solution/Stop Mix be dispensed in a fume hood and, upon completion of the assay, the contents of the plate be disposed of into organic waste.

4.B. Color Development and Recording of Data (continued)

4. **SAME DAY METHOD:** One hour after the addition of the Solubilization Solution/Stop Mix, the contents of the wells may be mixed to get a uniformly colored solution. Mixing can be done using a multichannel pipette; however, care should be taken to avoid bubble formation. Bubbles on the surface may interfere with the accurate recording of absorbance values.

OPTIONAL OVERNIGHT METHOD: Allow the plate to stand overnight in a sealed container with a humidified atmosphere to completely solubilize the formazan crystals. Incubation at 37°C will accelerate solubilization of crystals and is recommended for assays with high cell numbers and a large quantity of formazan formation.

5. Record the absorbance at 570nm using an 96-well plate reader. The use of a reference wavelength will reduce background contributed by cell debris, fingerprints and other nonspecific absorbance. Any reference wavelength between 630–750nm may be used; greater sensitivity is obtained by using a reference wavelength of 650nm or above. If your plate reader does not have dual wavelengths, the reference wavelength may be eliminated with only a minimum effect on accuracy among replicate samples. If the plate reader is equipped with a shaking device to mix the contents of the wells, it is recommended that the plate be shaken prior to reading to ensure a uniformly colored solution.

Note: The absorbance maximum for formazan in the Solubilization Solution/Stop Mix is 570nm, but the absorbance can be read between 550–600nm (see Figure 4).

4.C. Calculations

1. The average of the absorbance values for the positive control (100% lysed cells) may be used as a blank value and subtracted from all other absorbance values to yield Corrected Absorbance Values.
2. Plot Corrected Absorbance Values 570nm (Y axis) versus concentration of cytotoxic agent (X axis, log scale), and determine the IC_{50} value by locating the X-axis value (ng/ml) corresponding to one-half the maximum absorbance value (see Figure 6).

Note: IC_{50} = one unit of bioactivity or the inhibitory concentration of cytotoxic agent necessary to kill half of the cell population.

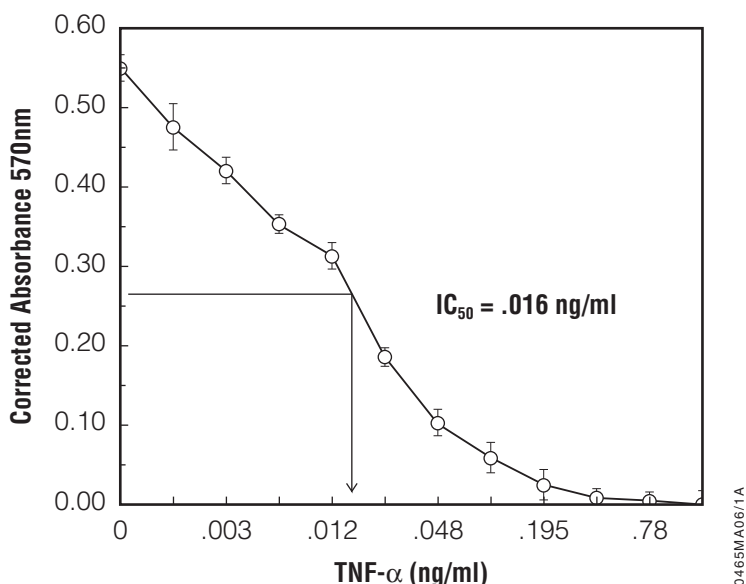


Figure 6. L929 assay of TNF- α .

5. Larger Volume Assay Protocol

This system also can be used for plates with larger wells. For this application, record the absorbance values using a conventional spectrophotometer. Use the same proportions of the Dye Solution to culture medium (i.e., 15:100 ratio) as in the directions for the 96-well plates above. As an example, if the assay is done in a 24-well plate with a final volume of 1ml, add 150 μ l of the Dye Solution. Return the plate to the incubator for 4 hours, then add 1ml of the Solubilization Solution/Stop Mix to each well. Allow approximately 1 hour for the formazan crystals to solubilize, then mix the contents of each well with a Pasteur pipette to obtain a uniform color. Transfer the solution to a cuvette and read at 570nm in a spectrophotometer. The negative control wells (cells cultured without growth factor) can be used as a reference point to zero the spectrophotometer.

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7. Related Products

ATP-Based Cell Viability Assay System

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

Resazurin-Based Cell Viability Assay System

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

MTS-Based Cell Viability Assay Systems

Product	Size	Cat.#
CellTiter 96® AQueous One Solution Cell Proliferation Assay	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay	1,000 assays	G5421
	5,000 assays	G5430
	50,000 assays	G5440
CellTiter 96® AQueous MTS Reagent Powder	250mg	G1112
	1g	G1111

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

7. Related Products (continued)

Protease-Based Cell Viability Assay

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

Viability and Cytotoxicity Assay

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

Cytotoxicity Assay Systems (LDH)

Product	Size	Cat.#
CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH release)	1,000 assays	G1780
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH release)	200–800 assays	G7890
	1,000–4,000 assays	G7891
CytoTox-Glo™ Cytotoxicity Assay (LDH release)	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
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
DeadEnd™ Colorimetric TUNEL System	40 reactions	G7130
	20 reactions	G7360
CaspACE™ Assay System, Colorimetric	100 assays	G7220
	50 assays	G7351

7. Related Products (continued)

Apoptosis Reagents

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

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