CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

3D Culture 96 Well Collagen I Cell Proliferation Assay

Reagent kit for investigating cell proliferation in 3D Culture

96 samples

Catalog #: 3447-096-K

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Table of Contents

I. Quick Reference Procedure for Trevigen's 3D Culture Collagen I Cell Proliferation Assay (including the Cell Toxicity Protocol; also see the Tumorigenesis protocol in section VI):

Read through the complete Instructions for Use prior to using this kit. Determine the optimal seeding density for each cell line used. In general, 5,000 cells per well (25,000 cells/cm^2) in 100 µl cell culture medium is a good starting point.

1. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 80% confluence.
2. Prepare Collagen I on ice (for 8 wells = 1 stripwell column); add components in order:
    a. Add 275 µl of cold cell culture medium.
    b. Add 5 µl 350 mM NaOH, and pipet to mix.
    c. Add 70 µl Collagen I, and pipet to mix.
3. Working on ice, coat each well of the 96 well plate with 35 µl of 3D Culture Collagen I. Centrifuge plates at 300 x *g* at 4°C for 10 minutes in a swinging bucket rotor to eliminate bubbles and evenly disperse Collagen I across well bottoms.
4. Transfer plate to a CO_2 incubator set at 37˚C for one hour to promote gel formation.
5. Prepare seeding media on ice (for 8 wells = 1 stripwell column):
    a. Add 1 ml of cold cell culture medium.
    b. Add 20 µl of 1 mg/ml Collagen I (from step 2), and pipet to mix.
    c. Warm to 37°C for step 7.
6. After one hour, harvest and count cells.
7. Centrifuge cells at 200 x g for 3 min, remove supernatant, and wash with ice cold PBS. Dilute cells to desired concentration in pre-warmed seeding medium from step 5.
8. Add 100 µl of cells (in 2% Collagen I) per well on top of the gel plug.
9. Incubate at 37˚C in CO_2 incubator for 48 - 96 hours.
10. Assay remaining cells for a standard curve (section VI. B.); each cell type will require a separate standard curve.
11. Dilute test compounds to desired concentration in pre-warmed (37°C) cell culture medium, and add 100 µl per well.
12. Add 15 µl of cell proliferation reagent per well, and incubate at 37˚C in a CO_2 incubator.
13. Remove 96 well plate, and read absorbance at 450 or 490 nm. Readings may be taken between 1 to 4 hours after the addition of reagent.

II. Background

Recent studies indicate that the composition of the extracellular environment influences cellular responses to apoptosis inducing agents[1,2] implicating a role for extracellular proteins in influencing both toxicity and drug resistance. As a result, this environment must be mimicked during the course of cell-based studies to provide the most accurate translation to animal models. **Cultrex® 3D Culture Cell Proliferation Assays** were created in an effort to provide more physiologically relevant assessment when using cell models in the screening process for compounds that influence toxicity, cell survival, tumorigenicity, and new tumor formation. These assays offer a flexible, standardized, high-throughput format for quantitating the degree to which pharmacological compounds influence toxicity or tumorigenicity in an *in vivo*-like environment. The **Cultrex® 3D Culture Cell Proliferation Assay** has been adapted to multiple formats so that cell proliferation may be evaluated against different extracellular matrices; the assay is available in the following formats:

- Basement Membrane Extract (BME)
- Laminin I
- Collagen I
- No matrix

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. **CULTREX® 3D Culture Cell Proliferation Assays** contain reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

IV. Materials Supplied

Component	Quantity	Storage	Catalog#
3D Collagen I	1 ml	4˚C	3447-001-01
Cell Proliferation Reagent	3 ml	-20˚C	3445-096-02
3D Culture 96 Stripwell Plates	2 plates	Room Temp	3445-096-03

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl pipettor, 20 - 200 µl pipettor, and 200 - 1000 µl pipettor
2. Laminar flow hood or clean room
3. 37˚C CO_2 incubator

4. Low speed swinging bucket 4°C centrifuge and tubes for cell harvesting
5. Hemocytometer or other means to count cells
6. -20˚C storage
7. ice bucket
8. standard light microscope (or inverted)
9. pipette aid
10. timer
11. 96-well plate reader (450 nm or 490 nm)
12. Computer and graphing software, such as Microsoft® Excel®.

Reagents

1. Cell Harvesting Buffer; EDTA, trypsin, or other cell detachment buffer.
2. Tissue Culture Growth Media, as recommended by cell supplier.
3. Pharmacological agents for addition to culture medium, if necessary.
4. Sterile PBS or HBSS to wash cells.
5. Trypan blue or equivalent viability stain

Disposables

1. Cell culture flask, 25 cm^2 or 75 cm^2
2. 50 ml tubes
3. 1 - 200 µl and 200 - 1000 µl pipette tips
4. 1, 5 and 10 ml serological pipettes
5. gloves

VI. Assay Protocol

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

A. Cell Harvesting

Culture cells per manufacturer's recommendation. The following procedure is suggested and may need to be optimized to suit the cell type(s) being studied.

1. Cells should be passaged 2 or 3 times prior to use in the assay, and adherent cells need to be 80% confluent. Each well requires approximately 5,000 cells. 25 and 75 cm^2 flasks yield at least 1 x 10^6 and 3 x 10^6 cells, respectively. Determine the number of cells needed to perform a standard curve for each cell type (Section VI.B) and the cell proliferation assay (Section VI. C) .
2. Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.
3. Wash cells two times with sterile PBS or HBSS. Use 5 ml per wash for a 25 cm^2 flask and 10 ml per wash for a 75 cm^2 flask.

4. Harvest cells. For 25 cm^2 flask or 75 cm^2 flask, add 1 ml or 2 ml, respectively, of Cell Harvesting Buffer (see Materials Required But Not Supplied), and incubate at 37˚C for 5 to 15 minutes until cells have dissociated from bottom of flask.
5. Transfer cells to a 15 ml conical tube, and add 5 ml of cell culture medium.
6. Centrifuge cells at 200 x *g* for 10 minutes to pellet cells, remove medium, and resuspend cells in 2 ml of cell culture medium. Cells may need to be gently pipetted up and down with serological pipette to resuspend cells.
7. Count cells, and dilute to 1 x 10^6 cells per ml in cell culture medium.

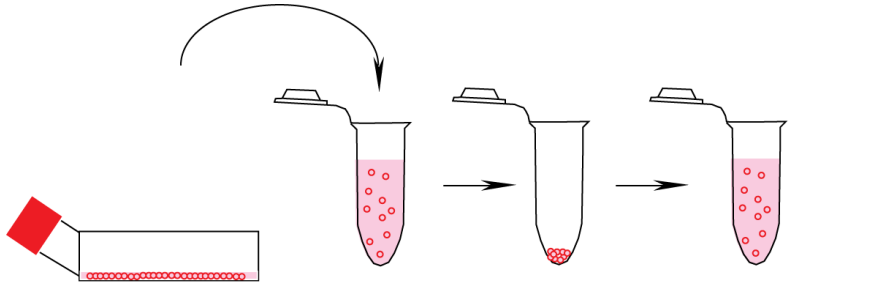

Figure 1. Harvesting and resuspending cells for 3D culture.

B. Standard Curve

The standard curve is necessary to translate OD 450/490 nm to number of cells. A separate standard curve should be run for each cell type, and conditions should be performed in triplicate.

1. Determine maximum range of standard curve (eg. 100,000 cells), and develop conditions for standard curve (eg. 100,000 cells, 50,000 cells, 25,000 cells, 10,000 cells, 5,000 cells, 2,000 cells, 1,000 cells, 500 cells, and 0 cells).
2. Determine the total number of cells needed for standard curve:
   = cells/well x wells/condition = cells/condition
   sum(cells/condition) = total number of cells needed
3. Calculate the volume of harvested cells needed:
   = Total number of cells needed/1 x 10^6 cells/ml
4. Transfer volume of harvested cells needed to a 15 ml conical tube, and centrifuge at 200 *x g* for 10 minutes to pellet cells.
5. Remove supernatant, and resuspend cells in culture medium at 1 x 10^6 cells/ml.

6. Dilute cells to highest condition for a final volume of 200 µl (eg. 100,000 cells/ 200 µl = 5.0 x 10^5 cells/ml) with cell culture medium. Add 200 µl/well, and serially dilute remaining stock with cell culture medium to generate the desired number of cells per well (in 200 µl of cell culture medium). Repeat dilutions until all conditions have been satisfied. Omit cells from at least three wells to calculate background.
7. Add 25 µL of Cell Proliferation Reagent to each well, and incubate at 37°C, 5% CO_2.
8. Read absorbance of plate at 450 nm or 490 nm at incubation periods of 1, 2, 3, and four hours (see Table 1 for sample data).
9. Average values for each condition; then subtract background from each value (see Table 2).
10. Plot standard curve of absorbance vs. number of cells (see Figure 2).
11. Insert a linear trendline (best fit) with y intercept at zero, Determine optimal incubation period based on minimum standard deviations and maximum R^2 values.
12. Use the line equation for each cell line to calculate number of cells in each well.

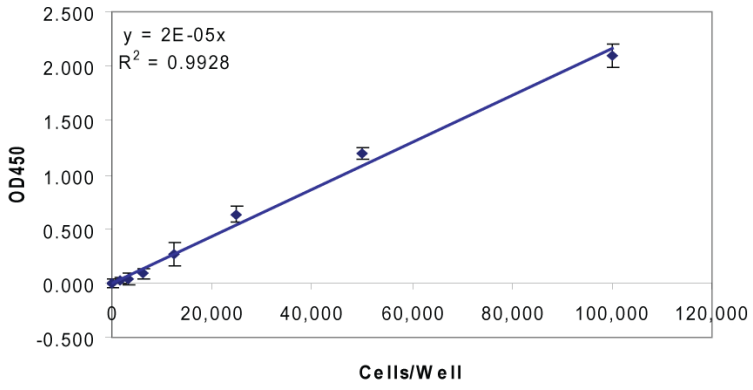

Figure 2. Standard curve for MDA-MB-231 cells. MDA-MB-231 cells were serially diluted and seeded in uncoated stripwells as indicated on the graph, and 15 µl of 3D Culture Cell Proliferation Reagent was added to each well. Absorbance at 450 nm was recorded after 2 hours.

Sample data for standard curve:

Table 1. Raw data for MDA-MB-231 standard curve.

Cells/Well	OD 450 nm			
100,000	2.211	2.362	2.458	2.432
50,000	1.457	1.520	1.477	1.397
25,000	1.006	0.833	0.873	0.890
12,500	0.581	0.583	0.375	0.593
6,250	0.414	0.367	0.319	0.319
3,125	0.382	0.309	0.297	0.243
1,563	0.337	0.291	0.281	0.260
0	0.316	0.276	0.216	0.265

Table 2. Values corrected for background.

Cells/Well	OD 450 nm (corrected)			
100,000	1.942	2.093	2.190	2.164
50,000	1.188	1.252	1.208	1.129
25,000	0.737	0.565	0.604	0.621
12,500	0.313	0.315	0.107	0.325
6,250	0.146	0.098	0.050	0.051
3,125	0.113	0.041	0.029	-0.026
1,563	0.068	0.022	0.013	-0.009
0	0.048	0.008	-0.052	-0.003

Table 3. Summary of averages for corrected values for MDA-MB-231 standard curve.

Cells/Well	Average	Std Dev
100,000	2.097	0.111
50,000	1.194	0.051
25,000	0.632	0.074
12,500	0.265	0.105
6,250	0.086	0.046
3,125	0.039	0.057
1,563	0.024	0.032
0	0.000	0.041

C. Cell Proliferation Assay

Prior to Day 1

1. Determine optimal seeding density for each cell line used. In general, 5,000 cells per well (25,000 cells/cm^2) in 100 µl cell culture medium is a good starting point.
2. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no greater than 80% confluence.

Day 1

Coat Stripwells

3. Prepare Collagen I on ice (for 8 wells = 1 stripwell); add components in order:
    a. Add 275 µl of cold cell culture medium.
    b. Add 5 µl 350 mM NaOH, and pipet to mix.
    c. Add 70 µl Collagen I, and pipet to mix.
4. Working on ice, coat each well of the 96 well plate with 35 µl of 3D Culture Collagen I. Centrifuging plates at 200 x *g* at 4°C for 10 minutes in a swinging bucket rotor will eliminate any bubbles resulting from pipetting and disperse the coating evenly across the bottom of the wells.
5. Transfer plate to a CO_2 incubator set at 37˚C for one hour to promote gel formation.

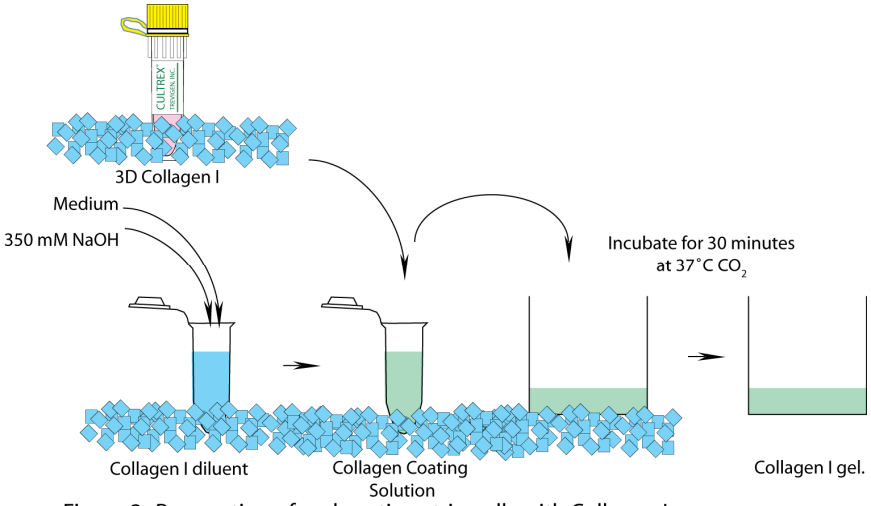

Figure 3. Preparation of and coating stripwells with Collagen I.

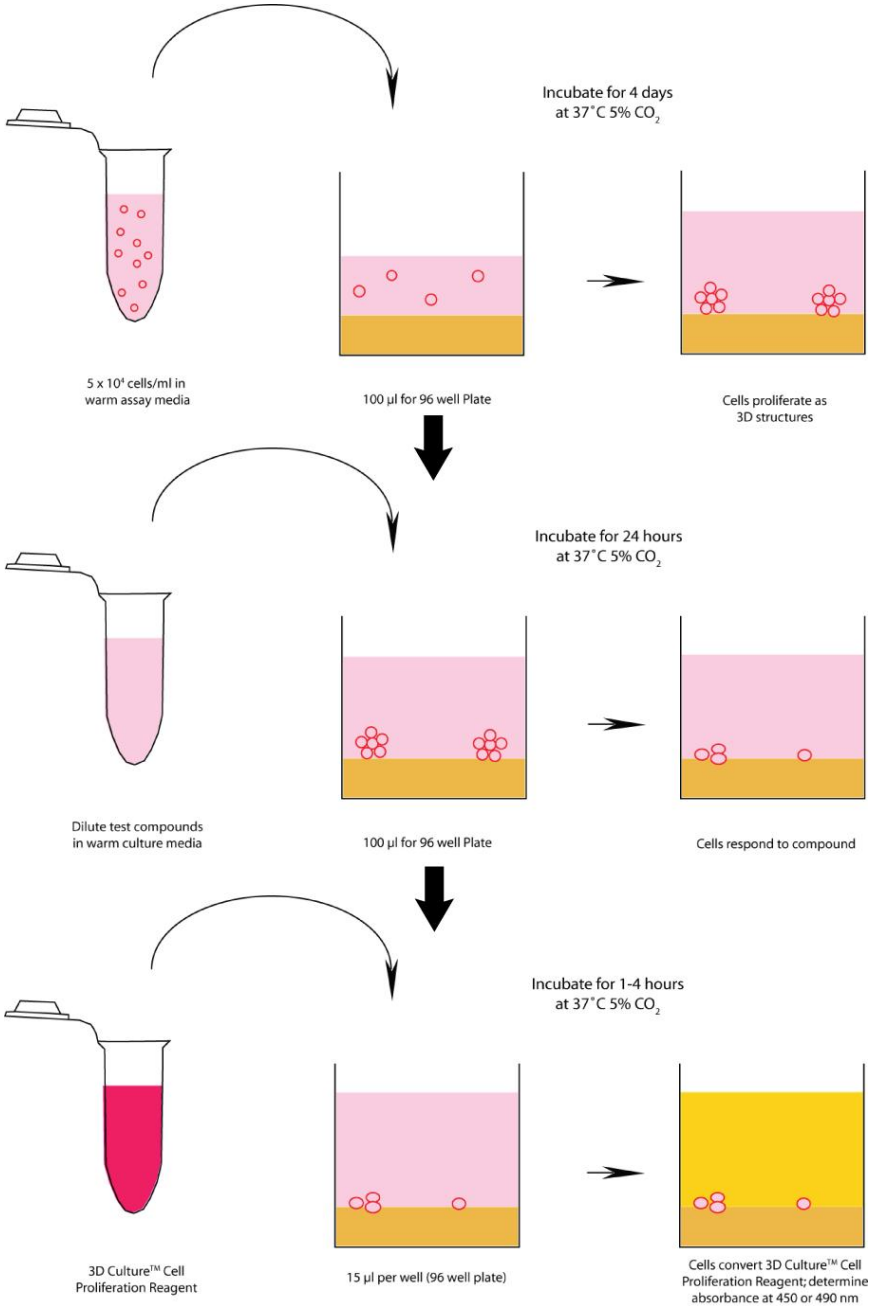

Figure 4. Illustration of protocol for assessment of toxicity in 3D culture.

6. Prepare seeding media on ice (for 8 wells = 1 stripwell):
    a. Add 1 ml of cold cell culture medium.
    b. Add 20 µl of 1 mg/ml Collagen I (from step 3), and pipet to mix.
    c. Warm to 37°C for step 7.
7. After one hour, harvest and count cells, as directed in section VI, A.
8. Centrifuge cells at 200 x g for 3 min, remove supernatant, and wash with ice cold PBS; then dilute cells to the desired concentration in cell seeding medium from step 6 warmed to 37°C.
9. Add 100 µl of cells to each stripwell.
10. Assay remaining cells for standard curve (section VI, B); each cell type will require a separate standard curve.
11. Continue to either Toxicity Assay or *in vitro* Tumorgenicity Assay.

3D Culture Toxicity Assay:

This assay tests the ability of compounds to induce cell death after the establishment of 3D structures.

1. Culture cells at 37˚C in CO_2 incubator for 48 - 96 hours to promote the formation of 3D structures (Figure 4).
2. When structures have reached desired size/morphology, dilute test compound(s) in cell culture medium.
3. Add 100 µl of media containing test compound to each well, and incubate at 37˚C in CO_2 incubator for 24 hours.
4. Once incubation is complete, add 15 µl of 3D Culture™ Cell Proliferation Reagent per well.
5. Assess absorbance at 450 or 490 nm between 1-4 hours. Optimal incubation times should be determined when performing the standard curve, based on linearity of curve (lowest R^2) accompanied by the lowest standard deviations.

***In Vitro* Tumorgenicity/Toxicity Assay:**

In the body, metastasizing cells can enter the circulation and emerge as single cells that can establish tumors. This assay tests for the inhibition of proliferation and formation of 3D structures, starting from single cells.

1. Dilute test compounds as desired in warm (37°C) cell culture medium, and add 100 µl per well.
2. Incubate at 37˚C in CO_2 incubator for 48 - 96 hours.
3. Once incubation is complete, add 15 µl of 3D Culture™ Cell Proliferation Reagent per well.
4. Assess absorbance at 450 or 490 nm between 1-4 hours. Optimal incubation times should be determined when performing the standard curve, based on linearity of curve (lowest R^2) accompanied by the lowest standard deviations.

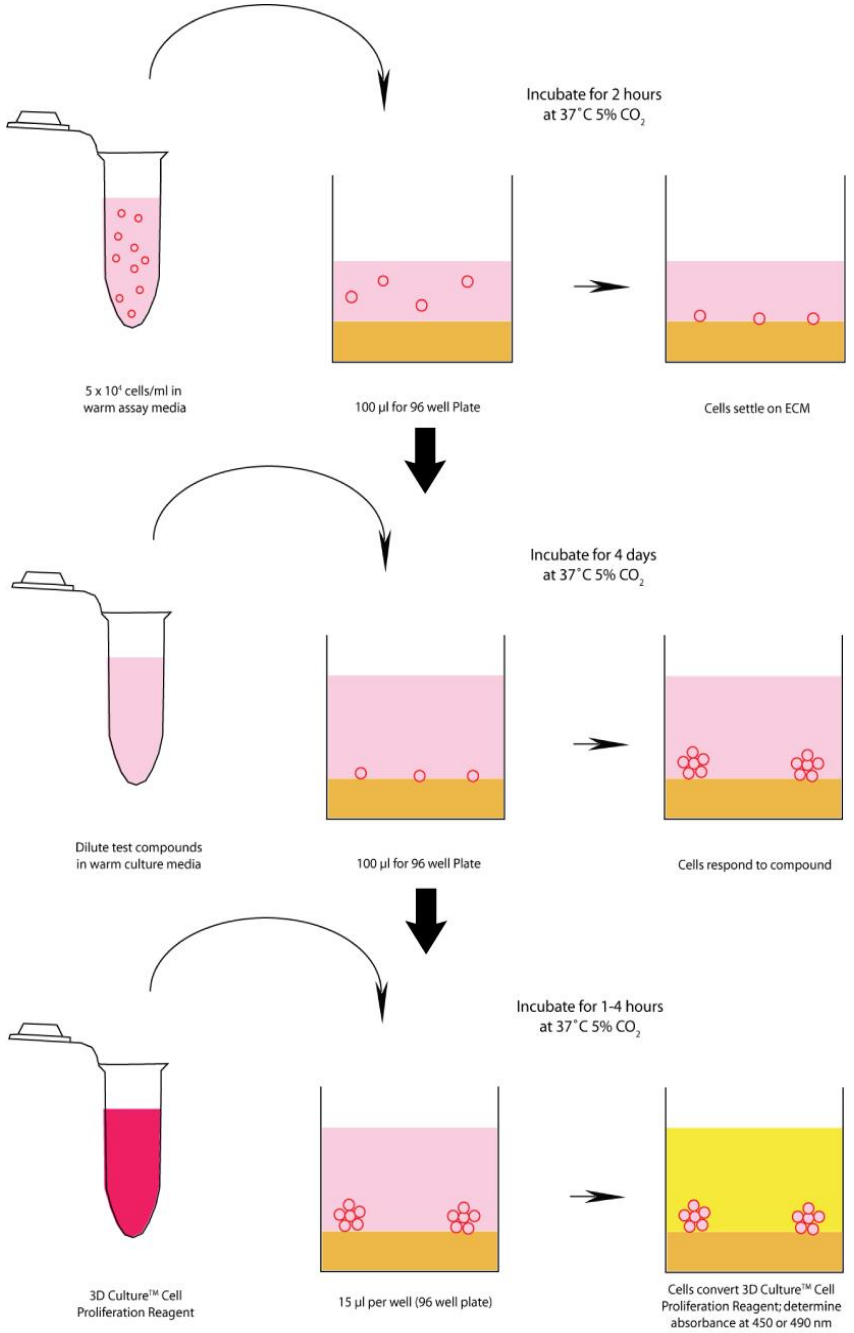

Figure 5. Illustration of protocol for assessment of tumorigenicity in 3D culture.

VII. Data Interpretation

1. After plotting a standard curve (section VI, B) with Y intercept at zero, insert trendline, equation, and R-square value (coefficient of determination), as demonstrated in Figure 2.
2. For assay samples, first average all wells for each condition, in the same fashion as was done for the standard curve, Table 1.
3. Next, subtract background from averages, similar to Table 2.
4. Use the trendline equation from the standard curve to determine the number of cells present in each well; for the equation, y = mx + b, replace Y value with OD 450 or 490 nm, and solve for X. See an example of a trendline and equation in Figure 2.
5. For each test sample, the number of cells may be compared to the number of cells present in the untreated control.
6. Figure 6 demonstrates that there is no significant effect of 3D hydrogels on readout for this assay.
7. Figure 7 demonstrates the practical application of this assay in determining ECM-modulated resistance of an apoptosis inducing reagent, etoposide.

Sample Data:

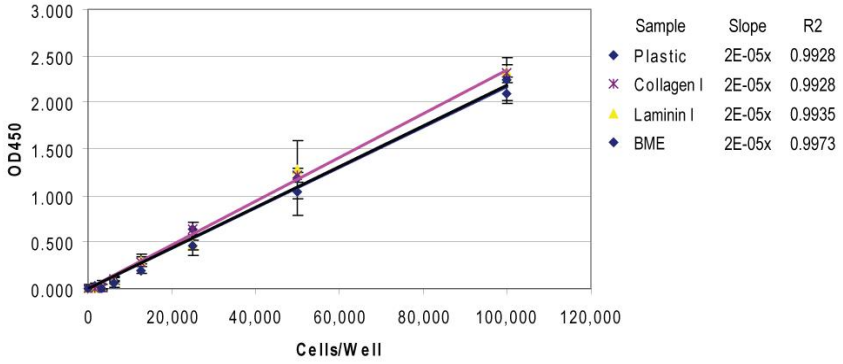

Figure 6. The presence of 3D hydrogels has no significant effect on readout. Stripwells were coated with 35 µl of either Collagen I, Laminin I, or BME, and allowed to polymerize at 37°C. MDA-MB-231 cells were serially diluted and seeded in both coated and uncoated stripwells as indicated on the graph, and 15 µl of 3D Culture Cell Proliferation Reagent was added to each well. Absorbance at 450 nm was recorded after 2 hours.

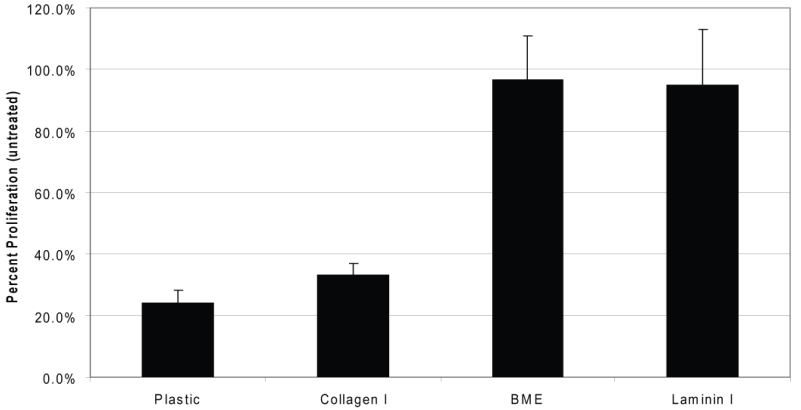

Figure 7. Proliferation of MDA-MB-231 cells in different extracellular environments in the presence of 50 µM Etoposide. 3D Culture was conducted using the tumorigenicity protocol. Briefly, cells were seeded in the presence or absence of ECM proteins and treated after two hours. Cell cultures were incubated at 37°C 5% CO_2 for 4 days. Then 15 µl of 3D Culture Cell Proliferation Reagent was added to each well, and absorbance at 450 nm was determined at 2 hours. Values were assessed as a percentage of untreated controls.

VIII. Troubleshooting

Problem	Cause	Solution
Low/No signal	Instrument not set up properly.	Read absorbance at 450 or 490 nm; adjust gain for optimal sensitivity, if applicable.
	Insufficient cell number	Increase cell number.
	Insufficient incubation period with substrate	Increase incubation period.
	Cells may have died as a result of treatment.	Test cells for viability in treatment regimen (dose response curve).
Well to well variability	Inconsistent pipetting	Calibrate pipettors, and monitor pipette tips for air bubbles.
	Poor cell dissociation	Pipette cells up and down to create single cell suspension.

Problem	Cause	Solution
High background	Instrument not set up properly.	Read absorbance at 450 or 490 nm; adjust gain for optimal sensitivity, if applicable.
	Contamination - proteases released by bacteria or mold may affect Cell Proliferation Reagent	Start a new culture from seed stocks, and re-assay. If seed stock is contaminated, then it may be prudent to get new cells.

IX. References

1. Aoudjit F, Vuori K. (2001) Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* **20**:4995-5004.
2. Hodkinson PS, Elliott T, Wong WS, Rintoul RC, Mackinnon AC, Haslett C, T Sethi. (2006) ECM overrides DNA damage-induced cell cycle arrest and apoptosis in small-cell lung cancer cells through β1 integrin-dependent activation of PI3-kinase. *Cell Death and Differentiation* 13, 1776–88.

X. Appendices

Appendix A. Reagent and Buffer Composition

1. 3D Culture™ Collagen I

Collagen I is derived from murine rat tail tendons. Collagen I is a major conmponent of connective tissue which forms fibrillar networks responsible for cellular organization. It is provided in 20 mM acetic acid. No not Freeze.

2. Cell Proliferation Reagent

Water soluble tetrazoleum substrate with electron coupler.

3. 96 Stripwell Plates

Clear, TC-treated stripwell plates.

Appendix B. Related producst available from Trevigen.

Catalog #	Description	Size
4890-025-K	TACS™ MTT Cell Proliferation Assay	2500 tests
4891-025-K	TACS™ XTT Cell Proliferation Assay	2500 tests
4895-50-K	TACS™ 2 Hoechst CPA1 Kit (Vital)	2500 tests
4896-50-K	TACS™ 2 Hoechst CPA2 Kit (Fixed)	2500 tests

Catalog #	Description	Size
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests
4817-60-K	FlowTACS™ Apoptosis Detection Kit	60 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 tests
4830-01-K	TACS™ Annexin V FITC Kit	100 samples
4835-01-K	Annexin V Biotin Kit	100 samples
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 tests
6305-100-K	MitoShift™ Mitochondrial Potential Assay Kit	100 tests