**CULTREX® Instructions**

*For Research Use Only. Not For Use In Diagnostic Procedures*

# CultreCoat® Collagen I 96 Well Cell Adhesion Assay

## Reagent kit for investigating cell adhesion

## 96 samples

## Catalog #: 3492-096-K

# CultreCoat® Collagen I 96 Well Cell Adhesion Assay

## Catalog # 3492-096-K

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E12/8/08v1

# I. Quick Reference Procedure for Trevigen's CultreCoat® Collagen I 96 Well Cell Adhesion Assay:

Read through the complete Instructions for Use prior to using this kit. Determine the optimal seeding density for each cell line used. In general, 15,000 cells per well in 100 µl is a good starting point.

1. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 80% confluence.
2. Warm reagents to room temperature.
3. Prepare Rehydration Buffer, and warm to 37°C.
4. Rehydrate Collagen I 96 Well CA Plate with 100 µl per well of warm Rehydration Buffer for two hours.
5. Label cells with 2 µM Calcein AM for one hour.
6. Harvest cells, and wash with PBS.
7. Suspend cells in Adhesion Buffer, and dilute to optimal seeding concentration.
8. Aspirate Rehydration Buffer from Collagen I 96 Well CA Plates, and seed 100 µl of cells per well.
9. Incubate for 1 hour and 15 minutes at 37°C 5%$CO_2$.
10. Remove Collagen I 96 Well CA Plate from incubator, and read fluorescence at 485 nm excitation/520 nm emission. The fluorescence intensity is the total Relative Fluorescence Units (RFU) per well.
11. Aspirate Collagen I 96 Well CA Plate, and wash 3 times with Wash Buffer.
12. Add 100 µl of Adhesion Buffer per well.
13. Read fluorescence at 485 nm excitation/520 nm emission. The fluorescence intensity is the RFU per well after Wash.
14. Calculate percent cell adhesion, standard deviation, and non-specific binding.



# II. Background

The extracellular matrix (ECM) provides a structural and signaling framework for tissue morphogenesis, homeostasis, and wound healing by regulating cellular activities. These activities are modulated through interactions between specific transmembrane cell surface receptors, which are most commonly integrins, and their coordinate ECM ligands, resulting in cellular adhesion and signal transduction. During this process, the extracellular domain of the integrin binds to the ECM, and the intracellular domain binds to the cytoskeleton, forming a bridge across the plasma membrane. Concurrently, this interaction controls a wide variety of cellular processes, including cell cycle progression [1, 2], differentiation [3, 4], cell migration & invasion [5, 6], and apoptosis [7, 8].

Trevigen's cell adhesion assays provide a simple, standardized, high throughput format for assessing factors that influence cell-matrix interactions. During the assay, cells absorb Calcein AM and convert it to Calcein, providing a fluorescent label for each cell. The stripwells are rehydrated, and the cells are then seeded in the coated stripwells and allowed to adhere to the ECM proteins. The total fluorescence for each stripwell is assessed, providing a loading control for each group, and then the stripwells are washed 3 times to remove non-adherent cells. The final fluorescence for each well is assessed, and the percent cell adhesion is calculated.

This assay provides many advantages over existing kits. The black stripwell format minimizes background, providing greater sensitivity, and it affords flexibility for the number of samples assessed. Multiple experiments can be conducted simultaneously using the same kit. Also, Calcein labeling allows direct comparisons between the number of cells that are loaded and the number that adhere, providing a loading control, unlike dyes that only detect the total number of cells that adhere, such as Cyquant®. In addition, controls are provided for determining background and non-specific binding. The **CultreCoat® Cell Adhesion Assays** have been adapted to multiple formats so that cell adhesion may be evaluated against different extracellular matrices; the assay is available in the following formats:

- Adhesion Protein Array
- Basement Membrane Extract (BME)
- Laminin I
- Collagen IV
- Fibronectin
- Vitronectin
- Collagen I

# 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. **CultreCoat® Cell Adhesion 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# |
|---|---|---|---|
| Collagen I 96 Well CA Plate | one | 4˚C | 3492-096-P |
| 2X CA Assay Buffer | 100 ml | Room Temp | 3490-096-01 |
| 10X CA Blocking Agent | 3 ml | 4˚C | 3490-096-02 |
| Calcein AM | 50 µg | -20˚C | 4892-010-01 |

## 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. 4˚C storage
7. standard light microscope (or inverted)
8. pipette aid
9. timer
10. 96-well fluorescence plate reader (485 nm excitation/520 nm emission)
11. 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
6. DMSO (Cell Culture Grade or above)

### 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. 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 the assay, and plan accordingly.
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 the bottom of the flask. Non-enzymatic buffers are recommended if cells are compatible; if not, monitor and minimize enzymatic reactions to reduce loss of cell surface receptors.
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 3 minutes to pellet cells, remove medium, and resuspend cells in 2 ml of Adhesion Buffer (Step VI.C.6). Cells may need to be gently pipetted up and down with serological pipette to resuspend cells.
7. Count cells, and dilute to optimal seeding concentration in Adhesion Buffer.

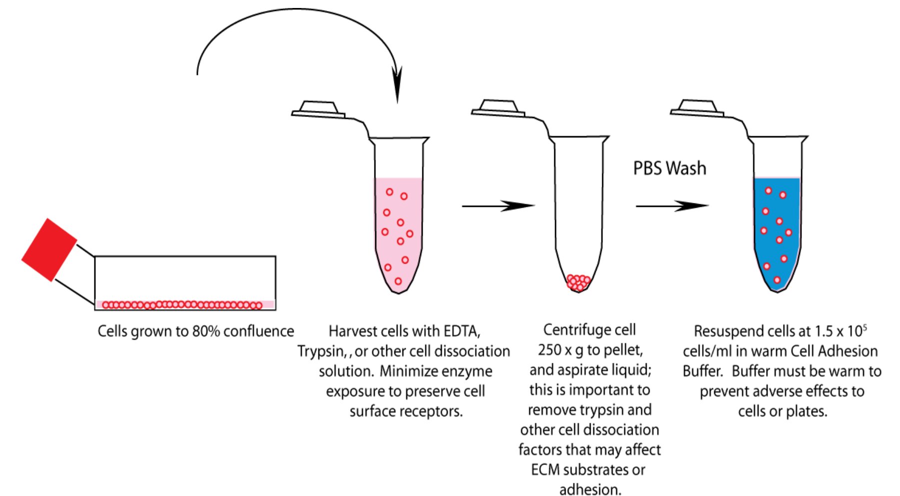


Figure 1. Harvesting and resuspending cells for CultreCoat® Cell Adhesion Assays.

## B. Prior to Starting the Assay

1. Determine optimal seeding density for each cell line used. In general, 15,000 cells per well 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.

## C. Cell Adhesion Assay

1. Prepare Rehydration Buffer:

| Component | One Strip = 8 wells | One Plate = 96 wells |
|---|---|---|
| 2X CA Assay Buffer | 500 µl | 6 ml |
| 10X CA Blocking Agent | 100 µl | 1.2 ml |
| diH$_2$O | 400 µl | 4.8 ml |
| Total | 1 ml | 12 ml |

2. Invert to mix, and warm to 37˚C.
3. Dispense 100 µl of Rehydration Buffer into the required number of stripwells from the Collagen I 96 Well CA Plate, and incubate for one hour at 37˚C in a $CO_2$ incubator (total rehydration time will be 2 hours). This step provides for protein rehydration and blocks non-specific binding, so buffer is needed in every well. Store unused stripwells desiccated at 4°C.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| B | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| C | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| D | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| E | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| F | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| G | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 | COL1 |
| H | None | None | None | None | None | None | None | None | None | None | None | None |

Figure 2. Pre-Coating for Collagen I 96 Well (COL1) CA Plate.



4. Prepare 2 mM Calcein AM by adding 25 µl of DMSO to the 50 µg lyophilized Calcein AM. Store unused Calcein AM at -20˚C.
5. After one hour, add Calcein AM to cells at a final concentration of 2 µM (1:1000), and incubate for one hour at 37˚C in a $CO_2$ incubator.
6. Prepare Adhesion Buffer:

| Component | One Strip = 8 wells | One Plate = 96 wells |
|---|---|---|
| 2X CA Assay Buffer | 1500 µl | 18 ml |
| 10X CA Blocking Agent | 100 µl | 1.2 ml |
| diH$_2$O | 1400 µl | 16.8 ml |
| Total | 3 ml | 36 ml |

7. Invert tube to mix, and warm to 37˚C. Save one third volume of this solution for step 20.
8. After one hour, harvest and count labeled cells, as directed in section VI, A.
9. Centrifuge cells at 200 x g for 3 min, remove supernatant, and wash with PBS.
10. Resuspend cells in Adhesion Buffer, and count cells.
11. Dilute labeled cells to the desired concentration in Adhesion Buffer. Cells may be treated with test compounds at this point.
12. Upon completion of rehydration (Step 5), aspirate Rehydration Buffer, and add 100 µl of cells to each well. Add 100 µl of Adhesion Buffer without cells to Row A to account for background fluorescence.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | No Cells - Background Control | | | | | | | | | | | |
| B–H | Calcein-Labeled Cells | | | | | | | | | | | |

Figure 3. Cell Seeding for the CultreCoat Collagen I 96 Stripwell Cell Adhesion Assay.

13. Incubate stripwells for one hour and 15 minutes at 37˚C in a $CO_2$

incubator; optimal incubation periods may vary with cell line.

14. Prepare Wash Buffer:

| Component | One Strip = 8 wells | One Plate = 96 wells |
|---|---|---|
| 2X CA Assay Buffer | 2 ml | 24 ml |
| $diH_2O$ | 2 ml | 24 ml |
| Total | 4 ml | 48 ml |

15. Invert to mix, and warm to 37˚C.
16. Place Collagen I 96 Well CA Plate in fluorescence plate reader, and assess fluorescence at 485 nm excitation/ 520 nm emission. This is the Total RFU. Record your gain setting.
17. Wash each well with 100 µl of warm Wash Buffer using a multichannel pipette, and aspirate wash. Repeat 2 times.
18. Add 100 µl of warm Adhesion Buffer to each well.
19. Place Collagen I 96 Well CA Plate in fluorescence plate reader, and assess fluorescence at 485 nm excitation/ 520 nm emission. This is RFU per well after Wash. **Use the same Gain setting as used in step 16.**
20. For each well, calculate Percent Adhesion:
    = (RFU after Wash – Background) / (Total RFU – Background)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | No Cells - Background Control | | | | | | | | | | | |
| B | Assay Samples | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | No Coating - Non-specific Adhesion Control | | | | | | | | | | | |

Figure 4. Samples and Controls for the CultreCoat Collagen I 96 Stripwell Cell Adhesion Assay.

## VII. Data Interpretation

1. For each sample, subtract background.



2. Determine Percent Adhesion for each sample by dividing RFU after Wash by Total RFU.
3. Average Percent Adhesion for each sample and determine standard deviations. Also determine non-specific binding.
4. Graph data.

Sample Data:

**Raw Data (RFU):**

| Row | Total RFU | RFU After Wash | Coating |
|---|---|---|---|
| A | 584 | 565 | Background |
| B | 47201 | 38300 | Collagen I |
| C | 49751 | 40809 | |
| D | 49360 | 41268 | |
| E | 47756 | 39799 | |
| F | 46362 | 35959 | |
| G | 48131 | 40010 | |
| H | 41820 | 1902 | None |

**Subtract Background from Each Well (RFU):**

| Row | Total RFU | RFU After Wash | Coating |
|---|---|---|---|
| B | 46617 | 37735 | Samples |
| C | 49167 | 40244 | |
| D | 48776 | 40703 | |
| E | 47172 | 39234 | |
| F | 45778 | 35394 | |
| G | 47547 | 39445 | |
| H | 41236 | 1337 | None |

**Calculate Cell Adhesion for Each Well (%)**

| Row | Percent Adhesion | Coating |
|---|---|---|
| B | 81% | Samples |
| C | 82% | |
| D | 83% | |
| E | 83% | |
| F | 77% | |
| G | 83% | |
| H | 3% | None |

**Summarize Data**

| | |
|---|---|
| **Average Percent Adhesion** | 82% |
| **Standard Deviation** | 2.3% |
| **Non-Specific Binding** | 3% |

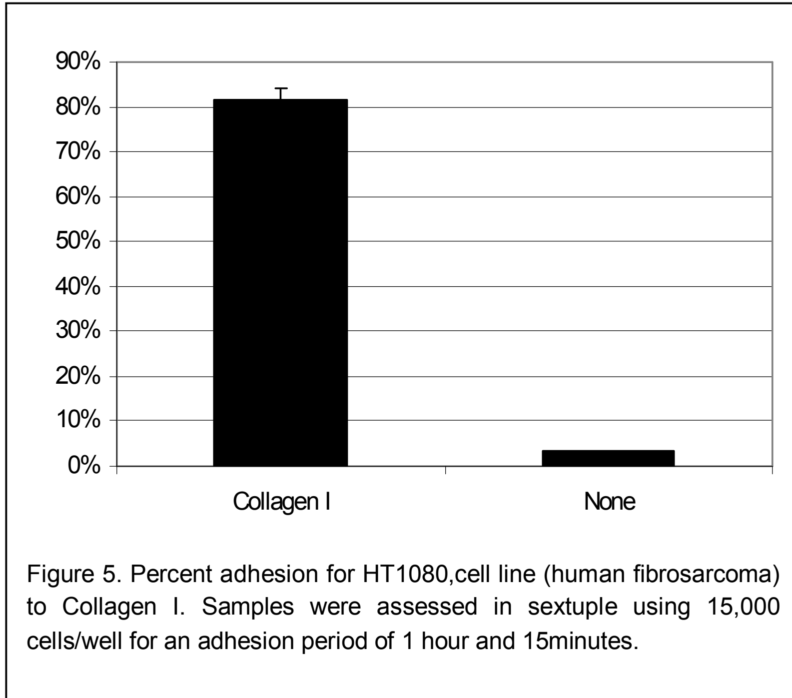


Figure 5. Percent adhesion for HT1080,cell line (human fibrosarcoma) to Collagen I. Samples were assessed in sextuple using 15,000 cells/well for an adhesion period of 1 hour and 15minutes.



## VIII. Troubleshooting

| Problem | Cause | Solution |
|---|---|---|
| Low/no signal | Instrument not set up properly. | Read fluorescence at 495 nm excitation/ 520 nm emission; adjust gain for optimal sensitivity, if applicable. |
| | Insufficient cell number. | Determine optimal cell number needed for detection using Calcein AM. Smaller or less metabolically active cells may require higher seeding concentrations. |
| | Poor cell viability; Calcein AM is a metabolic substrate. | Do not perform assay with viability below 90%. |
| High background | Instrument not set up properly. | Read fluorescence at 495 nm excitation/ 520 nm emission; adjust gain for optimal sensitivity, if applicable. |
| | Contamination - proteases released by bacteria or mold may affect Calcein AM | Start a new culture from seed stocks, and re-assay. If seed stock is contaminated, then it may be prudent to get new cells. |
| Lower than expected/ published percent adhesion | The cell seeding concentration is too high; cells exceed binding capacity of the ECM. | Decrease/ optimize seeding concentration. |
| | Poor cell health or over-trypsinization. | Examine cells for viability at the onset of assay, and minimize exposure to trypsin. |
| | The incubation period is suboptimal. | Assess multiple incubation periods to determine optimal parameters. |
| Well to well variability | Inconsistent pipetting | Calibrate pipettors, and monitor pipet tips for air bubbles. |

## IX. References


1. Dike, L.E. and D.E. Ingber, *Integrin-dependent induction of early growth response genes in capillary endothelial cells.* J Cell Sci, 1996. 109 (Pt 12): p. 2855-63.
2. Zhu, X., et al., *Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein.* J Cell Biol, 1996. 133(2): p. 391-403.
3. Takeuchi, Y., et al., *Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells.* J Biol Chem, 1997. 272(46): p. 29309-16.
4. Xiao, G., et al., *Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells.* J Bone Miner Res, 2002. 17(1): p. 101-10.
5. Cary, L.A., J.F. Chang, and J.L. Guan, *Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn.* J Cell Sci, 1996. 109(7): p. 1787-1794.
6. Hu, X.-W., D. Meng, and J. Fang, *Apigenin Inhibited Migration and Invasion of Human Ovarian Cancer A2780 Cells through Focal Adhesion Kinase.* Carcinogenesis, 2008: p. bgn244.
7. Boudreau, N., et al., *Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix.* Science, 1995. 267(5199): p. 891-3.
8. Haraguchi, M., et al., *Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins.* J. Biol. Chem., 2008: p. M801125200.


## X. Appendix: Reagent and Buffer Composition

### 1. Collagen I 96 Well CA Plate

Collagen I is derived from Rat Tails. The Collagen I is coated to rows A-G of a black, 96 stripwell plate, and row H is left uncoated as a control for non-specific binding. See Figure 2 for plate layout.

### 2. 2X CA Assay Buffer

Physiological buffer solution.

### 3. 10X CA Blocking Agent

Blocking agent that prevents non-specific interactions between ECM proteins and cells.

### 4. Calcein AM

A non-fluorescent, hydrophobic compound that easily permeates intact, live cells. Once in the cell, Calcein AM is hydrolyzed by intracellular esterases to produce Calcein, a hydrophilic, strongly fluorescent compound that is retained in the cell cytoplasm.



## XI. Related products available from Trevigen.

| Catalog# | Description | Size |
|---|---|---|
| 3455-024-K | Cultrex® 24 Well BME Cell Invasion Assay | 24 inserts |
| 3480-024-K | CultreCoat® 24 Well BME-Coated Cell Invasion Assay | 24 inserts |
| 3465-024-K | Cultrex® 24 Well Cell Migration Assay | 24 inserts |
| 3455-096-K | Cultrex® BME Cell Invasion Assay | 96 samples |
| 3456-096-K | Cultrex® Laminin I Cell Invasion Assay | 96 samples |
| 3457-096-K | Cultrex® Collagen I Cell Invasion Assay | 96 samples |
| 3458-096-K | Cultrex® Collagen IV Cell Invasion Assay | 96 samples |
| 3465-096-K | Cultrex® 96 Well Cell Migration Assay | 96 samples |
| 3471-096-K | Cultrex® In Vitro Angiogenesis Assay Endothelial Cell Invasion | 96 samples |

### Accessories:

| Catalog# | Description | Size |
|---|---|---|
| 3400-010-01 | Cultrex® Mouse Laminin I | 1 mg |
| 3400-010-02 | PathClear® Mouse Laminin I | 1 mg |
| 3440-100-01 | Cultrex® Rat Collagen I | 100 mg |
| 3442-050-01 | Cultrex® Bovine Collagen I | 50 mg |
| 3410-010-01 | Cultrex® Mouse Collagen IV | 1 mg |
| 3430-005-02 | Cultrex® BME with Phenol Red, PathClear® | 5 ml |
| 3431-005-02 | Cultrex® BME with Phenol Red, Growth Factor Reduced, PathClear® | 5 ml |
| 3432-005-02 | Cultrex® BME, No Phenol Red, PathClear® | 5 ml |
| 3433-005-02 | Cultrex® BME, No Phenol Red, Growth Factor Reduced, PathClear® | 5 ml |
| 3430-005-01 | Cultrex® BME with Phenol Red | 5 ml |
| 3432-005-01 | Cultrex® BME; no Phenol Red | 5 ml |
| 3431-005-01 | Cultrex® BME with Phenol Red; Reduced Growth Factors | 5 ml |
| 3433-005-01 | Cultrex® BME; no Phenol Red; Reduced Growth Factors | 5 ml |
| 3416-001-01 | Cultrex® Bovine Fibronectin NZHD | 1 mg |
| 3417-001-01 | Cultrex® Bovine Vitronectin NZHD | 50 μg |
| 3438-100-01 | Cultrex® Poly-L-Lysine | 100 ml |
| 3443-050-03 | Cultrex® Murine VEGF | 1 μg |
| 3443-050-02 | Cultrex® Human FGF-2 | 5 μg |
| 3443-050-01 | Cultrex® Human EGF | 50 μg |
| 3443-050-04 | Cultrex® Human β-NGF | 2 μg |
| 3437-100-K | Cultrex® Cell Staining Kit | 100 ml |
| 3439-100-01 | Cultrex® Cell Recovery Solution | 100 ml |
| 3450-048-05 | CellSperse™ | 15 ml |

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for research use only and is not intended for
diagnostic purposes or for use in humans.

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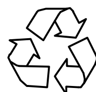

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