DATA SHEET

CultreCoat® Adhesion Protein Array Kit

Reagent kit for investigating cell adhesion

96 samples

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Catalog #: 3496-096-K

Table of Contents

I. Quick Reference Procedure for Trevigen's CultreCoat® Adhesion Protein Array Kit:

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 Adhesion Protein Array Plate with 100 µl per well of warm Rehydration Buffer for two hours.
5. Meanwhile, 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. For most epithelial cells, this is approximately 15,000 cells/well.
8. Aspirate Rehydration Buffer from Adhesion Protein Array 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 Adhesion Protein Array Plate from incubator, and read fluorescence at 485 nm excitation/520 nm emission. The fluorescence intensity is the total RFU per well.
11. Aspirate Adhesion Protein Array 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. **CultreCoat® Cell Adhesion Assay kits** have been adapted to multiple formats so that cell adhesion may be evaluated against different extracellular matrices; the assay is available in the following 96 well 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#
Adhesion Protein Array Plate	one	4˚C	3496-096-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. Each well requires approximately 15,000 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 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. For most epithelial cells, this is around 150,000 cells/ml = 15,000 cells/well.

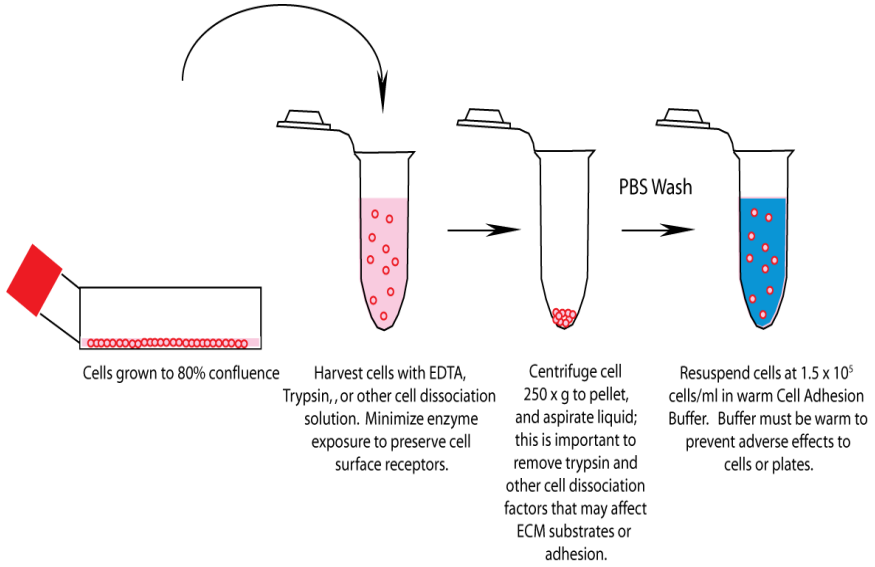

Figure 1. Harvesting and resuspending cells for CultreCoat® Cell Adhesion Assays.

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_2O	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 Adhesion Protein Array Plate, and incubate for one hour at 37˚C in a CO_2 incubator. 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	Col4	Col4	Col4	Col4	Col4	Col4	Col4	Col4	Col4	Col4	Col4	Col4
B	LN1	LN1	LN1	LN1	LN1	LN1	LN1	LN1	LN1	LN1	LN1	LN1
C	BME	BME	BME	BME	BME	BME	BME	BME	BME	BME	BME	BME
D	Col1	Col1	Col1	Col1	Col1	Col1	Col1	Col1	Col1	Col1	Col1	Col1
E	FN	FN	FN	FN	FN	FN	FN	FN	FN	FN	FN	FN
F	VN	VN	VN	VN	VN	VN	VN	VN	VN	VN	VN	VN
G	None	None	None	None	None	None	None	None	None	None	None	None
H	None	None	None	None	None	None	None	None	None	None	None	None

Figure 2. Pre-Coating for BME 96 Well CA Plate.

4. Prepare 2 mM Calcein AM by adding 25 µl of DMSO to the 50 µg tube of 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_2O	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 Controls		Calcein-Labeled Cells									
B												
C												
D												
E												
F												
G												
H												

Figure 3. Cell Seeing for the CultreCoat Adhesion Protein Array 96 Well 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 Adhesion Protein Array 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 Adhesion Protein Array 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 Controls		Assay Samples									
B												
C												
D												
E												
F												
G			No Coating - Non-specific Adhesion Controls for Assay Samples									
H												

Figure 4. Samples and Controls for the CultreCoat Adhesion Protein Array 96 Well 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.

Sample Data:

Raw Data (RFU):

	Total RFU			RFU After Wash			
Row	Strip 1	Strip 2	background	Strip 1	Strip 2	background	Coating
A	34266	32643	669	19876	21145	635	Collagen IV
B	32252	32026	576	17497	17862	756	Laminin I
C	30725	31578	577	18695	20136	594	BME
D	51100	49206	499	43509	40617	735	Collagen I
E	48201	50707	653	19211	26236	549	Fibronectin
F	44804	44659	502	36463	36341	553	Vitronectin
G	38102	37562	584	605	853	579	None
H	43854	41444	422	456	962	565	

Subtract Background from Each Well (RFU):

	Total RFU		RFU After Wash		
Row	Strip 1	Strip 2	Strip 1	Strip 2	Coating
A	33597	31974	19241	20510	Collagen IV
B	31676	31450	16741	17106	Laminin I
C	30148	31001	18101	19542	BME
D	50601	48707	42774	39882	Collagen I
E	47548	50054	18662	25687	Fibronectin
F	44302	44157	35910	35788	Vitronectin
G	37518	36978	26	274	None
H	43432	41022	-109	397	

Calculate Cell Adhesion for Each Well (%)

Row	Strip 1	Strip 2	Coating
A	57%	64%	Collagen IV
B	53%	54%	Laminin I
C	60%	63%	BME
D	85%	82%	Collagen I
E	39%	51%	Fibronectin
F	81%	81%	Vitronectin
G	0%	1%	None
H	0%	1%	

Summarize Data

	Percent Adhesion	
Coating	Average	Std Dev
Collagen IV	61%	5%
Laminin I	54%	1%
BME	62%	2%
Collagen I	83%	2%
Fibronectin	45%	9%
Vitronectin	81%	0%
None	0%	1%

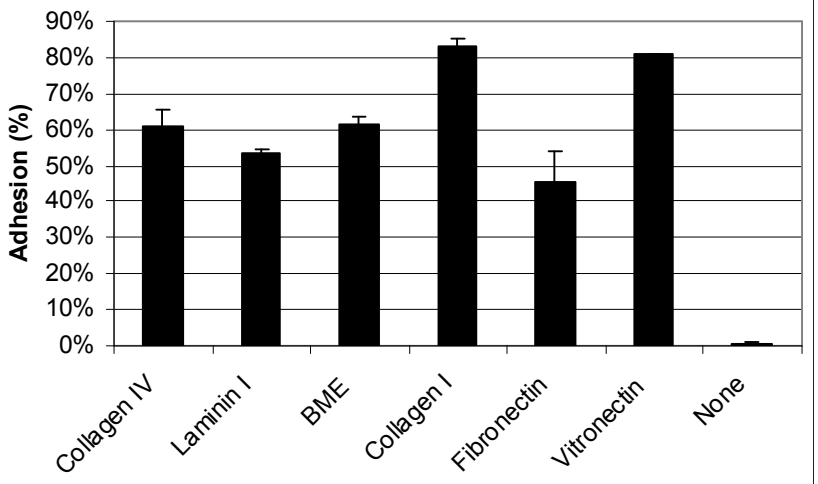

Figure 5. Percent adhesion for MG63, human osteosarcoma, to Collagen IV, Laminin I, and BME, and HT1080, human fibrosarcoma, to Collagen I, Fibronectin, and Vitronectin. Samples were assessed in duplicate using 15,000 cells/well for an adhesion period of 1 hour and 15 minutes.

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

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X. Appendix: Reagent and Buffer Composition

1. Adhesion Protein Array Plate

Basement membrane extract, Laminin I, and Collagen IV are derived from murine EHS sarcoma cells. Collagen I is isolated from rat tails. Fibronectin and Vitronectin originate from bovine blood. Each protein is coated to a different row of a black, 96 stripwell plate, and rows G and H are 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
3415-005-02	Cultrex® Human BME, PathClear®	1 mg
3400-010-01	Cultrex® 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
3420-001-01	Cultrex® Human Fibronectin, PathClear®	1 mg
3416-001-01	Cultrex® Bovine Fibronectin NZHD*	1 mg
3421-001-01	Cultrex® Human Vitronectin, PathClear®	50 µg
3417-001-01	Cultrex® Bovine Vitronectin NZHD	50 µg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3439-100-01	Cultrex® Poly-D-Lysine	100 ml
3439-100-01	Cultrex® Cell Recovery Solution	100 ml
3450-048-05	CellSperse™	15 ml

*NZHD = New Zealand Herd Derived

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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