Appendix B. Detailed experimental protocols

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HUVEC maintenance and non-enzymatic passaging

Daily/Weekly Maintenance

Check liquid nitrogen level in cryo-container.
Check the water light on the incubator. If illuminated, add distilled, de-ionized water to the opening at the top and back of the incubator until the light goes out.
Check the temperature of the incubator door. (If there is condensation on the clear door of the incubator, then the temperature is either too high or too low.)
Check the level of CO_2 in the tank.
Check the level of water in the tray in the incubator. This tray should be cleaned out weekly and disinfected.
Make sure counters are clean and dust-free.

Monthly/Quarterly Maintenance

Scrub shelves and water trays with 10% Rocal-D, rinse with water. Autoclave for 25 minutes. Replace shelves and trays. Allow the incubator to equilibrate overnight.

Changing Media

(For all of these protocols, "ethanol" refers to a mixture of 70% ethanol and 30% water.)
Making fresh media:
Turn on the flow-hood for 5 minutes. Turn on the water bath and warm up the basal media and the "bullets" to 37°C. Be sure to match up the appropriate "bullet kit" with the right basal media (e.g., EBM-2 with EGM-2 and FBM with FGM). For Cell Applications medium, the “bullet kits” are already added.

Put on gloves and spray the outsides with ethanol. Wipe down the inside of the flow-hood with ethanol. Dry off the bottles, wipe down with ethanol, and place them in the flow-hood. Place the "bullets" into the tube rack to keep them from falling over. Ethanol the entire rack and place in the flow-hood. Open the "bullets". Use a pipet to add the "bullets" to the media. Add the serum to the media if desired. Close the bottle and mix well.

Aspirating:
Using a new pipet, aspirate the used medium from the dish and discard into a beaker.
Change pipets and add 10 mL fresh medium to the dish.

Non-Enzymatic Cell Passaging

Preparation:
Check HUVEC using phase contrast microscopy. Cells should be 75-95% confluent.
Turn on flow-hood for 5 minutes.
Turn on water bath, warm up PBS and media to 37°C.

Rinsing:
Do the rinse steps as quickly as possible. Only rinse one or two plates at a time.
Do two rinses with 5 ml PBS, then add 5 ml cold Versene. While waiting for the cells to round up and detach, begin rinsing the next two plates, etc.

Detachment:
Place the plate on the phase contrast microscope. Watch to see the cells begin to round up and detach (5-15 minutes). Once the cells are rounded, rap the plate gently against the table to dislodge them. Put the plate back into the laminar flow-hood and swirl gently. Pipet the versene and cells into a labeled centrifuge tube. Be sure to make a counter-weight centrifuge tube with water.

Quantification:
Centrifuge the cells at 1050 rpm (200 g) for 5 minutes; a small cell pellet should be visible. Carefully aspirate off the versene. Resuspend the cells in fresh media using 2ml media/plate. (Be sure to use serum free media or PBS+ if the cells will be used in subsequent attachment assays.) Place 10 microliters of the cell suspension onto one side of the hemacytometer. Mix 10 microliters of the cell suspension with 5 microliters of trypan blue and add to the other side of the hemacytometer. Count the number of cells/square on both sides and the number of blue stained cells. Calculate the concentration (10^4 cells/ml = #cells/square) and viability. Calculate the total number of living cells in the centrifuge tube and the concentration of living cells. Determine how many new plates ($1x10^5$ cells/plate) or cryovials ($3.5x10^6$ cells/cryovial) to make.

Seeding new plates:
Add fresh media and cell suspension to new plates. Should have a total volume of 10ml and a total of $1x10^5$ cells in each plate. Label plates with cell strain, passage number, date, and initials. Transfer plates to incubator.

Making cryovials
Take cell culture grade DMSO out of the refrigerator and allow to melt. Label cryovials with cell strain, passage number, date, and initials. Re-centrifuge a known number of cells to make them more concentrated. Aspirate off media and redilute in new media to a total volume of 0.9 ml/$3.5x10^6$ cells. Add 0.1 ml/$3.5x10^6$ cells of DMSO to the cell suspension. Mix thoroughly and add 1ml of the solution to each cryovial. Wrap cryovials in glass wool and place in a beaker inside of a styrofoam box. Place the box in the -80°C freezer for at least 2 hours (but can be up to several days). Once they are frozen, quickly transfer the cryovials to the liquid nitrogen container without allowing them to melt. Wearing gloves, remove a colored cryocane from the container and snap the vial into place. Replace the cryocane and fill the container back up with liquid nitrogen. Make a record of which color cryocane was used and how many vials were added.

Thawing Cryovials

Warm up the water bath and the media to 37°C. Place 10 ml fresh media into plate. Allow plate to equilibrate in the incubator. Wearing gloves and safety glasses, remove the cryovial from the liquid nitrogen container. Immediately place the vial in the 37°C water bath. Swirl gently until the mixture is completely thawed. Wipe down the outside of the vial with ethanol. Carefully pipet the cells from the vial into the fresh plate. Place the plate in the incubator. Change media the following day.

For cells in suspension, follow the directions above to thaw, but pipet the cells into a centrifuge tube with 10 ml fresh media. Centrifuge at 800 rpm for 5 minutes. Aspirate off DMSO and resuspend in fresh media. If desired, can check viability and cell concentration before adding to a new plate. Place the plate in the incubator.

10X PBS Recipe

40 g NaCl
1 g KCl
5.75 g Na_2HPO_4-$7H_2O$
1 g KH_2PO_4
add 500 ml water, pH to 7.4, autoclave

aECM 10 L Fermentation

Day 1: Streak a plate, make 400 mL 2xYT

Day 2: Make 10 L of TB (Terrific Broth) medium
Add tryptone and yeast to 3 2L flasks
Add salt to 1 2L flask and pour into fermentor
Add glycerol to 1 2L flask
Mark tops of liquid volume on flasks to check volume after autoclaving.
Autoclave the medium along with 1 flask of extra water.

Check chiller - hook up water lines, turn on, flush lines.
Calibrate pH probe, fill DO probe with electrolyte.
Assemble fermentor: cover antifoam and acid port with foil, clamp off everything else, add 2L water plus salts.
Autoclave, liquid cycle, 35 minutes, make sure probes are immersed.
Charge the DO probe overnight.
Make antibiotics:
50 mg/ml chloramphenicol in ethanol
34 mg/ml kanamycin in water
Inoculate 400 mL 2xYT culture (be sure to add 400 µl antibiotics)
Make 1 L TEN buffer and find centrifuge tubes

TEN Recipe
5.8 g NaCl
1.21 g Tris
0.37 g EDTA
Add 1 L water, pH to 8.0

Day 3: Take OD of 400 ml flask
Fill medium into fermentor and heat to 37°C, set up agitation, pH, and foam feed-back loops.
Inoculate with entire overnight culture and add 1-2 ml antifoam. Monitor OD.
At OD 6-8, induce with 2.5 mM IPTG (5.96 g IPTG in 25 ml water)
Express protein for 2-4 hours. (RGD has better yields at 2 hr.)
Take OD samples every 30 or 60 min. (See SDS-PAGE protocol to see how to prepare these samples for gels.)
Harvest and centrifuge cells, 4°C, 7000 rpm, 10 min in 500 mL centrifuge tubes.
Combine cell pellets and weigh. Resuspend in 3 mL TEN/g wet cells. Freeze.

Purification of aECM with Terminal Lysines (from Eric Welsh, Alyssa Panitch, Kathleen Di Zio, and Paul Nowatzki)

1. Thaw frozen cells in TEN buffer in 37°C shaker. Add DNase, RNase, $MgCl_2$, and PMSF. Shake for a couple of hours (until runny).

2. Centrifuge in JA-20 rotor, 25°C, 17000 rpm, 60 min. Protein is in pellet. Decant supernatant and save to check for lost protein.

3. Resuspend pellet in 1.5 ml of 4 M urea per gram of wet cell mass. May require several hours, but if you leave it overnight, store at 4°C.

4. Centrifuge in JA-20 rotor, 4°C, 17000 rpm, 60 min. Protein is in supernatant. Keep the pellet for analysis.

5. Pour supernatant into dialysis tubing and dialyze against filtered water at 4°C for 5 days, changing water frequently.

6. Centrifuge dialysate, JA-20, 4°C, 17000 rpm, 60 min. Precipitate should be minimal.

7. Decant supernatant into tared 50 ml tube. Freeze at -20°C, move to -80°C. Remove cap, cover with Kimwipe, lyophilize about 2 days until dry.

OPTIONAL:

Instead of step 7, you can cycle the protein through the LCST again to increase purity.

7A. Centrifuge the supernatant in JA-20, 25°C, 17000 rpm, 60 min. Protein is in pellet. Keep supernatant for analysis.

7B. Resuspend pellet in a minimal amount of water. May take several hours at 4°C.

7C. Centrifuge in JA-20, 4°C, 17000 rpm, 60 min. Freeze and lyophilize supernatant.

These steps may reduce impurities by 50% but also reduce protein yield 15-50%.

Purification of aECM with Internal Lysines (from Kathleen Di Zio and Julie Liu)

1. Following 10 L fermentation, centrifuge in 500 mL bottles at 10,000 rpm for 15 min in tared bottles. Pellet weight should be between 200-300 g. Split pellet into two 1 L containers and redisperse in 2 ml/g TEN buffer. Freeze at –20°C.
2. Add a small amount of DNase, RNase, and PMSF. Thaw at room temperature for 1-2 hours. Transfer to 2 L flask and add water up to a final volume of 1.2 L. Stir at 4°C overnight.
3. Bring pH up to 9 using 6 N NaOH and stir for one hour. Spin in JA-14 rotor (250 mL bottles) at 14,000 rpm for 1-2 hours at 4°C. Save and record weight of pellet (P1C). Add NaCl to supernatant (S1C) to make final concentration of 1 M. Stir at 4C overnight.
4. Incubate S1C at 37°C for >3 hours. Pre-warm JA-14 rotor to 37°C. Spin at 14,000 rpm for 1-2 hours at 37°C. Save and record volume of supernatant (S1H). Record weight of pellet (P1H). Resuspend pellet in 1 g/10 mL water. Stir overnight at 4°C.
5. Bring pH of P1H up to 9 using 1 N NaOH and stir for one hour. Spin in JA-17 rotor at 17,000 rpm for 2 hours at 4°C. Save and record weight of pellet (P2C). Add NaCl to supernatant (S2C) to make final concentration of 1 M. Stir at 4°C overnight.
6. Incubate S2C at 37°C for >3 hours. Pre-warm JA-17 rotor to 37°C. Spin at 17,000 rpm for 2 hours at 37°C. Save and record volume of supernatant (S2H). Record weight of pellet (P2H). Resuspend pellet in 1 g/10 mL water. Stir overnight at 4°C.
7. Bring pH of P2H up to 9 using 1 N NaOH and stir for one hour. Spin in JA-17 rotor at 17,000 rpm for 2-3 hours at 4°C. Save and record weight of pellet (P3C). Add NaCl to supernatant (S3C) to make final concentration of 1 M. Stir at 4°C overnight.
8. Incubate S3C at 37°C for >3 hours. Pre-warm JA-17 rotor to 37°C. Spin at 17,000 rpm for 2 hours at 37°C. Save and record volume of supernatant (S3H). Record weight of pellet (P3H). Resuspend pellet in 1 g/10 mL water. Stir overnight at 4°C.
9. Transfer P3H to dialysis tubing (25 mm, 12-14,000 MW cut-off). Tubing can be cleared by microwaving in EDTA solution for 3 minutes, rinsing with water, and repeating. Dialyze at 4°C with 2 water changes per day for a total of 6 water changes.
10. Transfer P3H from dialysis tubing to 50 mL centrifuge tube. Centrifuge in JA-17 rotor at 17,000 rpm for 1 hour at 4°C. Save and record weight of dialyzed pellet (P4C). Supernatant (S4C) can be frozen at –20°C and lyophilized or further temperature cycled to reduce water volume.

SDS-PAGE and Western of aECM Proteins

1. Assemble gel caster, mark 3 cm from the top.
2. Mix separating gel (add TEMED last). Generally use 12% gel for aECM proteins.

Acrylamide Conc (%)	7.5	12	15
30% acryl/0.8% bisacryl	1.875	2.00	3.75
4xTrisCl/SDS pH 8.8	1.875	1.25	1.875
water	3.75	1.75	1.875
10% am. persulfate	0.05	0.05	0.05
TEMED	0.01	0.01	0.01

3. Pour separating gel up to 3 cm mark. Top with butanol. Once excess volume has formed a gel, rinse off butanol two times with water. Blot dry with filter paper.
4. While waiting for gel to harden, prepare samples (see next page for info)
5. Mix stacking gel, 3.9% acrylamide.

30% acryl/0.8% bisacryl	0.325
4xTrisCl/SDS pH 6.8	0.625
water	1.5
10% am. persulfate	0.025
TEMED	0.005

6. Pour stacking gel and add comb. Mark lanes. Once excess volume has formed a gel, remove comb. Wipe away excess gel.
7. While waiting for gel to harden, boil samples for 1 min.
8. Remove gel from caster. Assemble gel runner (be sure to remove excess gel from alumina plate so it forms a good seal with the voltage source.) Add buffer and check for leaks.
9. Load gel (10-14 μl sample and 5 μl ladder).
10. Run at 150 V for about 1 hour or until dye front runs off the bottom of the gel.
11. To stain: Add stain with 5X more dye than normal because aECM proteins are hydrophobic and difficult to stain. Leave on waver at least overnight. Destain for 1 hour.
12. To transfer: Assemble transfer apparatus in the following order: positive side, sponge, 2x filter paper, nitrocellulose membrane, gel, 2x filter paper, sponge, negative side. Hole vertically so gel is facing you when you load it into the transfer apparatus. Run for 30 V for 40 min. Check to see if pre-stained MW bands have transferred. If so, mark them on the membrane with lab marker.
13. Block with 50 mL of 1X PBS + 0.1% Tween + 5% milk for 45 min at room temp or overnight at 4°C.
14. Add 0.5 μl of T7-tag HRP-conjugated antibody for 30 min at RT or overnight at 4°C.
15. Rinse for two times for 15 min each with 50 mL of 1X PBS + 0.1% Tween.
16. Detect with 0.75 mL of ECL reagent 1 and 2. Seal in Saran wrap and tape to light-free cassette. Expose to x-ray film for 30 sec and develop.

Recipes

30% acrylamide/0.8% bisacrylamide
30.0 g acrylamide
0.8 g N,N' methylene bisacrylamide
bring volume to 100 mL with water, filter with 0.45 μm, store at 4°C in dark.

4X TrisCl/SDS pH 8.8 (1.5 M TrisCl + 0.4% SDS)
91 g Tris base
300 mL water
pH to 8.8 with 1N HCl
add water to make 500 mL, filter with 0.45 μm, add 2 g SDS, store at 4°C.

4X TrisCl/SDS pH 6.8 (0.5 M TrisCl + 0.4% SDS)
6.05 g Tris base
40 ml water
pH to 6.8 with 1N HCl
add water to make 100 mL, filter with 0.45 μm, add 0.4 g SDS, store at 4°C.

SDS-PAGE Sample Preparation

Whole cell lysate
Centrifuge 1 mL cells at 14,000 rpm, 2 min, decant, resuspend in 50 μl water*OD.
Freeze, thaw, add 5 μl of 2 mg/ml RNase and DNase and incubate 15 min at 37°C

Purification pellets
weigh out 100 mg, dissolve in 1 mL water, add 1-2 urea pellets to help dissolve

Purification supernatants
use as is

Pure protein
weight out 10 mg, dissolve in 1 mL cold water

Sample buffer
To all of the above, add 15 μl sample buffer + β-mercaptoethanol to 15 μl sample and boil 1 min

To prepare sample buffer, add 300 μl sample buffer to 5 μl β-mercaptoethanol

Preparation for Edman Degradation/N-terminal Sequencing

1. Run SDS-PAGE gel as above.
2. Transfer to PVDF membrane using CAPS transfer buffer instead of standard transfer buffer.
3. Stain and destain membrane as above. Cut out stained bands and estimate sample concentration using molecular weight standards (see masses listed below).

CAPS Transfer Buffer

2.21 g cyclohexylaminopropane sulfonic acid (CAPS, free acid)
0.5 g DTT
150 mL methanol
water to 1L
pH to 10.5 with NaOH, store at 4°C
for proteins > 60 kD, use 1% methanol

See Blue Plus 2, pre-stained

	x mg per 10 µl loaded on gel
Myosin	2.2
Phosphorylase B	?
BSA	0.75
Glutamic dehydrogenase	1.25
Alcohol dehydrogenase	0.8
Carbonic anhydrase	0.9
Myoglobin red	1.5
Lysozyme	2.5
Aprotinin	1.8
Insulin, B chain	2.5

Mark 12, unstained

	x mg per 5 µl loaded on gel
Myosin	0.38
B-glactosidase	0.2
Phosphorylase B	0.35
BSA	0.2
Glutamic dehydrogenase	0.6
Lactate dehydrogenase	0.4
Carbonic anhydrase	0.22
Trypsin inhibitor	0.32
Lysozyme	0.25
Aprotinin	0.38
Insulin, A and B chains	1.12

LPS Quantification: Protocol for Pyrotell Endotoxin Assay

I. Preparation (can be completed several days in advance)

a. Check the lot numbers of CSE (Control Standard Endotoxin, Product number E0005) and Pyrotell (detection reagent, Product number G5250). See if we already have a Certificate of Analysis for these products. If not, call Associates of Cape Cod at 1-800-848-3248 and request one from Customer Service. Our FAX number is 626-793-8472. The Certificate of Analysis is required to determine the exact CSE concentration.

b. Count out the number of tubes that will be needed for the assay (usually about 60 tubes). Make sure the tubes are 10x75mm flint glass tubes (Product number 60825-402 from VWR). Place the tubes in a glass tray, cover with two layers of tin foil. Heat in an oven at 180ºC for 3 hours to depyrogenate (thermally degrade) the endotoxin. Remove from oven and allow to cool.

c. Check to make sure we have an adequate supply of endotoxin free water.

d. Autoclave one box of long (gel-loading) tips.

II. Make Sample Dilutions (can be completed one day in advance)

a. You will need to make 3 sets of dilutions for every sample being tested.

b. Remove protein from freezer and allow it to come to room temperature.

c. Rinse off tweezers with ethanol and allow to dry.

d. Tare one glass tube (only touch the outsides of the tubes with gloves on, or use tweezers to pick them up).

e. Place the protein inside the tube and weigh the amount. Calculate how much water is needed. (For sample calculations, see the Sample Preparations page.) Return the unused protein to the freezer.

f. Wipe the top of the water bottle with ethanol. Using a syringe, add water to the protein. Be careful not to touch the protein with the tip of the syringe (this will contaminate the end of the syringe!)

g. Place the tube in the cold room until the protein completely solubilizes (about 10-15 minutes). Place the long tips in the cold room as well.

h. Label the remaining sample tubes. Using a syringe, measure out endotoxin free water into each tube. (See the Sample Preparations page to see how much water to add to each tube.)

i. Cover the top of the water bottle with Parafilm and store at room temperature.

j. Place the tubes in the cold room to cool off for 5-10 minutes.

k. Using the long tips, add some of the protein solution to the first tube. Vortex the tube for 5 seconds. Repeat the procedure until all of the dilutions are made. (Again, see the Sample Preparations page for common concentrations.)

l. If the samples are going to be left overnight, cover with Parafilm and leave in the cold room.

III. Reconstitute the CSE (can be done up to 4 weeks before the assay)

a. Remove the CSE from refrigerator. Remove the metal seal from the vial. Note, the vial will probably look empty.

b. Add 5ml of endotoxin free water with a syringe through the stopper. The vial is sealed under vacuum, so the syringe should dispense automatically once the needle has penetrated.

c. Vortex the vial for one minute. Let the vial stand for 30 minutes at room temperature, vortexing for one minute at 10 minute intervals during this time.

d. Remove the rubber stopper and throw it away. Cover with Parafilm. Write the date on the bottle. Can be stored in the refrigerator like this for up to 4 weeks.

IV. Make the CSE Dilutions (must be done on the day of the assay)

a. Must make two sets of CSE dilutions.

b. Label the glass tubes.

c. Wipe the top of the water bottle with ethanol. Add the appropriate amount of water to each tube. (See the CSE Preparation page to determine the correct amount.)

d. Vortex the reconstituted CSE for 60 seconds. Add the appropriate amount to the first dilution tube. Vortex the tube for 10 seconds.

e. Continue making dilutions using the volumes from the CSE Preparation page. Vortex for 10 seconds between dilutions.

f. Make the second set of dilutions.

g. Cover the CSE vial with Parafilm and store in the refrigerator. Cover the endotoxin free water with Parafilm and store at room temperature. Cover the CSE dilutions wit Parafilm until the assay is performed

V. Make the Spiked Sample Dilutions (must be done on the day of the assay)

a. One full dilution set of each sample must be spiked.

b. In the cold room, use the long tips to add 2microliters of the 25EU/ml CSE dilution to each tube. Vortex for 10 seconds.

VI. Perform the Pyrotell Assay

a. Remove the Pyrotell from the refrigerator. Gently tap the vial to shake down all of the contents. Remove the metal seal and the rubber stopper.

b. Wipe the top of the water bottle with ethanol. Using a syringe add 5ml of endotoxin free water to the Pyrotell. Be sure not to touch the vial or the Pyrotell with the needle (this will contaminate the syringe!)

c. Gently mix the Pyrotell be swirling the vial on the countertop. Do not vortex or shake the vial! This will cause excessive foaming.

d. Cover the vial with Parafilm and wait 5 minutes.

e. Gently swirl the Pyrotell and check to make sure it has all gone into solution. Add 100 microliters of Pyrotell to each glass tube. (You can use the repeating pipettor to do this step more quickly.)

f. Shake the rack of tubes for 30 seconds.

g. Place the rack inside the incubator for 65 minutes.

h. Make a chart to write down the assay results.

i. Carefully remove the tubes from the incubator. Be careful not to tap the vials against the sides of the incubator or against one another.

j. Smoothly invert the vials one by one. A gel that does not collapse is a positive test result. No gel, or a gel that collapses, is a negative test result.

CSE Preparation Page

Check the Certificate of Analysis. If the concentration of CSE is reported to be 5000 EU/vial, the following dilution series can be made for the Pyrotell Assay with 0.25 EU/ml sensitivity.

The reconstituted CSE has a concentration of 1000 EU/ml.

Dilution Name	Volume Of Water	CSE Dilution To be Added	Volume Of CSE	Final Conc.	Final Volume
(#)	(□l)	(#)	(□l)	(EU/ml)	(□l)
1	975	Reconstitued CSE	25	25	1000
2	980	1	20	0.5	1000
3	100	2	100	0.25	200
4	100	3	100	0.125	200
5	100	4	100	0.0625	200
6 (just water)	100	- - -	0	0	100

Make two separate sets of these dilutions.

Dilution #1 will be used to make the spiked samples.

Dilutions #2,3,4,5, and 6 will be used in the assay. For the assay, you will need 100 microliters of each of these dilutions. Dilutions # 3, 4, and 6 already have exactly 100 microliters in each tube. Add 100 microliters of Dilution #2 to a clean, labeled tube. Remove 100 microliters from Dilution #5.

Sample Preparation Page – Concentration Range = 10-0.0032 mg/ml

Begin with a solution of 10mg protein plus 1ml water.

Dilution Name	Volume Of Water	Sample Dilution To be Added	Volume Of Sample Dilution	Final Conc.	Final Volume
(#)	(□l)	(#)	(□l)	(mg/ml)	(□l)
1	1000	10 mg protein	- - -	10	1000
2	100	1	25	2	125
3	100	2	25	0.4	125
4	100	3	25	0.08	125
5	100	4	25	0.016	125
6	100	5	25	0.0032	125

Make three separate sets of these dilutions.

One set will be used to make the spiked samples.

Dilutions #1,2,3,4,5, and 6 will be used in the assay. For the assay, you will need 100 microliters of each of these dilutions. Dilutions # 2, 3, 4, and 5 already have exactly 100 microliters in each tube. Add 100 microliters of Dilution #1 to a clean, labeled tube. Remove 25 microliters from Dilution #6.

Sample Preparation Page – Concentration Range = 8 – 0.25 mg/ml

Begin with a solution of 8mg protein plus 1ml water.

Dilution Name	Volume Of Water	Sample Dilution To be Added	Volume Of Sample Dilution	Final Conc.	Final Volume
(#)	(µl)	(#)	(µl)	(mg/ml)	(µl)
1	1000	8 mg protein	- - -	8	1000
2	100	1	100	4	200
3	100	2	100	2	200
4	100	3	100	1	200
5	100	4	100	0.5	200
6	100	5	100	0.25	200

Make three separate sets of these dilutions.

One set will be used to make the spiked samples.

Dilutions #1,2,3,4,5, and 6 will be used in the assay. For the assay, you will need 100 microliters of each of these dilutions. Dilutions # 2, 3, 4, and 5 already have exactly 100 microliters in each tube. Add 100 microliters of Dilution #1 to a clean, labeled tube. Remove 100 microliters from Dilution #6.

Sample Preparation Page – Concentration Range = 2 – 0.008 mg/ml

Begin with a solution of 10mg protein plus 1ml water.

Dilution Name	Volume Of Water	Sample Dilution To be Added	Volume Of Sample Dilution	Final Conc.	Final Volume
(#)	(µl)	(#)	(µl)	(mg/ml)	(µl)
1	1000	10 mg protein	- - -	10	1000
2	120	1	30	2	150
3	100	2	50	0.667	150
4	100	3	50	0.22	150
5	100	4	50	0.074	150
6	100	5	50	0.025	150
7	100	6	50	0.008	150

Make three separate sets of these dilutions.

One set will be used to make the spiked samples.

Dilutions #2, 3, 4, 5, 6 and 7 will be used in the assay. For the assay, you will need 100 microliters of each of these dilutions. Dilutions # 2, 3, 4, 5 and 6 already have exactly 100 microliters in each tube. Remove 50 microliters from Dilution #7.

Substrate Preparation

Adsorbed aECM films

1. Prepare 1 mg/ml solution of aECM/cold PBS (Positive control = 10 μg/ml fibronectin)
2. Sterile filter, 0.2 μm.
3. Completely cover bottom of tissue-culture polystyrene (75 μl/well).
4. Incubate at 4°C overnight.
5. Rinse three times with cold PBS (200 μl/well).
6. Prepare BSA, fraction V, 0.2% in PBS. Heat at 85°C for 10 min. Sterile filter.
7. Block with BSA (50 μl/well).
8. Rinse three times with cold PBS (200 μl/well).

Crosslinked aECM films using BS3

1. Dissolve 10-30% w/v protein in PBS at 4°C. May take a few hours. Centrifuge to be certain that all of the protein is dissolved. (For cell culture, usually use 20% w/v).
2. Prechill pipet tips, molds, and water.
3. Immediately before crosslinking, weigh out crosslinker.
4. Dissolve crosslinker in water (1 mg BS3 in 6 μl water).
5. Stir together quickly, fill the mold using a prechilled tip. (Sometimes its easier to do this if the end of the tip is cut off first.) For a 96 well plate, use 15 μl. For teflon molds, use 300 μl.
6. For teflon molds: sit on hotplate at 60°C overnight.
   For 96 well plates, centrifuge at 800 rpm, 15 min either at room temp or 4°C. If you want films to be transparent, centrifuge at 4°C, cover with sealing tape, let reaction continue at 4°C for 1 day.
7. IMPORTANT: Store BS3 under dry nitrogen or argon. Parafilm cap. Store in container with Dri-rite at 4°C.

BS3 MW = 572.43 g/mol, 2 active groups
CS5 MW = 37.120 kDa, 14 active groups
1:1 stoichiometry 40 mg CS5:4.31 mg BS3

Crosslinked aECM films using HMDI

1, Dissolve 10-30% w/v protein in DMSO. (Usually use 10%, 100 mg/ml.)
2. Dilute HMDI in DMSO 10X.
3. Add crosslinker quickly and fill mold. This must be done in about 30 sec.
4. Let react on hotplate at 60°C overnight.

HMDI MW = 168.19 g/mol, 2 reactive groups
HMDI density = 1.15
Kt43 MW = 42,974 Da, 4 reactive groups
1:1 stoichiometry 30 mg Kt43:0.22385 μl HMDI

For physical testing, pull to about 50% (should be extensible up to 300%). Use pull rate of 10% of film length/min. Aspect ratio of 8:1 is ideal, but 3:1 or greater is fine. Try to keep this standard for films you are comparing.

Bicinchoninic acid (BCA) Quantification of Protein Adsorption (from Julie Liu)

These directions are for use with QuantiPro BCA Assay Kit
Sigma, Product Code: QP-BCA

1. Prepare test substrates in triplicate. Usually performed overnight. Rinse three times with PBS. Do not block with BSA.
2. Prepare fresh standards, one for each test protein being analyzed. Do not use the BSA standard that comes with the QP-BCA kit. To make a 50 μg/ml standard, add 50 μl of a 1 mg/ml solution to 950 μl PBS. Follow the table below for other standards.

Concentration (μg/ml)	Vol. of 50 μg/ml Std. (μl)	Vol. of PBS (μl)
0.5	3	297
5	30	270
10	60	240
20	120	180
30	180	120

3. Add 50 μl PBS to each test well containing adsorbed protein. Add 50 μl of each protein concentration standard to a well. For the blank, add 50 μl of PBS to a clean well.
4. Prepare the working reagent: 4 ml QA, 4 ml QB, 0.8 ml QC
5. Add 50 μl working reagent to each well using a repeating pipetor.
6. Seal the plate with polyolefin tape (Nalge Nunc).
7. Incubate the plate at 60°C for 1 hour.
8. Allow the plate to equilibrate to room temperature for about 20 minutes with the sealing tape still in place.
9. Read the absorbance at 562 nm. To obtain optimum reading, set the plate reader to mix/vortex for 5 seconds prior to reading.

Cell Adhesion (Flick Method)

1. Prepare substrates in clear 96-well plates.
2. Passage HUVEC non-enzymatically. Resuspend at $4x10^5$ cell/ml in PBS^+ or serum-free medium.
3. Add 0.1 ml cells/well. Add 0.5 ml PBS^+ or serum-free medium with or without competitive peptide or antibody.
4. Incubate for 30 min at 37°C.
5. Remove media by flicking the plate. Rinse once with PBS^+ or serum-free medium.
6. Fix in 70% ethanol for 10 min.
7. Stain with 0.1% crystal violet in water for 25 min. (Filter the crystal violet solution using 0.45 μm filter prior to use.)
8. Wash five times with water.
9. Solubilize the dye with 0.2% TritonX-100 in water (50 μl/well).
10. Read absorbance at 595 nm.

Cell Adhesion (Buoyant Centrifugation Method)

1. Prepare substrates in black 96-well plates. Prepare a control plate with fibronectin.
2. Passage HUVEC non-enzymatically. Resuspend in serum-free medium at 2 ml/plate.
3. Dissolve 1 aliquot of calcein acetoxymethyl ester in 5 µl DMSO. Add 1 µl of label/plate. Incubate 30 min at room temperature.
4. Centrifuge for 5 min, 200 *g*, remove supernatant. Resuspend in 2 ml/plate of PBS^+ or serum-free medium. Repeat centrifugation. Cell pellet will be yellow.
5. Resuspend at $2.67x10^5$ cell/ml in PBS^+ or serum-free medium.
6. Add 0.150 ml cells/well.
7. Incubate at 37°C for 30 min.
8. Add 0.2 ml Percol+PBS (add 11.1 ml 10X PBS to 100 ml Percol to match ion concentration) to each well using the next to slowest setting on the repeating pipetor.
9. Centrifuge the test plate for 10 min. Let the control plate sit for 10 min at room temperature.
10. Align the wicks in the harvesting frame by pushing them through the frame until they're flush with the posts. Remove the filters from the wicks. Use the harvesting frames to gently remove the detached cells.
11. Use a pipet to remove the trace amount of Percol+PBS.
12. Add 0.2 ml of PBS to each well.
13. Read the fluorescence at an excitation of 485 nm and emission of 538 nm.
14. Calculate the cell adhesion index (CAI) as the fluorescence reading of a test well divided by the average of the fluorescence reading to fibronectin at 1 *g*.

The normal detachment force can be estimated using Archimedes' theorem, where the force equals the volume of the cell times the relative centrifugal force time the difference in density between the cell and the surrounding medium. Using the following estimates for these parameters,

Volume Cell (nl)	0.0005
Percoll density (g/ml)	1.123
Cell density (g/ml)	1.07
Medium density (g/ml)	1

the following centrifugal forces correspond to these normal detachment forces:

RCF g	*Buoyant F* µdynes	*Buoyant F* pN
1	0.02598749	0.2598749
10	0.2598749	2.598749
100	2.598749	25.98749
1000	25.98749	259.8749

Cell Adhesion (Inverted Centrifugation Method)

1. Prepare substrates in black 96-well plates. Prepare a control plate with fibronectin.
2. Record the background fluorescence of the substrates. (This is especially important if crosslinked films are used as substrates.) Excitation wavelength = 485 nm. Emission wavelength = 538 nm.
3. Passage HUVEC non-enzymatically. Resuspend in serum-free medium at 2 ml/plate.
4. Dissolve 1 aliquot of calcein acetoxymethyl ester in 5 µl DMSO. Add 1 µl of label/plate. Incubate 30 min at room temperature.
5. Centrifuge for 5 min, 200 *g*, remove supernatant. Resuspend in 2 ml/plate of PBS^+ or serum-free medium. Repeat centrifugation. Cell pellet will be yellow.
6. Resuspend at $2.67x10^5$ cell/ml in PBS^+ or serum-free medium.
7. Add 0.150 ml cells/well.
8. Incubate at 37°C for 30 min.
9. Add 0.3 ml of PBS^+ or serum-free medium. (Each well should have a slightly positive meniscus.) Take an initial fluorescence reading.
10. Seal the plate with polyolefin tape (Nalge Nunc). Be careful to not form any air bubbles. Place a lid on the plate and gently invert the whole assembly. Centrifuge the test plate for 10 min.
11. Align the wicks in the harvesting frame by pushing them through the frame until they're flush with the posts. Remove the filters from the wicks.
12. Remove the plates from the centrifuge and keep them inverted. Carefully remove the lid and the sealing tape. (This step should be done over a sink or a tray.) Use the harvesting frames to remove any remaining medium.
13. Read the final fluorescence.
14. Calculate the cell retention percentage as:
    (final - background)/(initial - background)*100%.

The normal detachment force can be estimated using Archimedes' theorem, where the force equals the volume of the cell times the relative centrifugal force time the difference in density between the cell and the surrounding medium. Using the following estimates for these parameters,

Volume Cell (nl)	0.0005
Percoll density (g/ml)	1.123
Cell density (g/ml)	1.07
Medium density (g/ml)	1

the following centrifugal forces correspond to these normal detachment forces:

RCF g	*Inverted F* µdynes	*Inverted F* pN
1	0.0343231	0.343231
10	0.343231	3.43231
100	3.43231	34.3231
1000	34.3231	343.231

Cell Adhesion (Flow Chamber Method)

1. Prepare substrates on 3"x1" sterilized glass slides. Use 1-well LabTek II chamber slides (Nalge Nunc).
2. Hook up flow chamber to medium reservoir and peristaltic pump. Align the silicone membrane on the bottom half of the flow chamber, align a blank slide onto each of the four flow cells, align and tighten down the top half of the flow chamber.
2. Passage HUVEC non-enzymatically. Seed the slides at $5x10^4$ cells/cm^2.
3. Incubate the slides for 60 min at 37°C.
4. While waiting, prime the entire flow circuit with medium heated to 37°C. Use a clamp positioned in between the pump and the flow chamber inlet to maintain pressure in the flow lines and quickly turn off the pump.
5. Remove the top half of the flow chamber and the blank slides. Be sure the silicone membrane is still properly aligned. Use a pipet to fill each flow cell until it has a slight positive meniscus.
6. Remove the chambers from the chamber slides. Carefully position one short end of the slide at the inlet of a flow cell. Gently lower the other side of the slide. This should push out the excess medium and form a seal between the slide and the silicone membrane without forming any air bubbles.
7. Replace the top half of the flow chamber and tighten it in place.
8. Take initial images of the cells along the length of the slide at 5-mm intervals.
9. Turn on the peristaltic pump (I used setting "39" on a Henry high speed cassette pump (Manostat) which corresponds to 7 ml/min, resulting in a maximum shear stress of 200 dyne/cm^2) and immediately release the clamp. Flow medium for 2 min.
10. Take final images of the cells along the length of the slide at 5-mm intervals. Use initial images to help align the microscope stage so the same region of the slide is being imaged before and after flow.
11. Determine the percent detachment by subtracting the number of cells visible after flow from the number of initial cells, and then normalize to the number of initial cells.
12. To clean the flow chamber, add four blank slides to the four flow cells. Align and tighten the top half of the flow chamber. Flow sterile water through the system until no traces of medium are visible. Disassemble the flow chamber and let the pieces air dry.
13. DO NOT USE ETHANOL TO CLEAN THE FLOW CHAMBER. Instead, use soap and water to gently wash. All pieces should be okay in the autoclave, but I generally try not to autoclave it very often, because the pieces could eventually warp.

Notes: The flow chamber is made of Thermalux Polysulfone (a sample was obtained form Port Plastics, City of Industry, CA) Silicone sheeting (thickness = 0.005") was purchased from Specialty Manufacturing Inc (Saginaw, MI) and is also available from Bioplexus (Saticoy, CA). Cole-Parmer and McMaster Carr sell teflon and stainless steel valves and tubing connectors that can be autoclaved.

Detoxi-Gel Columns (Pierce)

1. Prepare solutions:
    A. Detergent: 1% sodium deoxycholate in water
    B. AB buffer: 0.1 M ammonium bicarbonate, pH 7.8
    C. 10 mg/ml protein in AB buffer
2. Rinse Detoxi-Gel columns with 5 ml detergent and 5 ml water at room temperature.
3. Rinse column with 5 ml AB buffer in cold room.
4. Tare 7 eppendorf tubes for each Detoxi-Gel column.
5. Add 1 ml of 10 mg/ml protein in AB buffer to each column at 4°C.
6. Drain 0.5 ml void volume in eppendorf tube 1 and save as sample 1.
7. Wait 30 min. Drain 0.5 ml into tube 2 and save as sample 2.
8. Add 1 ml AB buffer to top of tube. Wait 30 min.
9. Drain 0.5 ml as sample 3. Wait 30 min.
10. Drain 0.5 ml as sample 4.
11. Repeat steps 8-10 until 7 samples have been taken. You can check for the presence of protein by taking the absorbance at 280 nm, but normally 7 samples is enough to elute most of the protein off the column.
12. Freeze and lyophilize the samples. AB buffer is volatile and should sublime away. If you're worried about it, you can dialyze first, but yield will be lower. Yields without dialysis were 63-87%.
13. Wash the column with 5 ml detergent and 5 ml water. Add 2 ml of 0.25% ethanol and allow 1 ml to drain. Cap and store the columns at 4°C.

PAI and tPA ELISAs

1. Prepare substrates. Seed non-enzymatically harvested HUVEC at $1x10^4$ cells/cm^2 and grow for 1-2 days until confluent in 2% serum EGM-2 (Clonetics).
2. If desired, challenge the cells with LPS (serotype O55:B5, Sigma) at a final concentration of 10 □g/ml.
3. Take 600 □l sample at each time point and replace with 600 □l EGM-2. My time points were 1, 6, 12, 24, 48 and 72 hours. Immediately freeze the samples.
4. Based upon my results and the previous reports of others, 500 □l of the following dilutions need to be made from each sample so that the levels of tPA and PAI are about 10 ng/ml:

Sample Time (hr)	tPA dilution	PAI dilution	PAI + LPS challenge dilution
1	1/1	1/10	1/10
6	1/1	1/100	1/100
12	1/2	1/250	1/250
24	1/4	1/500	1/1000
48	1/8	1/1000	1/2000
72	1/15	1/1000	1/2000

Note: The values listed above are the *final* dilution values. For example, the tPA "dilution" at 1 hour (1/1) should not be diluted at all while the PAI "dilution" (1/10) should be diluted with 9 parts medium. Also, tPA secretion should not be affected by LPS challenge, so the tPA + LPS challenge dilutions are identical to those for tPA without LPS challenge.

5. Let the entire tPA and/or PAI kit sit at room temperature for 15 min.
6. For 2 test strips (32 wells), add 54 ml water to 6 ml reagent 4. (Sometimes reagent 4 crystallizes upon storage, so be sure to vortex the vial and check for crystals.) Add 54 ml water to 6 ml reagent 7. Let both solutions stand for 30 min. Swirl prior to use.

Note: The standards and controls take up 16 wells for each assay. I normally run the samples in duplicate. So 2 test strips can analyze (32-16)/2 = 8 samples while 3 test strips can analyze (48-16)/2 = 16 samples, etc.

7. Dilute reagent 6 with reagent 4 in the following fractions: 1/1, 1/2, 1/4, 1/8, and 1/16. Add 1 ml reagent 6 to an eppendorf tube for fraction 1/1. Add 0.5 ml of reagent 4 in the other tubes and make a 0.5 ml dilution series with reagent 6.
8. Open up the test strips and snap them into the plate. Fill each well with 0.2 ml of each sample, control, or standard. For a negative control, use both reagent 4 and non-conditioned medium. For a positive control, use reagent 7. Cover the plate and incubate at room temperature (2 hours for the tPA assay and 1 hour for the PAI assay).

9. While the plate is incubating, prepare reagent 2. For 2 test strips, add 8 ml reconstituted reagent 4 to reagent 2. Let stand for 30 min and swirl prior to use. Then add 16 ml reagent 5 to 304 ml water to make the washing solution for 2 test strips.
10. Wash the wells 5 times with 0.25 ml reagent 5. Be sure to remove all of the fluid (by flicking the entire plate) after the final wash.
11. Add 0.2 ml reagent 2 to each well using a repeating pipetor. Incubate at room temperature (2 hours for the tPA assay and 1 hour for the PAI assay).
12. Prepare the OPD/H_2O_2 solution 5 min before washing. For 2 test strips, dissolve 2 tablets of reagent 3a and 2 tables of reagent 3b in 8 ml water.
13. Wash the wells 5 times with 0.25 ml reagent 5. Remove all liquid after final rinse.
14. Add 0.2 ml reagent 3 to each well using a repeating pipetor. Incubate at room temperature for exactly 6 min for the tPA assay or 3 min for the PAI assay.
15. Add 0.1 ml of 1 M HCl to each well using a repeating pipetor.
16. Incubate 10 min at room temperature.
17. Read absorbance at 492 nm.
18. Use the standards to calculate the concentration of tPA or PAI in each sample. Be sure to correct for the replacement volume of medium at each time sample, if necessary. Record the data as mass/10^5 cells/min, assuming a confluent monolayer of HUVEC contains $1x10^5$ cells/cm^2.

Note: ELISA kits are sold by American Bioproducts Company (Parsippany, NJ) and manufactured by Diagnostica Stago under the names Asserachrom tPA, product number 0248 and Asserachrom PAI-1, product number 0249. They are often on back-order for several months, so try to order well in advance. Chemicon has also begun to market a PAI-1 Activity Assay, but I have not used it.

HUVEC on *p*N_3Phe aECM Patterns

1. In a 24 well plate, add 1 ml of HUVEC ($4x10^5$ cells/ml) in Endothelial Defined Medium (Cell Applications) to patterned coverslip.
2. Incubates 6 hr at 37°C. Replace medium with serum-containing EGM-2 (Clonetics).

For all labeling steps, use filtered PBS.

3. Rinse very gently with 2 ml PBS. Move to a fresh well.
4. Fix with 1 ml ice cold acetone for 1 min. Aspirate immediately.
5. Rinse very gently 3 times with 2 ml PBS.
6. Block with 1 ml 10% BSA (NOT heat-denatured) for 30 min.
7. Add 0.5 □l T7 tag-HRP antibody to BSA. Incubate 6 hr - overnight at room temp.
8. Rinse 3x with 2 ml PBS for 5 min, no agitation.
9. Add 100 □l of secondary Ab, 38 □l of phalloidin, and 862 □l PBS. Incubate in dark 1 hr.
   (Secondary Ab is antimouse-Cy2 reconstituted to 1 mg/ml with water and then diluted with an equal volume of glycerol for storage in small frozen aliquots.)
10. Rinse with 2 ml PBS, aspirate immediately. Rinse with 2 ml PBS on waver for 10 min. Rinse two times with 2 ml PBS, 5 min, no agitation.
11. Add 1 ml of $3x10^{-7}$ M solution DAPI, 5 min, room temp.
    (Make two 100-fold dilutions of 10.9 mM stock solution: 10 □l + 990 □l PBS to make $10.9x10^{-7}$ M solution. Then add 275 □l of this to 725 □l of PBS.)
12. Rinse three times with 2 ml PBS, no waiting or agitation.
13. Blot sides and back of coverslip. Add filtered mounting medium and seal to a slide with clear nail polish.

Kinetics of Elastase Degradation

1. Make protein samples and standards using sodium borate buffer, pH 8 at 4°C.
2. Fill each well with 0.1 ml protein solution at 4°C or on ice.
3. Take background absorbance reading at 4°C at 450 nm.
4. Heat to 40°C.
5. Prepare elastase stock solution (purchased from Elastin Products, product number CK828, 0.1 mg/vial). Dilute 0.1 mg in 1 ml water to make a 1600 U/ml solution. Store at 4°C.
6. Prepare elastase working solution. Need 0.05 ml/well at 300 U/ml.
7. At t=0 hr, start a timer and add 0.05 ml elastase working solution to T=6 hr wells.
8. At t=2 hr, start a timer and add 0.05 ml elastase working solution to T=4 hr wells.
9. At t=4 hr, start a timer and add 0.05 ml elastase working solution to T=2 hr wells.
10. At t=5 hr, start a timer and add 0.05 ml elastase working solution to T=1 hr wells.
11. Prepare TNBS (2,4,6-trinitrobenzene sulfonic acid) working solution. I purchased TNBS in a 5% solution from Sigma and mixed 48.6 ml of this 5% solution with 4951.6 ml of sodium borate buffer to make a 1.5 mM working solution.
11. At t=6 hr, add 0.05 ml water to T=0 hr wells and standards. Add 0.05 ml TNBS working solution to each well.
12. Incubate at 4°C for 20 min.
13. Take absorbance reading at 4°C at 450 nm.
14. Use the standards to determine the number of N-termini in each test well. If the sodium borate buffer has a pH of 8, only the N-termini primary amines (and not the lysines) will affect the absorbance.

Recipe for Sodium Borate Buffer
50 mM boric acid, H_3BO_3 (3.9 g)
25 mM sodium hyhdroxide, NaOH (1.0 g)
Bring to 1 L with water, pH to 8

Sample Information
For a full kinetic analysis, I took readings at 6, 4, 2, and 1 hrs at concentrations of 6, 4, 2, 1, 0.8, and 0.5 mg protein/ml buffer. Each concentration and time point was repeated in triplicate and averaged.
Standards included protein concentrations of 1, 0.6, 0.4, 0.2, 0.1, 0.08, 0.05, and 0.0 mg/ml buffer in triplicate.

LCST Analysis

Standard Assay for aECM Comparison
10 mg/ml protein in PBS (PBS pH = 7.4 initially, will change upon aECM addition.)
Increase temperature at 30°C/hr. Record transmission (dynode voltage) at 300 nm.

Sample Preparation
- Weight out desired amount of protein in a weigh boat with tweezers (To get most accurate mass, lyophilize protein overnight prior to weighing.)
- Transfer protein to eppendorf tube, taking care to retain full mass of the protein

Perform following steps in cold room (approx. 4°C) to maintain solubility:
- Entire mass desired for solution dissolved in a smaller amount of PBS (approx. 700 μL for 1 mL solution) using P1000 pipet.
- Centrifuge approx. 1 min @ approx. 12000g at 2°C.
- Transfer to appropriate volumetric flask using P1000 pipet.
- Remove any excess sample remaining in eppendorf tube by using P200 pipet to fill eppendorf tube with relatively small volumes (e.g., 10 μL) and then transferring to volumetric flask. Total volume of PBS should never exceed 1000 μL for 1 mL flask.
- Transfer back to original eppendorf tube. Keep tube and cuvette holder on ice.

Sample Testing on CD
- Transfer approx. 400 μL to clean, pre-chilled, dry cuvette. Avoid any possible contamination of cuvette. Lens paper used to clean edges.
- Follow warm-up procedures for turning on lamp as listed on CD.
- Insert cuvette.
- Follow software setup procedures as listed on CD.
- Wavelength of light = 300 nm
- Follow shut-off procedures as listed on CD.

Glassware Cleaning
- Rinse twice with HCl (~3N).
- Rinse three times with dH_2O from tap.
- Rinse twice more with ddH_2O.

For cuvettes, if finished using for a period of time, store filled with ddH_2O.

Gelatin Zymography

1. Prepare substrates in 12-well plates. Seed non-enzymatically harvested HUVEC at $5x10^4$ cells/well, about $1.5x10^4$ cells/cm^2.
2. Allow to grow for 16 hr in full medium. Can also grow for shorter time periods in medium that has been pre-conditioned (i.e., medium that was in contact with HUVEC).
3. Take 0.1 ml samples at desired time points. Centrifuge 10 min, 14000 rpm, 4°C. Remove supernatant. Add 0.09 ml supernatant to 0.01 ml 10X non-reducing, SDS loading buffer, recipe below. Store at -20°C.

Ingredient	Final Concentration	Amount needed to make 100 ml
glycerol	50%	50 ml
SDS	10% (w/v)	10 g
4XTrisHCl, pH 6.8	50%	50 ml
bromophenol blue	0.04%	40 mg
water		fill to 100 ml

4. Prepare a 10% SDS-PAGE with 1 mg/ml gelatin. Make a 10 mg/ml gelatin stock solution (gelatin can be solubilized at 55°C) and store at -20°C in 1 ml aliquots.

Ingredient	Separating Gel Volume	Stacking Gel Volume
30% acryl/0/8% bisacryl	3.3 ml	0.325 ml
TrisCl/SDS pH 8.8	3.75 ml	- - -
TrisCl/SDS pH 6.8	- - -	0.625 ml
water	1.95 ml	1.5 ml
10% APS	50 µl	25 µl
TEMED	10 µl	5 µl
10 mg/ml gelatin	1 ml	- - -

5. Load 10 µl sample per lane and 5 µl molecular weight marker per lane.
6. Run at 4°C for 45 min at 200 V. (It's very important to not let the gel over-heat.)
7. Wash the gel 2 times with 50 ml of 2.5% TritonX-100 for 30 min.
8. Incubate the gel overnight at 37°C in activating buffer, recipe below.

Ingredient	Final Concentration	Amount needed to make 50 ml
TrisHCl, pH 7.6	50 mM	2.5 ml of 1 M soln
$CaCl_2$	5 mM	0.25 ml of 1 M soln
$ZnCl_2$	1 µM	5 µl of 0.1 M soln
TritonX-100	1%	0.5 g
NaN_3	0.02%	0.01 g
water		fill to 50 ml

9. Stain and destain gel as normal. MMP-9 appears at 96 kD. MMP-2 appears at 72 kD in an inactive form, 62 kD in a partially active form, and 59 kD in a fully active form.

Western Analysis of HUVEC Lysates (Detection of Integrins)

1. Grow HUVEC on prepared substrates. I used a full 100 mm dish at confluence.
2. Prepare lysis buffer, recipe at end, and chill on ice.
3. Rinse cells 2X with PBS.
4. Add 200 µl cold lysis buffer and incubate on ice 10-20 min.
5. If the lysate is clumpy, push through a syringe needle a few times (I did not need to do this step.)
6. Collect the lysate and centrifuge, 10 min, 14000 rpm, 4°C.
7. Save 50 µl of supernatant to run BCA analysis to "quantify" the number of cells. Store at -20°C.
8. Mix remaining supernatant with 10X non-denaturing sample buffer, recipe below. Store at -20°C.

Ingredient	Final Concentration	Amount needed to make 100 ml
glycerol	50%	50 ml
SDS	10% (w/v)	10 g
4XTrisHCl, pH 6.8	50%	50 ml
bromophenol blue	0.04%	40 mg
water		fill to 100 ml

9. Prepare 7.5% SDS-PAGE. Load 10 µl sample per lane and 5 µl molecular weight marker per lane. Run at 150 V.
10. Transfer to PVDF membrane. (Soak membrane in methanol for 2 sec and then soak in transfer buffer prior to assembling transfer apparatus.)
11. Transfer at 30 V for 30 min, then 60 V for 45 min. (Check for MW bands.)
12. Block overnight in TTBS + 0.2% casein at 4C, recipe for TTBS below.

Ingredient	Final Concentration
TrisCl, pH 7.5	100 mM
NaCl	0.9% (w/v) or 150 mM
Tween 20	0.1%

13. Add 3 ml TTBS + 0.2% casein and the appropriate primary antibody. Seal the gel in a zip-loc bag. Incubate on waver for 3 hr at room temperature. For β_1 sub-unit, add 10 µl of monoclonal antibody clone JB1A, Chemicon product number MAB1965. For β_4 sub-unit, add 30 µl of polyclonal antibody, Chemicon product number AB1924. (Also tested anti-β_4 monoclonal antibody, clone P1H4, but did not see Western staining.)
14. Rinse with PBST (PBS with 0.1% Tween 20) four times, 15 min each, 50 ml, room temperature, on waver.
15. Add 30 ml TTBS + 0.2% casein and secondary antibody. For β_1 sub-unit, add 15 µl of anti-mouse HRP conjugate (1:2,000 dilution). For β_4 sub-unit, add 1 µl of goat anti-rabbit HRP conjugate, Chemicon product number AP132P (1:30,000 dilution)
16. Rinse with PBST (PBS with 0.1% Tween 20) four times, 15 min each, 50 ml, room temperature, on waver.

17. Detect with 0.75 ml each of ECL-1 and ECL-2. The α_1 sub-unit normally runs at 130 or 125 kDa. The α_4 sub-unit runs as bands at 70 and 80 kDa or a single band at 150 kDa. This band has also been observed by others to shift up to 180 kDa at some gel conditions.

Lysis Buffer Recipe

Ingredient	Final Concentration in lysis buffer	Amount to make ~ 5 ml lysis buffer	Notes
water		2.55 ml	
1M Tris, pH 7.5	50 mM	0.25 ml	1M = 12.11g in 100 ml water, pH with 6N HCl
5M NaCl	150 mM	0.15 ml	5M = 11.7g in 40 ml water
20% TritonX-100	1%	0.25 ml	20% = 5 ml + 15 ml water
10% NP-40	0.5%	0.25 ml	
10% NaDeoxycholate	0.25%	0.125 ml	
0.5M β-glycerophosphate, pH 7.3	50 mM	0.5 ml	0.5M = 4.32g in 40 ml water, pH with 1N HCl
0.1M NaPP	10 mM	0.5 ml	0.1M = 1.78g in 40 ml water
0.5M NaF	30 mM	0.3 ml	0.5M = 0.84g in 40 ml water
100 mM benzamidine	1 mM	0.05 ml	100mM = 0.63g in 40 ml water
250 mM EGTA, pH 8	2 mM	40 µl	250mM = 7.6g in 40 ml water, pH will increase, pH before use with 50% NaOH
100 mM Na orthovanadate	100 µM	5 µl	100mM = 0.74g in 40 ml water, see instructions below
1M DTT	1 mM	5 µl	1M = 1.54g in 10 ml 0.01M NaOAc, pH 5.2, store -20°C
5mg/ml aprotinin	5 µg/ml	5 µl	Store -20°C
5mg/ml leupeptin	5 µg/ml	5 µl	Store -20°C
1mg/ml pepstatin	1 µg/ml	5 µl	Store -20°C
500X PMSF	1 mM	10 µl	500X = 0.87g in 10 ml ethanol or isopropanol

Stock solutions can be stored at room temperature unless otherwise noted. Make the lysis buffer fresh each time.

To make 100 mM sodium orthovanadate, prepare the solution and pH to 10.0 using either 1 N NaOH or 1 N HCl. (Starting pH will vary depending on lot. Solution will be yellow.) Boil until solution turns colorless, usually 10 min. Cool to room temperature. Readjust pH to 10.0 and boil and cool. Repeat until the solution remains colorless and the pH stabilizes at 10.0. Store as aliquots at -20°C.

Enterokinase Cleavage (to remove T7 and His tags from aECM proteins)

1. Weigh out 200 mg protein. Solubilize in 10 ml of 50 mM Tris, pH 8 (final concentration is 20 mg/ml.)
2. Prepare enterokinase stock solution by adding 100 μl water to 1 enterokinase vial (purchased from Roche). Add 50 μl of enterokinase stock solution to 10 ml of solubilized protein to yield 15 μg/ml.
3. Incubate at room temperature for 2-3 days.
4. Prepare EKapture Agarose (Novagen). Add 1.5 ml EKapture slurry to a 15 ml Falcon tube. Resuspend two times with 7.5 ml buffer, centrifuge, discard supernatant. Buffer is 20 mM Tris, 50 mM NaCl, and 2 mM $CaCl_2$.
5. Resuspend agarose with protein/enterokinase solution. Do not vortex.
6. Incubate at 4°C for 2-3 days on spinning wheel.
7. Centrifuge at 4°C.
8. Dialyze supernatant using 12-14,000 MWCO tubing.
9. Freeze dialysate in tared tube and lyophilize.