TROPO BINDING ELISA PROTOCOL

I. ELISA Workup

1. Buffers and Media Needed
   1.1. DME (LB) + 10% CS + HEPES (=DME-LB)
   - DME (HB/LB) AS A BASE
   - 10% Calf Serum
   - -30mM HEPES pH 7.4
   - Optional: P/S, NEAA, 1-glutamine

2. PBS-Tween
   - 1 liter PBS + 5 mls of 10% Tween 20 (polyoxyethelene Sorbitan Monolaurate - Sigma #P1379)
   - (PBS = 34 mls 1M K_2HPO_4 ;(Dibasic) + 6mls 1M KH2PO4 (Monobasic) +35g NaCl/4L)

A. Addition of Samples and Tropo Standards

- Use ELISA plates (Costar #3595) that are acid washed by soaking for 30 min. or more in 0.1N HOAC then rinsed with d H_20 and air dried.
- Dilute samples (with unknown amounts of tropo) in DME-LB. If you have no idea how much tropo is in samples, first try 1:5 serial dilutions of 1 replicate from each group before running all of samples.
- Add samples to plate at 100 μl per well (in duplicate) and keep track with "ELISA" Plate Key" printout. Try to avoid wells bordering on edge of plate.
- Run a tropo standard curve on each plate. This consists of tropo elastin diluted in DME-LB from 8 ng/100ul to 0.25 ng/100 ul, as well as a 0 ng point (=DME-LB), added in duplicate at 100 μl per well. Also include a total of 4 wells that will be used the NRS controls: 2 well of Blank Media (=DME-LB) and 2 wells of 8 ng (or highest standard point).

B. Incubation and Washing of Plate

- Cover plate with parafilm and incubate at 37ºC for 1.5 hours. Pour out wells.
- Wash 2-3 times with PBS/Tween. Pour out and dry (fill all wells).

C. Primary Antibody

- Dilute Antibodies 1:500 with PBS Tween. Add 100 μl of NRS diln to 0 & 8 ng wells in duplicate and 100 ul of Tropo antibody diln to all other wells.
- Repeat Step B.

D. Secondary Antibody

- GAR-HRP (Sigma #A6154, Rack 1-5). Dilute 1:8000 in PBS/Tween and add 100 ul to all wells used.
- Repeat Step B with an extra wash.

E. Substrate Reaction

- Remove needed amounts of ABTS stocks (KPL Cat #50-62-00) from bottle and let warm to room temperature. Mix equal volumes of A and B.
- Add 100 μl/well and let color develop for 15 min. If want to extend or shorten development, record time developed.
- Stop reaction by adding 50 μl/well of 10mM Sodium Azide (NaN_3).
- Read on ELISA Reader with 410 nm filter.

F. Calculations

- Plot standard curve and drop nonlinear points at upper end of curve (usually 8ng and sometimes 6ng). Determine equation of line and perform linear regression on samples (only those with absorbance values within linear range).
- Calculate amount of tropo in samples figuring in dilutions used:
  ie. Ng Tropo/ml = (ng tropo/100 ul) x (10) x Dilution from regression

I. Preparation of Standards

The following is a step by step procedure for making 12 sets of standards between 0.25 and 10 ng/100 ul, which is the typical range of sensitivity for the assay. You may want to make different dilutions or make more than 12 sets at a time.

- Once tropo is thawed, make up standards without delay to prevent unnecessary degradation. Also, do all measurements for dilutions containing tropo with polypropylene (pp) tubes and pipette tips to minimize tropo loss to glass or plastic ware. It is not a bad idea to use similar precautions with all samples used for ELISA but degradation and sticking of tropo does not seem to be as much of a problem in the presence of serum.
- Make up a stock solution of 2 μg/ml purified tropoelastin in DMC-LB.
- Add 1 ml of stock to 11.5 mls of DME-LB for highest standard of 16ng/100μl. Then do 1:2 serial dilutions (6 mls of DME-LB + 6 mls of previous standard) through 0.25ng/100μl.
- The 6 ng/100μl standard is made separately by adding 200μl of 2μg/ml stock to 6.5 mls of DME-LB.
- You now have 6 mls or more of each standard. Aliquot into labeled microtiter tubes at 450 μl per tube, so that you have a complete rack (12 sets) of standards and freeze until needed. Each set of standards is enough to run on two plates in duplicate.
- Save the remaining 2μg/ml stock frozen in 1.5 ml aliquots (enough to make another rack of standards).

II. Preparation of Samples

1. Media and Solutions Required

1.2. Cell Growth Medium (See Gibco catalogue for detailed recipes)

- DME (HG/HB) as a base
- 10% Calf Serum (or FCS)
- P/S

- NEAA
- 1-glutamine

2. Treatment or Conditioning Medium
Same as Growth Medium except cross-linking inhibitors should be added to keep Tropoelastin soluble:

- 100 μg/ml ßAPN (ß-amino propionitrile)
- 50 μg/ml penicillamine (or abrev. To Penm.)

Both ßAPN and pencillamine should be added fresh. Make 1000X stocks (10% and 5% respectively) in PBS, filter sterilize aliquot into sterile tubes and freeze.

Add any treatment chemicals, such as Heparin, SMEF, growth factors etc. to this media.

3. 0.25 mgs/ml Pepstatin A (100x for extraction)

- 25 mgs Pepstatin A per 100 mls

Dissolve in 100% EtOH and store at -20ºC in 3 ml aliquots.

4. HOAc Extraction Buffer
Final Concentration for 1 L

- 0.5 N HOAc - 20mls Glacial Acetic Acid
- 2.5μg/ml Peps.A - 10 mls Pepst. A stock (add fresh)

Only add Pepstatin A to the volume of 0.5N HOAc that will be used that day. (i.e. 500 ul Pepst. A for 50 mls)

III. General Information

- In most cell types used (FBC, FCL & SMC for instance) virtually all of the elastin is secreted into the media when the cross-linking inhibitors ßAPN and Penicillamine are added. Therefore, only the cell conditioned culture media needs to be tested under these conditions. However, if a cell type or treatment has not been looked at before, the cell matrix should also be looked at for elastin levels. For example, heparin increases the percent of tropo in matrix to greater than 50% of total. It is essential to add ßAPN and Penicillamine to cell conditioning medium for FBCs and other cells that are efficient elastin cross-linkers in order to keep most of the elastin soluble in the culture medium. FCLs do not cross-link elastin under normal culture conditions; however, in most cases it will not hurt to add ßAPN and penicillamine as a precaution.

IV. Cell Culture

- General Information: Typically, elastin-producing cells (FBCs, SMCs or old FCLs for example) can be passed into 24 well plates in the range of 4 x 10^4 to 1.5 x 10^5

(SMCs, FCLs or similar sized cells plated at 1 x 10^5 will be confluent in 1 to 2 days). Just after visual confluency, elastin-producing cells should produce enough elastin in 1.0 ml of media per day to be easily detected on EIA. Low elastin producing cells (such as Control Adventitia or younger FCL cells) may need longer conditioning times, smaller volumes of conditioning media or larger numbers of cells (in larger T.C. dishes) for the EIA to be sensitive enough.

- Most of our experiments are set up once the cells are confluent.
- Cell Maintenance: Pass cells into 24 well T.C. plates (or other appropriate T.C. dish). Feed cells biweekly with Cell Growth Medium (adding any treatment chemicals, if applicable) until they are ready to be assayed (i.e. cells are confluent).
- Cell Treatment: Typically, experimental treatment of cells done in triplicate if only the medium needs to be assayed. In that case, the media from all wells are assayed and 2 or 3 of the cell layers are used to normalize the elastin levels. If elastin in the cell matrix needs to be determined, then 4 or more wells are done per treatment. The conditioned media from 3 or 4 wells and the cell matrix extracts from 2 or more wells are assayed for elastin. The remaining 2 wells of cells are used to normalize to cell number or total protein content.
- Once confluent, feed each well with Treatment or Conditioning Medium and record volume and time added (800 μl - 1.0 ml works well). Leave medium on for 1 to 3 days, depending on experiment and keep track of the number of hours conditioned.

V. Assaying for Tropo Levels from Various Culture Fractions

- All volumes etc. for following assays are based on cells grown in 24 well plates. If using other sized T.C. ware, adjust volumes appropriately.

- Cell Conditioned Medium: After treatment, remove medium and transfer to microtiter tubes (BioRad Cat. #223-9390) or another small sized polypropylene tubes. Freeze, unless there is time to set up an ELISA that day. Feed cell layers with 1 ml EBS and store in T.C. incubator (up to a few hours) until ready to process cell layer for any of the following assays.
- Nothing (besides thawing and mixing) needs to be done with medium unless running undiluted and color (pH) is off. In that case, add 30 μl of 1M HEPES (or 12 μl of 2.5M) per ml after removing from cells, but before adding to ELISA plate.
- If there is something added to the conditioning medium (i.e cells treated with a chemical) that may interfere with EIA, run the std curve under the same conditions (or at least a couple of points on the std. curve to make sure that it doesn't effect the assay).

- Cell Matrix Extract: Wash each well with 1 ml of PBS.
- Scrape cell layer from each well with cell scraper into 200 μl of HOAc Extraction Buffer and transfer to a small polypropylene tube (microtiter tubes are easiest for subsequent steps if there are a lot of samples).
- Extract overnight in a rotor rack at 4ºC. Samples can be frozen before or after this step if desired.
- After extraction, neutralize samples by adding the following 200 μl of sample:

200 μl DME *HG/LB) + 20% CS + Hepes
100 μl 0.91 N NaOH (1 ml dH_20 + 10 mls 1N NaOH)

- After addition of first 2 reagents, check color. If yellow add NaOH (5 μl at a time). If pinkish purple, pH is on alkaline side and needs to be adjusted with HCl. If the color is orangish red the pH is just right. Don't worry if sample is not exactly the right color because the following reagents should bring it to the correct pH if sample is within the sensitive range of the color indicator, phenol red.
  50 μl 1M Hepes pH 7.4
  50 μl DME (HG/LB) + 20% CS + Hepes
- This will give a 600 μl sample in 100mM Hepes + ~10% CS.

- Trypsinized Cells:

VI. Assays for Normalizing Tropo Levels

- Cell Counts:

- Burton DNA Assay

- Total Protein Synthesis