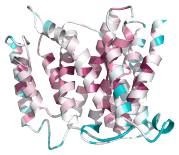
PROVOST & WALLERT RESEARCH

Investigating the Biochemistry & Cellular Physiology of NHE1
EST. 1998

MMP Zymogen Gel Assay
OPTIMIZATION TEST



INTRODUCTION: *Proteolytic degradation and remodeling of the extra cellular matrix (ECM) is largely controlled by a superfamily of Zn(2+)-dependent extracellular enzymes called matrix metalloproteinases (MMPs). So far, 15 MMPs have been cloned and characterized from humans. Gelatinases A and B, also known as 72 kDa and 92 kDa type IV collagenases (MMP2 and **MMP9**) digest denatured collagen (gelatin) and other ECM components including basement membranes. Processing of the precursor yields different active forms of 64, 67 and 82 kDa. Sequentially processing by MMP3 yields the 82 kDa matrix metalloproteinase-9.*

One method to assay MMP activity is to concentrate conditioned (serum free media – serum contains protease inhibitors) by ultrafiltration or lyophilization and running the protein on a native SDS PAGE gel saturated with gelatin (B-Casein). After electrophoresis, the gel is incubated in a buffer which includes triton X-100. The detergent and buffer allows the protein to renature and then the protease will hydrolyze the gelatin. After staining and destaining, clear bands indicate a protease is present. Molecular weight standards are required to learn the molecular weight of the active band.

Basic problem – these are enzymes and are fairly dilute so this presents two different challenges.

1. Being proteins, they will easily lose activity if not treated in a timely and appropriate manner.
   - ✓ Keep the solutions at or as close to ice cold (4°C) – in not on the ice and or in the fridge not on the bench at room temp.
   - ✓ Delays in using the enzyme will lead to a loss in activity and inconsistencies between assays/experiment.
   - ✓ If freezing the samples – only do one freeze thaw. i.e. once you freeze and thaw the sample, they must be used right away and not re-frozen.
   - ✓ Be consistent and follow protocol exactly – derivations will lead to inconsistent and loss of your time and our resources.
2. The enzymes are dilute – therefore to assay them we must concentrate them down. Using small volumes it is possible to lyophilize (dehydrate) the samples. BUT this is harder on the enzyme and can only be done with small volumes. If done with larger volumes, salts and metabolites increase in concentration along with the proteins and the gels do not run correctly. So we will work with a filtration method. Because the signal is low, we need to culture with more cells than 35 mm dishes. We will start with T25 flasks and if that isn't enough cells to give a good signal, we will then use T75 or 100 mm dishes.

PROTOCOL:

Preparing MMP Sample

1) Culture T25 flask to about 70% confluent. *See below for experimental details.*
2) Rinse cells with PBS and add ***exactly*** 4.5 ml of serum free media (see provost's notes on what to use). Add agonists as indicated. *We will see if a pre-starve is needed later.*
3) Incubate 24 hrs at 37 °C.
4) Collect exactly 4.00 ml of media into 15 ml falcon tube.
5) Centrifuge cells in (max RPM for 10 min at 4°C) to remove cell debris. Transfer media to new tubes.
6) Concentrate using the samples 2 ml at a time. Follow instructions for the devices. Spin at 4°C only. USE THE FIXED ANGLE CENTRIFUGE HEAD!
7) Adjust each sample to 50µl each using a pipet and PBS.
8) Add 10 µl of Zymogen Loading Dye. This is not the same buffers used for SDS PAGE GELS OR DNA GELS. **DO NOT HEAT SAMPLES BEFORE RUNNING GEL.**

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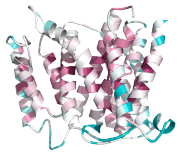

Zymogen Gel

1) Load samples onto zymogen using SDS-PAGE Running Buffer. Run until the blue Zymogen Loading Dye has reached the bottom of the gel (~90 min).
2) After electrophoresis, incubate gel at room temperature in 0.01% Triton X-100 for 1 hr with gentle rocking.
3) Decant solution and incubate gel in Zymogen Buffer 24 hours at 37°C.
4) Incubate 1 hour in Coomassee Blue Stain. *A stain with 0.5% (w/v) Coomassie Blue-R 250 may give better contrast.*
5) Incubate 1 hour in Destain or until the contrast of bands is obtained. Then remove destain and incubate in ddH_2O.

Zymogen Buffer (1L):
0.1M Tris-HCl pH 7.4
10 mM $CaCl_2$ (1.1g) check these
50 mM NaCl
2% Triton X-100
(Note – some use 0.02% Brij 35 buffer and or 1 μM Zn+)

Zymogen Loading Dye (5mL):
0.0625 mL 0.5M Tris pH 6.8
2mL 20%SDS
2g sucrose
5mg bromophenol blue dye
QS with ddH_2O

Experiment 1 – Centricon Filtration.

Day 1 Cell Culturing.

- ✓ 1 T25 (50-60%) – 3 T25 keep all three. Do with H460, H358.

Day 2/3 Starving and agonist stim.

- ✓ Cells should be about 70% overall confluent.
- ✓ Remove media, rinse as above with PBS and replace with RPMI base (now called serum free RPMI)
- ✓ For each cell line:
  - o One flask is control and gets no stimulation/agonist
  - o One flask will get PE – Add PE to final conc of 50 μM. (should be 7.5 μl)
  - o One flask will get 5 μl of uPA
- ✓ Incubate exactly 24 hrs.

Day 4 Cell Culturing.

- ✓ Harvest media as described above
- ✓ Max g force (RCF) is 5,000g – need to calculate rpm for the centrifuge
  - o RCF = 1.118×10^{-5} X radius X $(RPM)^2$
  - o Radius = distance in centimeters from center of rotation to base of filtrate vial
- ✓ Add 2 ml of cleared media to centricon and centrifuge at 4000 x g for 10-30 min.
- ✓ When run down, add enough media until all 4 ml are being concentrated down to less than 50 μl.
- ✓ 45o rotor will have a final stop of 25 μl and a 28 o rotor is 50 μl.
- ✓ Don't forget to remove filtrate between spins
- ✓ Follow experiment as above