Secretome analysis : general recommendations and protocol

Version 3 Friday, 04 November 2016

Experimental design

We consider here the preparation of conditioned medium (CM) as proxy for the "secretome" of a cell line. Typically such experiments are performed in a comparative fashion, with as a minimum a control and a test condition in which we expect changes in the secretome.

Key factors to obtain suitable samples for MS analysis of secretomes are :

1) The reduction of serum protein background (BSA etc)
2) The minimization of cell death/cell lysis to reduce the background of proteins of intracellular origin leaked out
3) Maximizing amount of secreted proteins in the CM, while attaining goals 1) and 2)

For this, conditioned media are prepared by growing cells in DMEM or RPMI medium without FBS/FCS and without phenol red (PR). Cells should be kept in this medium for 12h-24h to allow secretion of factors in sufficient amounts.

If possible a given phenotype or secreted factor should be known for the system under study and can be used as positive control to monitor sample preparation and see if the desired phenotype is conserved in these culture conditions. LDH or other proteins can be measured in the CM to assess degrees of cell death.

Preliminary tests :

1. Wash cells 3x in FBS-free, PR-free medium. Put them back in culture in this same medium.
2. Check the state of the culture after 12h, 24h and measure % of death cells and amount of debris in the supernatant.
3. Assess the desired phenotype(s) of the cells after this treatment to see if it is the same as observed in normal culture conditions. If all these conditions are met it is possible to prepare samples for analysis.

Sample prep protocol - Derived from procedure by Sabine Waeber, Stamenkovich lab, 2013

Assume : starting samples are cells+conditioned media grown in FBS-free conditions. Typically 10-50 x 1e6 cells. The protocol below is for 15-20 ml CM.

NOTE !! Amicon steps may take a long time….start this sample prep in the morning !!

Concept : Concentrate SN, remove small molecules and exchange buffer using for example Amicon Ultra-15 Centrifugal Filter Units with a cutoff at 3 kDa (prod. Nr. UFC900308 Millipore)

Materials :

- Amicon Ultra-15 Centrifugal Filter Units
- Ammonium hydrogen carbonate (ammonium bicarbonate) buffer, 100 mM (pH is self-adjusted)

-------**steps** --------

Centrifugations (to be adapted to local equipment)

- 10’ at 300 x g (remove cells) -> swinging bucket rotor
- 10’ at 2000 x g (remove dead cells) -> swinging bucket rotor change 50ml tubes
- 30’ at 10000 x g (remove cell debris) -> fixed angle rotor for high speed (50 ml Falcon ok) Remember : collect SN gently with pipet, do not aspirate everything (keep the same tubes)
- (during 10 min spin at 300 x g) Wash cartridges with MilliQ H2O (12 ml, 30 min at 4000 rpm in cell culture room, 4°) CAVEAT : don’t let the membrane dry (1.5 ml residual)
- Start in Amicon with 10 ml SN 10 min at 4000 x g (swinging angle rotor), add 10’ + 10’ centrifugation to concentrate about 10x (until 1.5 ml) (keep flowthrough)
- Add the 10 ml residual SN, centrifugation 20’, 4000 x g, swinging rotor, add 6’+5’ (1.5 ml residual)
- Dilute in ammonium bicarbonate (100 mM) up to starting volume (add 12 mL)
- Centrifugation : start with 30 min at 4000 x g, swinging rotor, +5’ until 1.5 ml left
- Add again ammonium bicarbonate 12 ml -> 30 min at 4000 x g with swinging rotor, +10’ + 5’ + 5’ (+ 5’ for N1) + 4’ + 3’ (+3’ for N1 and N3) + 3’ + 3’ (+3’ for N1 and N3)-> repeat until 0.5 ml left (less than 0.5 ml) -> spin until 0.5 ml left = final sample
- Freeze sample at -20C and send for MS analysis
- *Remember : before trashing the Amicon columns, rinse membranes (every membrane twice) with 200 µl of ammonium bicarbonate and collect in a separate tube (=washes)*

Note : It is a good idea to prepare a total extract of the cells by solubilizing for example 2x10e6 cells in 100 ul SDS sample buffer.The extract can be analysed to have an overview of the total proteome and evaluate if proteins in the secretome are abundant or not in the cell. An enrichment factor can be calculated if desired.