Supplementary data – Protocol

An optimized approach to recover secreted proteins from fibroblast conditioned-media for secretomic analysis

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- Fibroblast cells extraction
  - Incubate the biopsies in 0.05% thermolysin overnight at 4°C.
  - Separate the epidermis from the dermis mechanically.
  - Isolate fibroblasts from the dermis after treatment with 0,2 IU/ml collagenase H.

- Fibroblast cells culture
  - Grow cells in DMEM (Dulbecco-Vogt 133 modification of Eagle's medium) supplemented with 10% Bovine Growth Serum (HyClone BGS), 100 IU/ml penicillin G and 25 µg/ml gentamicin in 5% CO_2 at 37°C from a concentration of 2,5 x 10^5 cells on tissue culture dishes (75 cm^2) to desired confluence (40 or 95% of confluence).

- Fibroblasts induction
  - Wash 3 times with 10 ml of phosphate-buffered saline (PBS).
  - Deprive fibroblasts of serum (DMEM supplemented with 100 IU/ml penicillin G and 25 µg/ml gentamicin).
  - Incubate for the desired time (24h or 48h) in 5% CO_2 at 37°C.
  - Collect the supernatants and centrifuge at 300g for 10 minutes (4°C). Keep at -80°C until use.

- TCA-DOC precipitation
  - Thaw on ice the supernatants in high-speed centrifuge tube.
  - Add 1% (v/v) of a 2% sodium deoxycholate solution to the supernatants and incubate on ice for 30 minutes after mixing.
  - Add 100% trichloroacetic acid to a final concentration of 7,5% (v/v) and incubate on ice for 60 minutes after mixing.
  - Precipitate the proteins by centrifugation (15,000g for 20 minutes at 4°C) and discard the supernatants.
  - Add 20 ml of 100% ice-cold (-20°C) acetone to the pellets, vortex gently and keep at -20°C for 5 minutes.
  - Centrifuge (15,000g for 5 minutes at 4°C) and discard the supernatants.
  - Add 5 ml of 100% ice-cold (-20°C) acetone, vortex gently and keep at -20°C for 5 minutes.
  - Centrifuge (15,000g for 5 minutes at 4°C) and discard the supernatants.
  - Air-dry the pellets in a chemical hood for 30 minutes and dissolve in 210 µl of Isoelectric Focusing (IEF) buffer, pH 8.5.
  - Vortex the samples, centrifuge at 15,000g for 10 minutes at room temperature and keep at -80°C until use.

- TCA-NLS-THF precipitation
  - Thaw on ice the supernatants in high-speed centrifuge tube.
  - Add 1% (v/v) of a 2% sodium layroyl sarcosine solution to the supernatants and incubate on ice for 30 minutes after mixing.
  - Add 100% trichloroacetic acid to a final concentration of 7,5% (v/v) and incubate on ice for 60 minutes after mixing.

- Precipitate the proteins by centrifugation (15,000g for 20 minutes at 4°C) and discard the supernatants.
- Add 10% of final volume of ice-cold (-20°C) tetrahydrofuran to the pellets, vortex until dissolution of the pellets and keep at -20°C for 5 minutes.
- Centrifuge (15,000g for 20 minutes at 4°C) and discard the supernatants.
- Add 10% of final volume of ice-cold (-20°C) tetrahydrofuran to the pellets, vortex until dissolution of the pellets and keep at -20°C for 5 minutes.
- Centrifuge (15,000g for 20 minutes at 4°C) and discard the supernatants.
- Air-dry the pellets in a chemical hood for 30 minutes and dissolve in 210 µl of Isoelectric Focusing (IEF) buffer, pH 8.5.
- Vortex the samples, centrifuge at 15,000g for 10 minutes at room temperature and keep at -80°C until use.

- Protein conjugation
  - Dilute the different CyDyes to a concentration of 400pmol/µl in dimethylformamide (DMF).
  - Adjust the samples to a pH of 8.5 with 2M NaOH.
  - Mix 30 µg of protein at 1 µg/ml with 1 µl of the desired CyDye.
  - Vortex the samples, centrifuge (12,000g for 30 seconds at room temperature) and keep on ice for 30 minutes in the dark.
  - Stop the reaction with 1 µl of 10mM L-lysine.
  - Vortex the samples, centrifuge (12,000g for 30 seconds at room temperature) and keep on ice for 10 minutes in the dark.
  - Immediately use or keep at -80°C for up to 3 months in the dark.

- 2D electrophoresis
  - Pool the conjugated proteins together (Cy2, Cy3 and Cy5).
  - Add 90 µl of the mix to 90µl of reduction solution, vortex and incubate for 15 minutes on ice in the dark (Table 2).
  - Centrifuge (21,000g for 30 seconds at room temperature) and collect the supernatant.

    - First dimension electrophoresis
      - Rehydrate the Immobiline 230 DryStrip pH 3-11 NL, 24 cm at room temperature overnight in 450 µl of a mix of the remaining reduced supernatant and rehydration solution (Table 2).
      - Migrate the strips following the protocol mentioned in table 3.
      - Recover the strips with Plus One DryStrip cover fluid during migration and keep them at -80°C until use.

    - Second dimension electrophoresis
      - Cast 10-18% gradient polyacrylamide gels (26 X 20 cm) with the DALT*six* gel caster and the DALT*six* gradient maker.
      - Cast the gels (light then heavy gel) then the moving solution following the specific recipe (Table 2).

- Cover the gels with 30% isopropyl alcohol and let polymerize for three hours.
- Wash the gels with apyrogenic water, cover them with conservation buffer (Table 2) and let polymerize for another two hours.
- Thaw at room temperature the strips and wash them 20 minutes under agitation in a DTT solution (Table 2).
- Discard the DTT solution and wash the strips 20 minutes under agitation in an iodoacetamide solution (Table 2).
- Discard the iodoacetamide solution and add the strips on top of the gels.
- Add an overlays solution over the strips to maintain them in place (Table 2).
- Migrate the gels with the Ettan DALT*six* large vertical system at a constant rate of 0.5W/gel for 1 hour then over night at a constant rate of 1.25W/gel in 1X running buffer (Table 2).
- Keep the whole system at 20°C with a water-cooling system.

- Gels reading
  - Read the gels with the appropriate reading system at the optimal photomultiplier voltage (PMT) for each one of them, for each gel analyzed.