2D-PAGE PROTOCOL FOR HeLa STANDARD

The samples - prepared by CILBiotech - are lysates from HeLa cells (kept growing at $5x10^6$ cells/ml under sonoperfused cytostat conditions using Ipratech software) in 'multiplex buffer' (30mM Tris pH 8.5, 7M urea, 2M thiourea, 4% CHAPS). Cells are lysed at 3E7 cells/ml, equivalent to around 8 mg/ml protein concentration. Thaw the samples at RT, do not heat. Occasionally, some apparently insoluble material is present at the bottom of the tube after thawing. This will dissolve quickly once the sample is vortexed at RT.

The protocol below describes the procedure for a 24 cm long, pH 4-7 gradient strip and fluorescent (Flamingo, SYPRO Ruby, Deep/Lava Purple,) post-staining. For other gradients/strip lengths or staining methods, other procedures might be optimal. Please contact us if you need advice.

1. Vortex the thawed samples and mix 35 µL of the sample with 465 µL of lysis buffer according to Rabilloud (see below) to which a trace of bromophenol blue has been added. Mix thoroughly and centrifuge for 5 min at 13'000 g (max speed Eppendorf).
2. For each 1st dimension IPG strip, distribute 480 µL of the sample into a slot of your rehydration chamber and apply the 24 cm pH 4-7 IPG strip on top (gel side down). Passive in-gel rehydration of the strips should take at least 6 hours, but recommended and most practical is rehydration overnight at room temperature.
3. Run IPG strips using the voltage gradient below. The IEF equipment is not critical but it is strongly recommended to at least initially stick to this voltage gradient..

   Voltage gradient: 3 h 300 V

   5h: linear gradient 300-3500 V

   18 h at 3500 V.
4. After completion of the IEF, remove strips from the IEF instrument and equilibrate 12 min with equilibration buffer (see below) to which DTT has been added to a final concentration of 2%, and then 6 min with equilibration buffer to which iodoacetamide has been added to a final concentration of 5%.
5. The second dimension for the reference images was run on 20 x 25 cm large, 1.5 mm thick Tris-Glycine/SDS gels with 12 % T, 2.6 % C in a Laemmli Tris/Glycine/SDS running buffer (detailed composition see below), run overnight at 15 °C. Running conditions were 2 h at 8 mA constant current per gel followed by 16 mA per gel until the bromophenol dye front reaches the end of the gel. Using this gel size and these acrylamide/crosslinker concentrations will facilitate - automatic - alignment and comparison with the reference images.
6. Reference gels were stained with SYPRO Ruby according to the following protocol:

   i) In all steps, incl. the staining itself, we use 5-6 gels per plastic tray in a total volume of approximately 1 liter, i.e. 150 - 200 ml

ii) Remove gels from cassettes and fix for 3 h in ethanol:acetic acid:H_2O (40:10:50)

iii) Wash 3 times 30 min in H_2O

iv) Transfer gels to a new tray filled with Sypro Ruby. Incubate overnight **in the dark** under careful shaking (50-80 rpm), just enough to see the gels move relative to each other.

v) Transfer gels to new tray with H_2O and wash **in the dark** for 60 minutes before scanning.

vi) Scan at 100 μ resolution and 16-bit on a fluorescence scanner using the appropriate settings, in our case the FLA-3000: excitation blue laser (473 nm), emission filter Y520, sensitivity F1000 (maximal). For other fluorescence scanners, such as PharosFX Molecular Imager System or Typhoon, similar settings can be used, with either the blue or green laser. **Again, for optimal automatic comparison, please use the indicated resolution (100 μ) and bit depth (16-bit)**.

Other fluorescent dyes such as Flamingo, Deep or Lava Purple can be used according to the manufacturers' instructions, with essentially the same result. Colloidal Coomassie will require a slightly higher loading for an optimal result. Adjust step 1 accordingly to use 50-60 μL of sample and 450 -440 μL of Rabilloud buffer, respectively.

For more detailed step-by-step protocols, tips and tricks please visit our protocol page on www.fixingproteomics.org

Reagent	Composition
Multiplex buffer	30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5
Rabilloud buffer	7 M urea, 2 M thiourea, 1% DTT, 4% (w/v) CHAPS, 2% Pharmalytes 3-10, (optional + a trace of Bromophenolblue)
Equilibration buffer	50 mM Tris, pH 8.8, 6 M urea, 2% SDS, 30% Glycerol, 0.01 % Bromophenolblue.
Laemmli system	
Gel composition	12%T, 2.6% C acrylamide (from a 30:0.8 acrylamide /bisacrylamide stock), 0.375 M Tris pH 8.8, 0.1% SDS
Running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3 (usually from a 10 x stock, **do not adjust the pH with acid or base**)