2-D Western: 7 cm strip (rehydration loading) for mini-gel

1. Sample preparation

- either lyse cells directly into IEF sample buffer, or perform precipitation, and resuspend pellet in sample buffer; keep salts and other ionic compounds to a minimum; load amount to get strong Western signal in 1D gel (if known)
- sample buffer should be freshly supplemented with maximally 10 mM DTT before use if using DeStreak, 20 mM DTT if not

2. Rehydration sample loading

- thaw rehydration buffer stock
- for each strip, combine rehydration buffer plus sample volume to make a total of 125 µl, including the following:
    - DTT (fridge): 1.25 mg (8.1 µl of 1M stock), final conc. 1 % (w/v)
    - IPG buffer (fridge): 1.25 µl / 125 µl; 1 % (v/v) final conc.
- pipette complete sample into slot of focusing tray, noting position in tray
- take strip package from freezer
- take out strips one at a time with tweezers, remove thin plastic cover
- lower strip on top of sample, gel side down, “+” end facing towards “+” end of tray; avoid trapping bubbles
- cover slot with mineral oil; no need to fill entire tray
- cover with lid, rehydrate strips for at least 10 hours at RT
- [for active rehydration loading, place tray in IEF machine and apply 30 to 50 V for duration of rehydration]

3. IEF run

- turn on BioRad Protean IEF unit (switch on back of instrument, by power cord)
- go to program from previous run and check parameters, or program new
- recommended conditions for 7 cm, pH 3-10 NL (GE handbook):

step	300 V	0:30 h (or longer)
gradient	1000 V	3000 Vh
gradient	5000 V	4000 Vh
step	5000 V	500 – 2000 Vh
[step]	[500 V]	[12:00 h]*
		(total of 5000 – 6500 Vh)

  * optional step in case you are not around when the program ends; note total Vh when run finishes

  (BioRad recommendations: rapid ramp, 0 – 4000 V, total 8 to 10 kVh)
- start run by pushing ‘Start’ button on instrument

- when run is complete, stop program by pushing 'Stop' if using 500 V step;; take out strips using tweezers, letting excess oil drip off, or blotting briefly on a fresh Kimwipe
- continue directly with second dimension, or store strips at -80˚C in 15 ml conical tubes or equilibration trays
- clean tray carefully

4. Gels for second dimension

- cast or purchase mini-gels with straight top (one "well"), or use comb to create one small sample well, and one large strip well
- thickness at least 1 mm, to accommodate strip
- stacking gel is not required

5. Equilibration for SDS-PAGE

- thaw Equilibration buffer stock at room temperature
- prepare 2.5 ml each of Equilibration buffers 1 and 2 for each strip:
    - buffer 1: 2.5 ml stock plus 12.5 mg DTT (5 mg/ml)
    - buffer 2: 2.5 ml stock plus 112.5 mg iodoacetamide (45 mg/ml)
- equilibrate strip for 15 min in each buffer, using conical tubes or equilibration tray on horizontal shaker at RT; (frozen strips go directly into buffer, no extra thawing)
- during equilibration, prepare or melt agarose for sealing strips

6. Loading strips on gels

- for loading, leave gels outside gel tank
- rinse well(s) with running buffer
- [clip ends of strip if using sample well comb]
- take strip with tweezers, acidic (plus) end to the left, place plastic backing side on longer glass plate, push into well until flush with top of gel, without disturbing gel surface or trapping bubbles
- overlay with 0.5% low melt agarose in running buffer
- allow one minute for agarose to set
- put gels into tank, fill with running buffer, [load marker, if using well]

7. Running the second dimension gels

- recommended run conditions for 1 mm thick gels:
    - entry phase, 10 mA/gel, 15 min, then separation, 20 mA/gel, 1:30 h, or until BPB front has almost run out (GE 2D Handbook)
    - **or** 200 V constant, about 35 min (BioRad protocol)

Recipes

Sample Buffer (Cell lysis solution)

Reagent	Quantity		Final concentration
Urea (MW 60.06)	10.5g	4.2g	7M
Thiourea (MW 76.12)	3.8g	1.52g	2M
CHAPS (MW 614.89)	1g	0.4g	4% (w/v)
Tris (1M, not pH'ed)	750µl	300µl	30mM
H_2O MilliQ	to 25ml	to 10ml	

Rehydration Buffer Stock

Reagent	Quantity	Final concentration
Urea (MW 60.06)	10.5g	7M
Thiourea (MW 76.12)	3.8g	2M
CHAPS (MW 614.89)	1g	4% (w/v)
Bromophenol Blue (1% stock)	50µl	0.002% (w/v)
H_2O MilliQ	to 25ml	

SDS Equilibration Buffer Stock

Reagent	Quantity		Final concentration
Tris (1.0M, pH8.0)	20ml	40ml	100mM
Urea (MW 60.06)	72.07g	144.14g	6M
Glycerol (99.5% [v/v], MW92.09, density 1.26g/cm^3)	60ml/75.6g	120ml/151.2g	30% (v/v)
SDS (MW288.33)	4g	8g	2% (w/v)
Bromophenol Blue (1% stock)	400µl	800µl	0.002% (w/v)
H_2O MilliQ	to 200ml	to 400ml	

SDS-PAGE Running Buffer, 10x

Reagent	Quantity	Final concentration
Glycine (MW 75.07)	1152g	1.92M
Tris (MW 121.1)	242g	250mM
SDS (MW 288.38)	80g	1% (w/v)
H_2O, destilled/RO	to 8l	