Proteomic Analysis Protocol (v2022) : GSU Proteomics Core Facility

Part I: Overview

Part II: Experiment Protocol

Part III: Working Protocol

Part I: Overview

2 Dimensional Gel Electrophoresis.

1. 1st Dimension **I**so**E**lectric**F**ocusing (IEF)
   a. Protein sample labeling with Cy-dye
   b. Load labeled sample into sample loading try
   c. Isoelectricfocusing

2. 2nd Dimension SDS PAGE
   a. Preparing 2nd Dimension SDS Gel
   b. Equilibrate IEF Strip
   c. Load onto 2nd dimension gel
   d. SDS-PAGE

3. Visualize Protein spots and Quantitation
   a. Scan gel with Cy3 and Cy5
   b. Disassemble gel sandwich
   c. Stain with Coomassie Blue and destain
   d. Scan for Coomassie blue staining

Part II: Experiment Protocol

Amount of protein needed for each gel is depended on the methods of visualization:

- Cydye labeling: (25~50μg/sample)
- Fluorescence Stain: (50~100 μg/sample)
- Coomassie blue Stain: (250~400μg/gel)

I. 1st dimention IEF (IsoElectricFocusing)

1. Sample Labeling with Cy-dyes

i. label tubes for Sample A and Sample B
ii. add 25μg of protein samples each tubes (sample A or Sample B)
iii. add 1μl of 500mM Tris-HCl(pH 8.8) to raise pH above 8.0
iv. add 1μl of Cy3 or Cy5 to each tube and briefly mix and spin down
v. incubate on Ice for 30 min
vi. stop labeling with 1.2 μl of 10mM Lysine and briefly mix and spin down
vii. leave on ice at least for 10 min
viii. add 2X SB to each tube to the equal volume of the total reaction volume
ix. combine two tubes into one tube

Experiment Protocol:

2. Optional (add unlabeled Protein Sample)

- If the gel is going to be stained with Coomassie blue after scanning for fluorescence.
  i. calculate volume of sample needed for total of 350µg sample
     - Sample (350µg -50µg (2 X 25 µg) =300µg
  ii. add appropriate amount of unlabeled sample(s)
  iii. add equal volume of 2X SB to unlabeled protein sample volume

3. IEF sample Preparation and Run.

i. add **Rehydration Buffer** to the final volume of 360µl (need to be calculated)
ii. load all combined sample (360µl) into gel-strip loading boat
iii. remove protective cover from dried gel-strip (**Do Not Touch Gel-side**)
iv. place gel-strip onto loading boat with gel-side facing down
(Do not let gel touch any dried surface)
v. remove any visible air bubbles if any
vi. cover with (700 µl/18cm, 850µl/24cm) cover fluid solution
vii. place the loading boat onto IFE apparatus
viii. Start IEF program (usually, 24hrs)

- **IEF Run Program:** GE_24_4-7_Kyu
  1. 75uAmp Rehydration 16 hrs
  2. Step, 500V, 500Vhr
  3. Grad, 1000V, 800Vhr
  4. Grad, 10000V, 16500Vhr
  5. Step, 10000V, 13700Vhr
  6. Step, 300V, 3:00 hr

II. 2nd Dimension SDS PAGE.

1. **Glass plate preparation**.

   *****(During glass plate treatment, avoid cross contamination between bind silane and repel treatments)**

   i. **<u>Bind Silane treatment</u>** (should wear gloves); shorter glass plate without spacer
   - clean glass plate with 100% ethanol and dd-H_2O
   - place marker sticker at the sides of glass plate (side and middle with spacer space)
   - apply ~4ml bind silane mixture to glass plate
   - spread evenly and let it dry for 45 minutes
   - buff with lint-free paper (until <u>no trace of haze</u>)

   ii. **<u>Repellant treatment</u>** (should wear gloves); longer glass plate with spacer
   - clean glass plate with 100% ethanol and dd-H_2O
   - apply 0.8ml of repellant and spread evenly
   - let it dry for 10-15 minutes
   - buff with lint-free paper tower
   - rinse with dd-H_2O and air dry

II. 2nd Dimension SDS PAGE.

2. Gel casting

- place the treated glass plates in sequence into casting chamber
- insert plastic separator between each pair of glass plates
- place cover plate and secure with screws and claps
- gently pour the gel mixture avoiding trapping air bubbles inside gel solution
- remove trapped air bubbles if any by gentle tapping.
- overlay with ~2 ml of **Isopropanol** to each gel with minimally disturbing the gel surface
- after ~1 hours, once gels are solidified, pour off Isopropanol and wash with ddH_20
- overlay with 1X SDS PAGE running buffer

3. Running 2nd Dimension SDS Gel.

i. Equilibration of IEF strip

- from overnight IEF, take the loading boat out from IEF instrument.
- pick the gel strip at one end and drip excess cover fluid solution
- place gel strip into a petri-dish (the gel side facing inward)
- equilibrate with 20ml/strip of **EB I** (Equilibration Buffer I) for 20 minutes with agitation.
- pour off the EB I.
- equilibrate with 20ml/strip of **EB II** (Equilibration Buffer II) for 20 minutes with agitation. (add a bit of Bromo phenol powder into EB II buffer before apply to gel strip)
- pour off the EB II.

ii. Loading to 2nd Dimension SDS and run

- wash equilibrated strip with 100 ml of SDS Running buffer
- place the gel strip onto top of the 2nd dimension SDS-PAGE gel
- overlay with 0.5% of low melting Agarose (0.5% agarose in SDS running buffer)
- place gel assembly into running chamber and fill the chamber with SDS running buffer
- start run, 2-10 Amp/gel over-night
- following day, gradually increase power up to 30 Amp/gel
- when running indicator dye (Bromo phenol) is reaching at the bottom of gel, stop sun

III. Visualization of Gel and Image Analysis

1. Scan the gel with Typhoon scanner for Cy3 and Cy5
2. Stain with Coomassie Brilliant Blue (Simple Blue).
    - separate glass plate with a wedge and place the gel into plastic container
    - wash gel with ~300ml of ddH_20 for 10 min, repeat 2 more times
    - Stain with ~ 300 ml of "Simply Blue" Coomassie staining dye for 1~2 hours
    - destain with ~ 300ml of ddH_20 until the background become clear with change of ddH_20

IV. Buffers and Reagents

- **S/R stock Buffer:** 7M Urea
  2M Thiourea
  4% CHAPS

- **2X Sample Buffer (2X SB):**
  - 3% DTT and 3% IPG in S/R stock buffer

- **Rehydration buffer:**
  - 1.5% DTT and 1.5% IPG in 1X SB
  - Dilute 2X SB 2 times with S/R stock buffer

- **Bind silane mixture (for 10 ml)**

100% Ethanol:	8ml
ddH_20:	1.8ml
Acetic Acid:	0.2ml
Bind Silane:	**20µl**

- **Repel solution**

- **SDS Gel mixture (10% Polyacrylamide solution, for 30ml)**

ddH_20:	11.9 ml
1.5M Tris-HCl (pH 8.8):	7.5ml
Protogel (30% PA):	10ml
10% SDS:	0.3ml
10% APS:	0.3ml
TEMED:	**12µl**

- **Equilibration Buffer:** 50mM Tris-HCl (pH 8.8)
  6M Urea
  30% glycerol
  2% SDS

- EB 1: (1% DTT in EB)

- EB 2: (4.5% Iodoacetamide in EB)

- **1X SDS PAGE Running Buffer**
  -dilute 10 times from 10X running buffer

  10X Running Buffer: 250mM Tris-HCl (pH8.8)
  1.92M Glycine
  1% SDS

Part III: Working Protocol

1. Experimental Conditions:

i. Sample(s): A: E. coli cell extracts CA5 (Concentration; 4µgµ/l)

B: E. coli cell extracts CG5 (Concentration; 4µg/µl)

ii. Cydye labeling: 25µg/sample

iii. Coomassie blue Stain: total of 350µg

2. Experimental Design:

i. Group 1:
- Sample A: CA5 (25µg) Cy3
- Sample B: CG5 (25µg) Cy5

ii. Group 2:
- Sample A: CA5 (25µg) Cy5
- Sample B: CG5 (25µg) Cy3

iii. Group 3:
- Sample A: CA5 (25µg) Cy3
- Sample A: CA5 (25µg) Cy5

3. Experiment:

Day 1.

1) Sample Labeling: (Samples are in 4µg/µl)

Sample	sample vol	500mM Tris-HCl (pH8.8)	Cy dye	10mM Lysine	2X SB Equal to reaction volume	**Final Rx volume**
Sample A	(_____)µl	1µl	Cy3-1µl	1.2µl	(_____)µl	**(_____)µl**
Sample B	(_____)µl	1µl	Cy5-1µl	1.2µl	(_____)µl	**(_____)µl**

Incubate on ice for 30 min **Incubate on ice for 10 min**

– **Combine 2 tubes into one**.

2) Add unlabled Protein Sample

– Calculate volume of sample needed for 350µg of sample(s) (for coomassie staining)

– Sample (350µg –50 (2x 25µg) = 300µg/ 4µg/µl _____**µl**

– add **37.5µl** (150µg)of each sample A and B into tube

– add **2X Sample buffer** (equal volume to unlabeled protein sample volume, _____**µl)**

3. Experiment:

Day 1.

3) IEF sample Preparation and Run.

- add Rehydration buffer, _____µl = **(360µl -** _____**µl** - _____**µl** - _____ µl)

Final Vol **2X labeling Rx** **sample vol** **2X SB vol**

- **2X Sample Buffer:(3%** IPG Buffer (pH range 4-7), 3% DTT in S/R buffer s)

  Total 2X Sample buffer: ( ) µl, → ( ) µl IPG (pH4-7), ( ) mg DTT

- **Rehydration buffer:**(1.5% IPG Buffer (the same pH range to 2X Sample buffer), 1.5% DTT in S/R buffer)

  1:1 dilution of 2X sample buffer with S/R stock buffer

  Total Rehydration buffer: ( ) µl; → 2X SB( ) µl and S/R Stock buffer ( ) µl

4) Loading sample to IEF

- load all combined sample (360µl) into gel-strip loading boat
- place gel-strip onto loading boat with gel-side facing down (**Do not let gel touch any dried surface)**
- remove any visible air bubbles if any
- cover with 850µl (24cm) cover fluid solution
- place the loading boat onto IFE apparatus
- Start IEF program: GE_24_4-7_Kyu

Day 2.

5) Glass plate preparation

*****(During glass plate treatment, avoid cross contamination between bind silane and repel treatments)**

i. Prepare Bind silane solution for 20ml

100% Ethanol:	8ml x2 =	ml
ddH_20:	1.8ml x2 =	ml
Acetic Acid:	0.2ml x2 =	ml
Bind Silane:	**20µl x2 =**	**µl**

ii. <u>Bind Silane treatment</u> (should wear gloves); glass without spacer

- clean glass plate with 100% ethanol and dd-H_2O
- spread evenly bind silane (~4ml) and let it dry for 45 minutes
- buff with lint-free paper (<u>no trace of haze</u>)

iii. <u>Repellant treatment</u> (should wear gloves); glass with spacer

- clean glass plate with 100% ethanol and dd-H_2O
- apply 0.8ml of repellant and spread evenly and let it dry for 10-15 minutes
- buff with lint-free paper tower
- rinse with dd-H_2O and air dry

6) **SDS gel casting**.

i. **Prepare 10% APS solution (5ml)**

- 5ml x 0.1 = ( ) g of APS, dissolve in 5 ml of ddH_20

ii. **Prepare 10% PA solution (330ml)**

ddH_20:	11.9 ml x11=
1.5M Tris-HCl (pH 8.8):	7.5ml x11=
Protogel (30% PA):	10ml x11=
10% SDS:	0.3ml x11=
10% APS:	0.3ml x11=
TEMED:	**12µl** x11=

iii. **Casting gel(s)**

- Assemble gel casting cassette
- immediately after adding TEMED solution, pour gel solution to caster avoiding air bubbles trapping.
- Remove trapped air bubbles if any by gentle tapping.
- Overlay with ~2 ml of Isopropanol to each gel with minimally disturbing the gel surface
- After ~1 hours, pour off Isopropanol and wash with ddH_20
- Overlay with 1X SDS PAGE running buffer and cover with plastic wrap

7) Running 2nd Dimension SDS Gel.

i. Prepare EB 1 and EB 2 solution

- EB 1: (1% DTT in EB)

( )ml X 0.01(1%) = ( )g DTT

- EB 2: (4.5% Iodoacetamide in EB), add a little of Bromo phenol powder

( ) ml X 0.045 (4.5%) = ( )g Iodoacetamide

ii. Equilibrate IEF strip(s)

- From overnight IEF
- Pick the gel strip at one end and drip excess cover fluid solution
- Place gel strip into petri-dish (the gel side facing inward)
- Equilibrate with 20ml/gel of **EB I** (Equilibration Buffer I) for 20 minutes with agitation.
- Pour off the EB I.
- Equilibrate with 20m/gel of **EB II** (Equilibration Buffer II) for 20 minutes with agitation.
- Pour off the EB II.
- Wash equilibrated strip with 100 ml of SDS Running buffer
- Place the gel strip onto top of the 2nd dimension SDS-PAGE gel
- Overlay with 0.5% of low melting Agarose (0.5% agarose in SDS running buffer)
- Start run, 2-10 Amp/gel over night and increase upto 30 Amp/gel.

Day 3.

8) Scan the gel with Typhoon

9) Stain with Coomassie Brilliant Blue (Simple Blue).

- separate gel sandwich a wedge.
- place the gel into plastic container
- wash gel with ~300ml of ddH_20 for 10 min, repeat 2 more times
- Stain with ~ 300 ml of "Simply Blue" Coomassie staining dye for 1~2 hours
- destain with ~ 300ml of ddH_20 until the background become clear (change water frequently).