Pre-programmed Protocols in Machine Code Format

Protocol 1

IPROT,1,7cm broad&basic,D,0:00,20,20,500,6000,0.5
IPROT,1,7cm broad&basic,1,G,500,H,0:30
IPROT,1,7cm broad&basic,2,G,1000,H,0:30
IPROT,1,7cm broad&basic,3,G,6000,H,0:30
IPROT,1,7cm broad&basic,4,S,6000,V,8000
IPROT,1,7cm broad&basic,5,S,1000,H,1:00
IPROT,1,7cm broad&basic,6,S,0,H,0:00
IPROT,1,7cm broad&basic,7,S,0,H,0:00
IPROT,1,7cm broad&basic,8,S,0,H,0:00
IPROT,1,7cm broad&basic,9,S,0,H,0:00

Protocol 2

IPROT,2,7cm const Watt,D,0:00,20,20,500,6000,0.5
IPROT,2,7cm const Watt,1,W,0.1,H,1:00
IPROT,2,7cm const Watt,2,W,0.5,V,8000
IPROT,2,7cm const Watt,3,S,1000,H,1:00
IPROT,2,7cm const Watt,4,S,0,H,0:00
IPROT,2,7cm const Watt,5,S,0,H,0:00
IPROT,2,7cm const Watt,6,S,0,H,0:00
IPROT,2,7cm const Watt,7,S,0,H,0:00
IPROT,2,7cm const Watt,8,S,0,H,0:00
IPROT,2,7cm const Watt,9,S,0,H,0:00

Protocol 3

IPROT,3,18cm broad&basic,D,0:00,20,20,500,12000,1.5
IPROT,3,18cm broad&basic,1,G,1000,H,1:00
IPROT,3,18cm broad&basic,2,G,12000,H,1:00
IPROT,3,18cm broad&basic,3,S,12000,V,25000
IPROT,3,18cm broad&basic,4,S,1000,H,1:00
IPROT,3,18cm broad&basic,5,S,0,H,0:00
IPROT,3,18cm broad&basic,6,S,0,H,0:00
IPROT,3,18cm broad&basic,7,S,0,H,0:00
IPROT,3,18cm broad&basic,8,S,0,H,0:00
IPROT,3,18cm broad&basic,9,S,0,H,0:00

Protocol 4

IPROT,4,18cm narrow,D,0:00,20,20,500,12000,1.5
IPROT,4,18cm narrow,1,G,1000,H,1:00
IPROT,4,18cm narrow,2,G,12000,H,1:00
IPROT,4,18cm narrow,3,S,12000,V,50000
IPROT,4,18cm narrow,4,S,1000,H,1:00
IPROT,4,18cm narrow,5,S,0,H,0:00
IPROT,4,18cm narrow,6,S,0,H,0:00
IPROT,4,18cm narrow,7,S,0,H,0:00
IPROT,4,18cm narrow,8,S,0,H,0:00
IPROT,4,18cm narrow,9,S,0,H,0:00

Protocol 5

IPROT,5,18cm const Watt,D,0:00,20,20,500,12000,1.5
IPROT,5,18cm const Watt,1,W,0.1,H,1:00
IPROT,5,18cm const Watt,2,W,1.5,V,25000
IPROT,5,18cm const Watt,3,S,1000,H,1:00
IPROT,5,18cm const Watt,4,S,0,H,0:00
IPROT,5,18cm const Watt,5,S,0,H,0:00
IPROT,5,18cm const Watt,6,S,0,H,0:00
IPROT,5,18cm const Watt,7,S,0,H,0:00
IPROT,5,18cm const Watt,8,S,0,H,0:00
IPROT,5,18cm const Watt,9,S,0,H,0:00

Protocol 6

IPROT,6,24cm broad&basic,D,0:00,20,20,500,12000,2.0
IPROT,6,24cm broad&basic,1,G,1000,H,1:00
IPROT,6,24cm broad&basic,2,G,12000,H,1:00
IPROT,6,24cm broad&basic,3,S,12000,V,45000
IPROT,6,24cm broad&basic,4,S,1000,H,1:00
IPROT,6,24cm broad&basic,5,S,0,H,0:00
IPROT,6,24cm broad&basic,6,S,0,H,0:00
IPROT,6,24cm broad&basic,7,S,0,H,0:00
IPROT,6,24cm broad&basic,8,S,0,H,0:00
IPROT,6,24cm broad&basic,9,S,0,H,0:00

Protocol 7

IPROT,7,24cm narrow,D,0:00,20,20,500,12000,2.0
IPROT,7,24cm narrow,1,G,1000,H,1:00
IPROT,7,24cm narrow,2,G,12000,H,1:00
IPROT,7,24cm narrow,3,S,12000,V,100000
IPROT,7,24cm narrow,4,S,1000,H,1:00
IPROT,7,24cm narrow,5,S,0,H,0:00
IPROT,7,24cm narrow,6,S,0,H,0:00
IPROT,7,24cm narrow,7,S,0,H,0:00
IPROT,7,24cm narrow,8,S,0,H,0:00
IPROT,7,24cm narrow,9,S,0,H,0:00

Protocol 8

IPROT,8,24cm const Watt,D,0:00,20,20,500,12000,2.0
IPROT,8,24cm const Watt,1,W,0.1,H,1:00
IPROT,8,24cm const Watt,2,W,2.0,V,45000
IPROT,8,24cm const Watt,3,S,1000,H,1:00
IPROT,8,24cm const Watt,4,S,0,H,0:00
IPROT,8,24cm const Watt,5,S,0,H,0:00
IPROT,8,24cm const Watt,6,S,0,H,0:00
IPROT,8,24cm const Watt,7,S,0,H,0:00
IPROT,8,24cm const Watt,8,S,0,H,0:00
IPROT,8,24cm const Watt,9,S,0,H,0:00

Protocol 9

IPROT,9,24cm cup load,D,0:00,20,20,500,12000,2.0
IPROT,9,24cm cup load,1,G,1000,H,4:00
IPROT,9,24cm cup load,2,G,12000,H,6:00
IPROT,9,24cm cup load,3,W,2.0,V,64000
IPROT,9,24cm cup load,4,S,1000,H,10:00
IPROT,9,24cm cup load,5,S,0,H,0:00
IPROT,9,24cm cup load,6,S,0,H,0:00
IPROT,9,24cm cup load,7,S,0,H,0:00
IPROT,9,24cm cup load,8,S,0,H,0:00
IPROT,9,24cm cup load,9,S,0,H,0:00

Appendix B: Reagents and Solutions

Sample Preparation

Samples prepared for 2D should be fully denatured, free of insoluble material, and low in overall ionic strength.

Some protein samples will readily solubilize while others are more difficult, requiring additional reagents (such as thiourea, special detergents, *etc.*) to encourage solubilization. The common classes of additives are listed below.

Common classes of additives

Denaturants
Unfold the proteins to expose the internal native charges.

Non Ionic Detergents
Make samples more soluble without altering the protein charge.

Reductants
Help break internal disulfide bonds to further unfold the proteins, and help reduce the negative effects of oxidation of proteins during rehydration and IEF.

Other
Carrier amphoytes, proteases, DNases, RNases.

Denaturants

Urea is the most common reagent used in IEF for disrupting the internal bonding of the protein, allowing it to unfold. Samples are prepared using urea concentrations from 8–9.5 M. In general, the higher the urea concentration the better a sample can be solubilized. Urea reaches its saturation point near 10 M at room temperature.

Thiourea is also used to better solubilize some samples. Frequently, 2 M thiourea is combined with 5–7 M urea as a reagent for sample prep and IPG rehydration.

Detergents

Several types of non-ionic or zwitterionic detergents can be used to solubilize samples (CHAPS, Triton X100, Nonidet NP-40, and alkylamidosulfobetaine detergents). CHAPS is the most widely used detergent for 2D electrophoresis. It is stable in solution. Detergents such as SDS are not compatible with IEF because they bind to the proteins and mask the proteins' native charge.

Reductants

Dithiothreitol (DTT) is commonly used to reduce proteins in IEF. DTT breaks down in solution, so it is normally prepared and added just before use. Other reductants such as 2-Mercaptoethanol, Dithioerythritol (DTE) and Tributylphosphine (TBP) can be used.

Other

Carrier ampholytes or IPG buffers can be added to aid in protein solubility and help prevent protein precipitation during focusing. Concentrations of 0.5%–2% (v/v) are typically used. Carrier ampholytes may interfere with certain labeling experiments. In those cases, the reagents should then be omitted from the sample extraction step.

General Guidelines

- Sample preparation procedures are being refined and standardized. It is best to consult the literature to determine if a particular sample preparation buffer is recommended for the sample type.
- IEF works best with pure protein samples that are solubilized and denatured, and free of interfering molecules.
- Remove insoluble material with centrifugation.
- Keep the salt content as low as possible.
- Use freshly prepared reagents of high quality, or reagent solutions that have been stored frozen.
- Do not leave urea solutions out at room temperature for extended periods of time.
- Never heat protein samples in urea solutions. Heating cause carbamylation of proteins and will alter the native charge of the proteins.
- Keep samples on ice to prevent degradation.
- Add protease inhibitors to prevent protease activity. Protease inhibitors such as PMSF or Pefabloc can be added to inhibit serine protease activity while pepstatin can inhibit aspartic proteases.
- Some protocols use basic carrier ampholytes or Tris to get a high pH in the sample buffer. This helps solubilize some proteins and lowers enzyme activity that would attack the proteins.
- DNA and RNA can frequently be removed by ultracentrifugation. The sample can also be treated with DNase and RNase solution to break down the contaminants.
- Keep in mind that some protease inhibitors, DNase and RNase are proteins themselves and may show up on a 2D map.
- The water used for making reagents should be the highest quality available. Water with a resistivity of >5 megaohm-cm is best. Water purified by reverse osmosis or deionized water is acceptable.

Note: Salts are perhaps the most common contaminant causing poor IEF results.

Recipes

IEF Sample Extraction Buffer for 2D

1A. Urea Sample Buffer Solution

Prepares 25 ml
9.5 M urea, 4% CHAPS

	Final Concentration	Amount
Urea (FW 60.06)	9.5 M	14.26 g
CHAPS	4% (w/v)	1.0 g
Deionized water		to 25 ml

Store in 1 ml aliquots at -20 °C or below.

Prior to use, add 6 mg/ml DTT to get a final sample buffer composition of 40 mM DTT.

Optional: Add carrier ampholytes, such as SERVALYTS, to a concentration of 2% v/v (20 µl per ml sample buffer solution).

— OR —

1B. Thiourea + Urea Sample Preparation Solution

Prepares 25 ml
7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT

	Final Concentration	Amount
Urea (FW 60.06)	7 M	10.51 g
Thiourea (FW 76.12)	2 M	3.8 g
CHAPS	4% (w/v)	1.0 g
Deionized water		to 25 ml

Store in 1 ml aliquots at -20 °C or below.

Prior to use, add 6 mg/ml DTT to get a final sample buffer composition of 40 mM DTT.

Optional: Add carrier ampholytes, such as SERVALYTS, to a concentration of 2% v/v (20 µl per ml sample buffer solution).

IPG Strip Rehydration Solution

2A. Urea Rehydration Stock Solution

Prepares 50 ml
8 M urea, 2% CHAPS, 0.002% bromophenol blue

	Final Concentration	Amount
Urea (FW 60.06)	8 M	24 g
CHAPS	2% (w/v)	1.0 g
Bromophenol blue	0.002%	1 mg
Deionized water	—	to 50 ml

Store in 3 ml aliquots at -20 °C or below. 3 ml is sufficient to rehydrate six 24 cm IPG strips.

Just prior to use for IPG strip rehydration:
- Add 0.5–2.0% (v/v) carrier ampholytes (SERVALYTS).
- Add 9 mg DTT per 3 ml aliquot of rehydration stock solution 20 mM DTT.
- Protein sample can also be added to the 3 ml of rehydration solution.

2B. Thiourea Rehydration Stock Solution

Prepares 50 ml
7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue

	Final Concentration	Amount
Urea (FW 60.06)	7 M	21 g
Thiourea (FW 76.12)	2 M	7.6 g
CHAPS	2% (w/v)	1.0 g
Bromophenol blue	0.002%	1 mg
Deionized water	—	to 50 ml

Store in 3 ml aliquots at -20 °C or below. 3 ml is sufficient to rehydrate six 24 cm IPG strips.

Just prior to use for IPG strip rehydration:
- Add 0.5–2.0% (v/v) carrier ampholytes (SERVALYTS).
- Add 9 mg DTT per 3 ml aliquot of rehydration stock solution 20 mM DTT.
- Protein sample can also be added to the 3 ml of rehydration solution.

3. SDS Equilibration Buffer Solution

This solution is used after IEF, and before second dimension PAGE. The IPG strips are immersed in excess solution to raise the pH of the strip buffer so that it is suitable for PAGE, and to coat the proteins in SDS uniformly so that they migrate properly in the second dimension gel.

Note: IPG strips should be equilibrated just prior to second dimensin PAGE. Do not equilibrate the IPG strips before storing at -20 °C.

Prepares 200 ml
6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue

	Final Concentration	Amount
Urea (FW 60.06)	6 M	72.1 g
1.5M Tris-HCl, pH 8.8 stock solution	75 mM	10.0 ml
Glycerol (87% w/w)	29.3% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophenol blue	0.002% (w/v)	4 mg
Deionized water		to 200 ml

Aliquot into 30 ml aliquots and store frozen at -20 °C or below.

24 cm IPG's require 5 –10 ml per strip per equilibration step. Shorter strips can use proportionately less volume per equilibration step.

Equilibration Procedure

1 Thaw two aliquots of the equilibration solution.

2 Add 10 mg/ml DTT to one solution.

3 Place the IPG strips in the rehydration/equilibration tray.

4 Add 6.5 ml of solution to each slot containing an IPG strip.

5 Place on rocker for 10–15 minutes.

After equilibration, discard the first equilibration solution in an appropriate manner.

6 Add 25 mg/ml Iodoacetamide (IAA) to the second aliquot of equilibration solution.

7 Add 6.5 ml of solution to each slot containing an IPG strip.

8 Place on rocker for 10–15 minutes.

After equilibration, discard the second equilibration solution in an appropriate manner.

Following equilibration, the IPG strips are placed on the top of the second dimension gel, and sealed into place with the agarose overlay.

Agarose Overlay
1% Agarose in 1X Electrophoresis Buffer

Prepares 100 ml
1% agarose, 25 mM Tris, 192 mM glycine, 0.1% SDS
Prepare in a 500 ml flask to allow room for foaming.

Caution! SDS may cause the solution to boil over so exercise caution when heating and prevent boiling over.

	Final Concentration	Amount
Agarose	1%	1 g
10X Electrophoresis Buffer (250 mM Tris, 1.92M Glycine, 1% SDS)	1X	10 ml
Bromophenol blue		3 mg
Deionized water		to 100 ml

Gently swirl to suspend agarose.
Heat at low power in a microwave oven until agarose is fully dissolved.
Store in 1.5 ml aliquots at 4 °C in plastic screw top tubes.
Reheat aliquots in heating block.

Appendix C: References IEF100

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