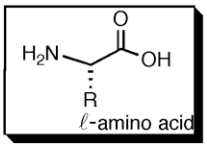
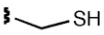
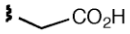
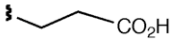
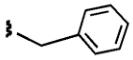
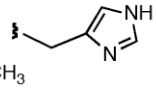
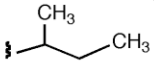
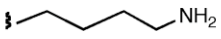
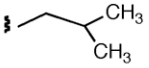
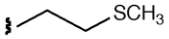
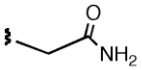
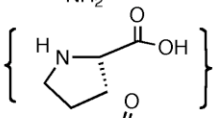
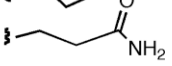
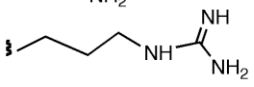
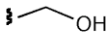
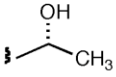
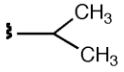
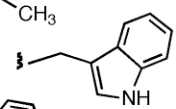
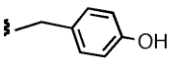
APPENDIX 1. AMINO ACIDS AND THE GENETIC CODE

Structures of the 20 amino acids



Abbreviations		Name	Side chain (R)
A	ala	alanine	
C	cys	cysteine	
D	asp	aspartic acid	
E	glu	glutamic acid	
F	phe	phenylalanine	
G	gly	glycine	
H	his	histidine	
I	ile	isoleucine	
K	lys	lysine	
L	leu	leucine	
M	met	methionine	
N	asn	asparagine	
P	pro	proline	 (this is the whole amino acid, not a side chain)
Q	gln	glutamine	
R	arg	arginine	
S	ser	serine	
T	thr	threonine	
V	val	valine	
W	trp	tryptophan	
Y	tyr	tyrosine	

Amino Acid Categories

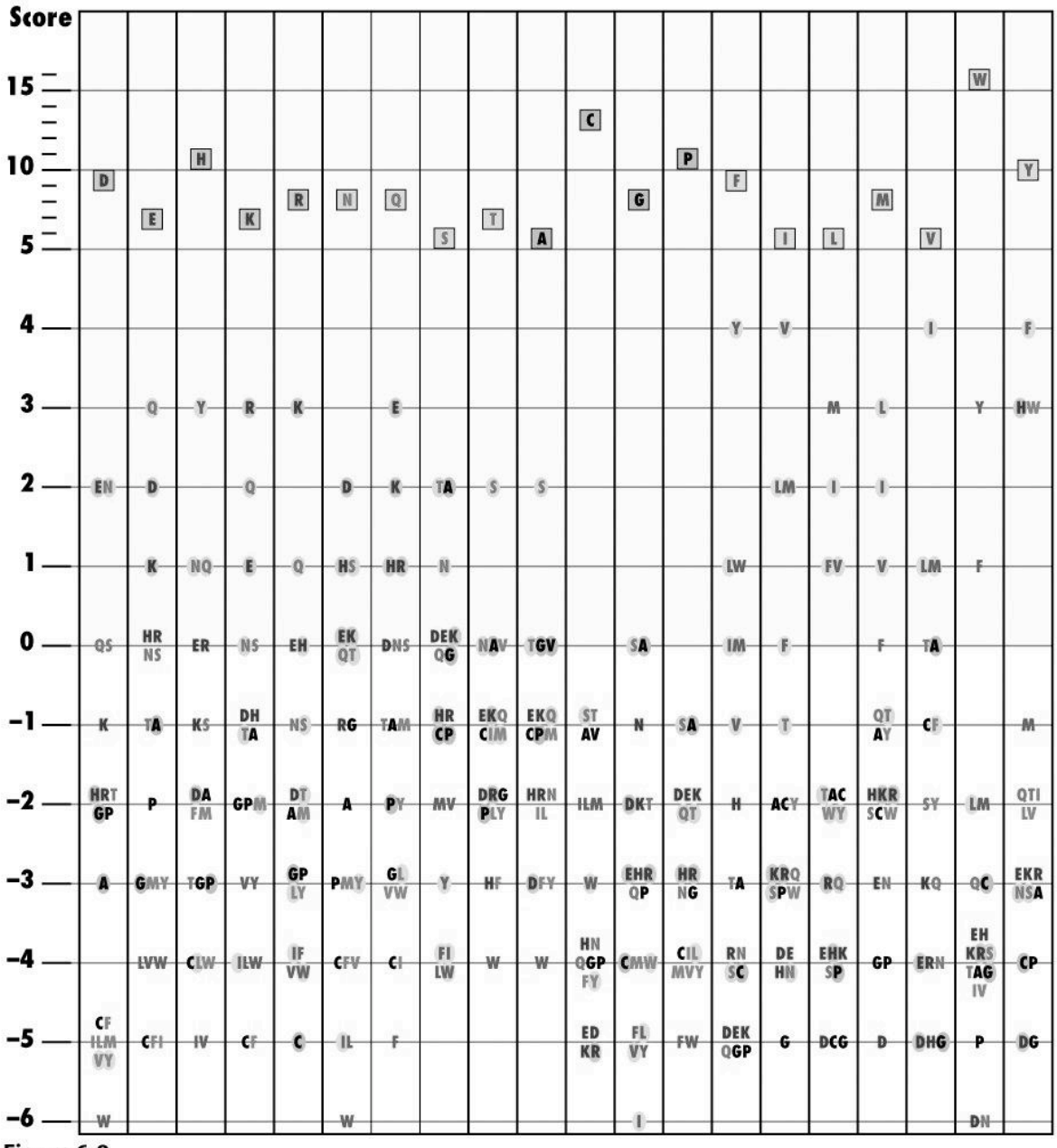
Hydrophobic: Ala, Val, Leu, Ile, Phe, Met, Trp, Gly
Acidic: Asp, Glu
Basic: Arg, Lys, His (pH of side group is 6)
Polar Uncharged: Ser, Thr, Cys, Tyr, Gln, Aln

Blosum-62 Substitution Matrix

The Blosum-62 substitution matrix shown on the next page describes the degree to which specific amino acids are substituted for other amino acids. The matrix was derived by examining substitutions that occur within aligned sequence blocks in related proteins. The matrix gives two different types of information: 1) how likely each amino acid is to be conserved and 2) if it is not conserved, which amino acids are most likely to replace it.

1. At the top of each column in the matrix is an amino acid that is boxed. The score of the boxed amino acid describes how likely it is that the boxed amino acid will be conserved. A higher score indicates that it is less likely for the residue to be substituted with another; the amino acid is more conserved. For example, aspartic acid (D, column 1) has a higher score than alanine (A, column 10) and a lower score than tryptophan (W, column 19). This means that aspartic acid is substituted less frequently than alanine but more frequently than tryptophan. In other words, tryptophan is more conserved than aspartic acid and alanine is less conserved than aspartic acid.

2. At the top of each column in the matrix is an amino acid that is boxed. The column describes how likely it is that the boxed amino acid will be substituted by an amino acid further down the column. The larger the score for a particular amino acid, the more likely the substitution is to occur. For example, in the first column, the boxed amino acid is aspartic acid (D). Because glutamic acid (E) has a higher score in this column compared to glutamine (Q), it is more common to see substitution of an aspartic acid with glutamic acid than with glutamine.


Score
15
10
5
4
3
2
1
0
−1
−2
−3
−4
−5
−6
D
E
H
K
R
N
Q
S
T
A
C
G
P
F
I
L
M
V
W
Y
EN
D
K
HR
NS
QS
K
TA
HRT
GP
A
GMY
LVW
CF
ILM
VY
W
CFI
Q
Y
R
K
NQ
E
Q
ER
KS
DA
FM
TGP
CLW
IV
DH
TA
GP
M
VY
ILW
NS
CF
E
K
EH
NS
DT
AM
GP
LY
IF
VW
C
D
Q
HS
HR
EK
QT
RG
A
PMY
CFV
IL
W
E
K
HR
DNS
TAM
PY
GL
VW
CI
F
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DEK
QG
HR
CP
MV
Y
FI
LW
S
NAV
EKQ
CIM
DRG
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W
T
S
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EKQ
CPM
HRN
IL
DFY
W
A
ST
AV
ILM
W
HN
QGP
FY
ED
KR
C
SA
N
DKT
EHR
QP
CMW
FL
VY
I
P
SA
DEK
QT
HR
NG
CIL
MVY
FW
F
IM
V
H
TA
RN
SC
DEK
QGP
Y
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LM
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ACY
KRQ
SPW
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G
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FV
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TAC
WY
RQ
EHK
SP
DCG
M
L
I
V
F
QT
AY
GP
EN
D
I
LM
TA
CF
SY
KQ
ERN
DHG
W
Y
F
LM
QC
EH
KRS
TAG
IV
P
DN
Y
HW
QTI
LV
EKR
NSA
CP
DG

Figure 6-9

The Genetic Code:

		First Base			
		T	**C**	**A**	**G**
Second Base	**T**	TTT Phe F TTC Phe F TTA Leu F TTG Leu F	CTT Leu L CTC Leu L CTA Leu L CTG Leu L	ATT Ile I ATC Ile I ATA Ile I ATG Met M (*start*)	GTT Val V GTC Val V GTA Val V GTG Val V
	C	TCT Ser S TCC Ser S TCA Ser S TCG Ser S	CCT Pro P CCC Pro P CCA Pro P CCG Pro P	ACT Thr T ACC Thr T ACA Thr T ACG Thr T	GCT Ala A GCC Ala A GCA Ala A GCG Ala A
	A	TAT Tyr Y TAC Tyr Y TAA *Stop* TAG *Stop*	CAT His H CAC His H CAA Gln Q CAG Gln Q	AAT Asn N AAC Asn N AAA Lys K AAG Lys K	GAT Asp D GAC Asp D GAA Glu E GAG Glu E
	G	TGT Cys C TGC Cys C TGA *Stop* TGG Trp W	CGT Arg R CGC Arg R CGA Arg R CGG Arg R	AGT Ser S AGC Ser S AGA Arg R AGG Arg R	GGT Gly G GGC Gly G GGA Gly G GGG Gly G

APPENDIX 2: SODIUM DODECYLSULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins can be separated according to size using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). A polyacrylamide gel consists of a solid gel matrix that is made by polymerizing acrylamide and bisacrylamide. A protein sample is denatured by mixing it with SDS, which is an anionic detergentand β-Mercaptoethanol which reduces disulfide bonds. The denatured protein is loaded onto the gel matrix and electrophoretically driven across the gel.

The rate at which a protein travels through the polyacrylamide is determined both by its size and by its mass-to-charge ratio. Proteins that are larger will travel more slowly through the gel, as will proteins with lower charge densities. SDS binds to protein in a ratio of approximately 1.4 g SDS per 1.0 g protein, resulting in an approximately uniform mass-to-charge ratio for most proteins. Furthermore, denaturing of the protein removes any three-dimensional structure that could affect movement of the protein through the gel. Thus, the rate of migration through the gel is (almost) directly related to the molecular weight of a protein. Smaller proteins migrate towards the anode (the anode is positively charged and therefore attracts anions) faster than larger proteins.

We will be using pre-cast SDS-PAGE gels from Bio-Rad. Basic directions for running a gel are given below. Each gel has 10 lanes. One will contain the molecular weight ladder and the rest will contain your samples. Before proceeding, determine the order in which your samples will be loaded in the lanes and record this clearly in your notebook. If you are running more than one gel, you might want to load the ladder in different lanes so you can differentiate the gels if they are switched.

Step 1: Preparing the gel and rig

The figure below shows the components of the gel-running apparatus (the rig). When putting the gel assembly together, the seal between the green gaskets on the electrode assembly (EA) and the cassettes must not have any leaks. It is not uncommon to have a slow leak that is not obvious. To make sure you do not have a leak, put the gel assembly together and fill with buffer well before (about 30 min) loading your samples. Before heating your samples, check whether the level of the buffer has changed, indicating the presence of a leak.

1. A gel cassette consists of a gel sandwiched between two plates. Remove the cassette from its packaging and rinse in water to remove azide (an anti-fungal preservative).
2. Use a razor to cut the tape on the gel cassette bottom. A black line indicates where to cut.

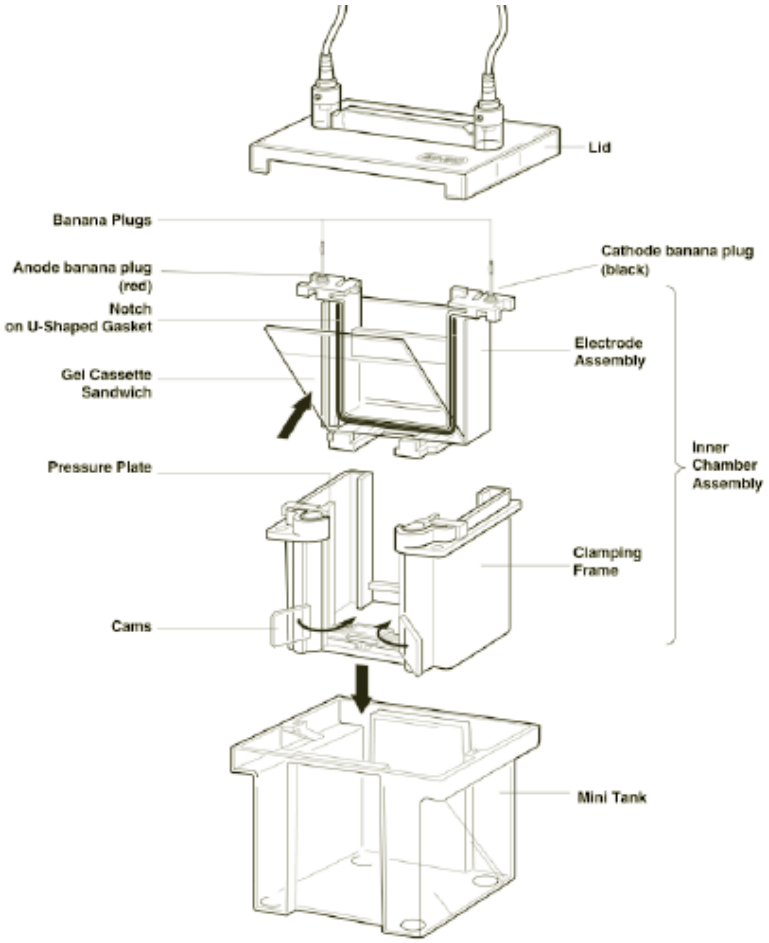


FIGURE 5 – *Bio-Rad protein-gel running assembly for running Ready gels*

3. Peel the plastic from the bottom of the cassette.
4. Carefully, remove the comb from the top of the cassette by gently pulling on one side of the comb, the other side, and repeating until the comb slides smoothly out of the gel. This will help you avoid damaging the lanes.
5. Place a gel cassette in the slots on each side of the Electrode Assembly (EA). The smaller plates of the cassettes should face inward. When running only one gel, place a Buffer Dam on one side of the EA.
6. Push the cassettes flush against the gaskets lining the sides of the EA.
7. Open the cam levers of the Clamping Frame. Slide the EA into the Clamping Frame, and close the cam levers. The cassettes now form a tight seal with the EA.
8. Lower the gel assembly into the Mini Tank.
9. Fill the inner chamber with SDS-PAGE running buffer until the level reaches halfway between the inner and outer plates of the gel cassette.
10. Add ~200 mL of the running buffer to the outer tank, until the wire at the bottom of the EA assembly is immersed.

Step 2: Preparing samples

1. Set a heat-block to 95°C and fill the wells with water using a squirt bottle. Prepare this early so that it is ready when you need it.
2. Add 62.5 µl β-Me to 500 µl 5X SDS-PAGE sample buffer (SB) to make SB+β (final concentration 2.5%). SB+ β must be made fresh daily.
3. Combine SB+β and sample in a 1:4 ratio (1 µL of buffer for every 4 µL of sample). The total sample volume must be ≤20 µL.
4. Heat samples to 95°C for 5 min.
5. Briefly spin the sample in a tabletop centrifuge to collect any liquid that has condensed under the lid.
6. Prepare 10 µL of the protein ladder per gel. This sample contains proteins with known molecular masses. To prepare 20 µl, combine 1 µl ladder, 4 µl SB+β, and 15 µl ddH_2O. Do not boil the ladder. The molecular weights of the proteins in the ladder are xxxxx.

Step 3: Running the Gel

1. Load the samples into the gel using a P20 pipetter. Pipet slowly to avoid losing sample.
2. Place the lid on the Mini Tank, matching the color of the jacks on the lid to the color of the banana plugs on the electrode assembly.
3. Connect the electrical leads into the power supply – being careful to match red-to-red and black-to-black.
4. Run gels at a constant voltage of 200 V for ~30 minutes. The blue dye will form a tight band that travels down the gel faster than (almost) all proteins. The tight blue band should run down the gel in a straight, horizontal line. If the band starts "smiling" (with the edges traveling slower than the middle), it is a sign that the current is heating up the middle of the gel. Immediately decrease the voltage by 30%.
5. Stop the voltage when the dye band is at the very bottom edge of the gel.

Step 4: Removing the gel

1. Turn off the power and remove electrical leads from the power supply.
2. Prepare one tray per gel (1 mL pipet-box lid) with about 100 ml ddH_20
3. Remove the tank lid, carefully remove the inner chamber, and discard the running buffer from the inner chamber. (Opening the cams before discarding the buffer lead to a mess).
4. Open the cams, remove the EA from the Clamping Frame, and remove the gel cassettes from the EA.
5. Place a razor between the two plates of the cassette, and gently separate the two plates by prying them apart. The gel will adhere to one of the two plates. The gel is susceptible to ripping, so be very careful.
6. Run the sharp edge of the razor between the gel and plate to detach the gel. Lift the gel gently off the plate and place it in the water. Wetting the gel and plate can help with this process.

Step 5: Staining the gel

We will use Simply Blue SafeStain from Invitrogen. Unlike home-made Coomassie stain, Simply Blue contains no methanol or acetic acid, and can thus be disposed of in the sink. The microwave procedure takes less than 15 min and yields very sensitive results. Do not allow the staining solution to boil.

1. Place the gel, which is in 100 ml of ddH_20, in a loosely covered container and microwave on High (950 to 1100 watts) for 1 minute, until the water almost boils.
2. Shake the gel for 2 min and discard the water.
3. Repeat Steps 1 and 2 two more times, for a total of 3 water washes.
4. After the last wash, discard the water and add 20 ml of SimplyBlue and microwave on High until the solution almost boils (45-60 sec). Do not overheat.
5. Shake the gel for 5 min and discard the stain.
6. Wash the gel in 100 ml of ddH_20 for 10 min while shaking, and discard the water. You will be able to see distinct bands now.
7. Add 20 ml of 20% NaCl and shake for at least 5 minutes. The gel can be stored for several weeks in the salt solution.

Step 6: Drying and preserving the gel

Drying the gel will allow you to store it in your notebook. The protocol can be used for IEF gels as well as SDS-PAGE gels. You will need:

- 2 pieces of cellophane, both just larger than the size of the gel-frames.
- Gel frame with its center removed (frame #1)
- Gel frame without its center removed (frame #2)
- 4-6 steel clamps

1. Wet one piece of cellophane thoroughly on both sides and place on frame #2.
2. Smooth out the cellophane. Remove *all* air bubbles between the cellophane and gel. Trapped air bubbles will cause your gel to crack. To remove stubborn air bubbles, lift up a corner of the cellophane and slowly bring it back down onto the frame. Let it re-adhere to the plastic frame, starting at the center of the frame and moving outward to the edge. Use a squirt bottle to add additional water between the cellophane and plastic frame. You might need a lot of water.
3. Center the gel on top of the cellophane. Use the squirt bottle to add an excess of water to the top of the cellophane.
4. Wet the second piece of cellophane thoroughly on both sides, and place on top of the first. The gel is now sandwiched between the two pieces of cellophane.
5. Smooth the second piece of cellophane, and remove all air bubbles (use the hints from step #2).
6. Place frame #1 on top of the second piece of cellophane. The gel and cellophane are now sandwiched between the two frames.
7. Clamp the assembly with the binders. Use one clamp on the short sides and two on the long sides.

8. Place the assembly in the hood. The circulating air will help the gel dry evenly. Let the cellophane dry overnight, or as long as is required to dry the cellophane.
9. Remove the binders and carefully remove the cellophane from the frames.
10. With scissors, cut away the excess cellophane from the preserved gel, leaving about half-an-inch of cellophane around the gel. Tape the preserved gel into your notebook.

APPENDIX 3: ISOELECTRIC FOCUSING GELS

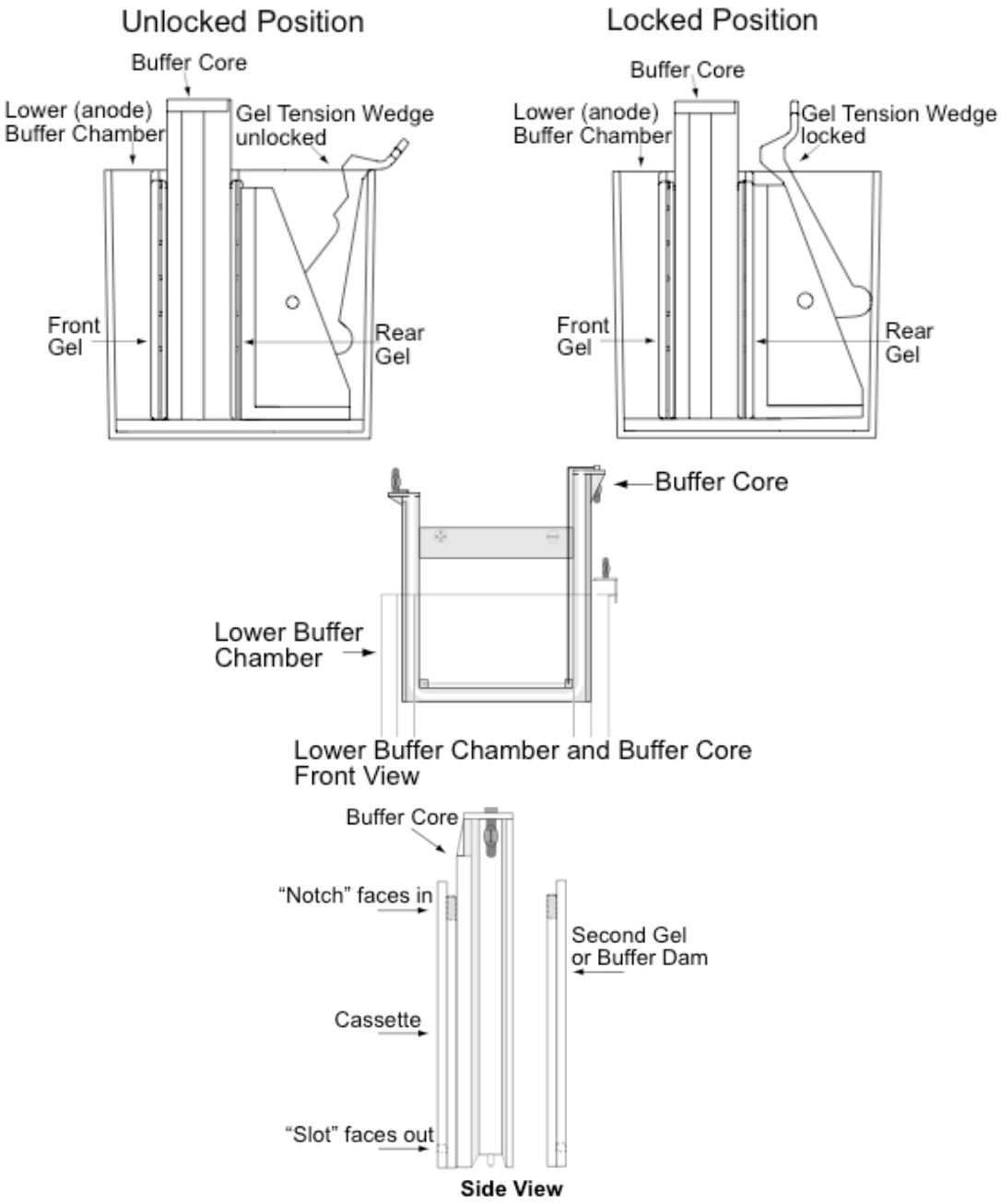
An isoelectric focusing gel (IEF gel) can be used to separate proteins with different isoelectric points (pI). The pI is the pH at which a protein has a net charge of zero, and it depends on the number of acidic and basic residues in the protein.

Step 1: Setting up the gel

We will be using the Invitrogen *SureLock Mini Cell* electrophoresis systems. Look at your rig and the figure on the next page, and make sure you can identify all parts. A gel cassette consists of a polyacrylamide gel sandwiched between two plates.

1. Cut open the gel cassette pouch and remove the cassette. Rinse the gel with water.
2. Peel off the tape covering the slot on the back of the cassette.
3. *Carefully*, remove the comb from the top of the cassette, avoiding damaging the lanes. This is best done by gently pulling on one side of the comb, and then the other. Repeat until the comb slides smoothly out of the gel.
4. Use a pipet to wash the wells of the cassette with *IEF cathode running buffer*.
5. Lower the gel apparatus Buffer Core into the Lower Buffer Chamber. The negative electrode will fit in the opening in the gold plate on the Lower Buffer Chamber.
6. Insert the Gel Tension Wedge into the gel chamber behind the Buffer Core. Be sure the Gel Tension Wedge is in its unlocked position.
7. Place the gels in the Lower Buffer Chamber, one behind and one in front of the Buffer Core. The wells of the cassettes should face towards the Buffer Core. Use the Buffer Dam instead of a gel if you are running only one gel.
8. Push the Gel Tension Wedge into the locked position. This forms a seal between the cassettes and Buffer Chamber.
9. Fill the Upper Buffer Chamber with ~200 mL of *IEF cathode running buffer*. The buffer must completely cover the wells.
10. Fill the Lower Buffer Chamber with ~600 mL of *IEF anode running buffer*. Pour the buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber.

When putting the assembly together, the seals between the cassettes and Buffer Core are prone to leaks. It is not uncommon to have a slow leak that is not obvious. To help identify such leaks, fill the Upper Buffer Chamber with the *IEF cathode running buffer*, and let the gel assembly sit for some time (~15 minutes). If the level of buffer in the chamber has lowered, you have a leak. Undo the assembly, remove the gel, rinse the gels and reassemble.


Unlocked Position
Buffer Core
Lower (anode)
Buffer Chamber
Gel Tension Wedge
unlocked
Front
Gel
Rear
Gel
Locked Position
Buffer Core
Lower (anode)
Buffer Chamber
Gel Tension Wedge
locked
Front
Gel
Rear
Gel
Buffer Core
Lower Buffer
Chamber
Lower Buffer Chamber and Buffer Core
Front View
Buffer Core
"Notch" faces in
Second Gel
or Buffer Dam
Cassette
"Slot" faces out
Side View

Step 2: Loading samples and running the gel

1. Prepare your samples. For each, combine 25 µl sample with 25 µl *IEF loading buffer*. You will load 25 µl of the resulting sample per gel. For the standard, add 4 µl of the undiluted stock to the lane.

   Because IEF samples contain no dye, the only way to see where your sample is going is through the *schlieren lines*. These are the wavy lines observed when mixing two liquids with different indices of refraction. The effect is caused by changes in the index of refraction of the mixing solution. Where your sample begins mixing with the buffer in the gel's lane, you will observe these lines. To help you see them more clearly, it may help to shine a light directly above the gel apparatus and look at the gel from a steep angle.
2. Place the lid on the Buffer Core. The lid is asymmetric, and can only fit onto the Core in one configuration
3. Connect the electrical leads into the power supply – being careful to match red-to-red and black-to-black.
4. Run gels at a constant voltage of 100 V for 60 minutes, 250 V for 60 minutes, and then 500 V for 30 minutes. The current should stay in the range of 5-15 mA/gel throughout.

Some of the proteins in the *IEF standard samples* are pre-stained and will be visible migrating down the gel.

Step 3: Removing and staining the gel

One of the gels will be stained for all proteins with *Coomassie + Crocein IEF gel stani.* The other will be stained for tyrosinase activity with catechol. Catechol is reduced by tyrosinase, and the product polymerizes into a brown product (melanins) that can be visualized on the gel. **Wear gloves throughout this procedure.**

1. Turn off the power and remove electrical leads from the power supply.
2. Before removing the gels from the gel cassettes, prepare protein gel stain. You will need an empty 1 ml pipette-tip box and lid for each gel.
   - For the protein-stained gel, pour 40 ml protein stain in a box.
   - For the catechol-stained gel, pour 40 ml loading buffer in a box. First incubating the gel in loading buffer adjusts the pH so that it is amenable to tyrosinase activity staining.
3. Remove the lid from the gel apparatus and unlock the Gel Tension Wedge. Remove the cassettes.
4. Lay the cassettes well-side up on a flat surface and gently insert the beveled edge of the gel knife into the gap between the plates. Push up and down until you hear cassette crack. Repeat this on all sides of the cassette until the two plates are completely separated.
5. Separate the plates. The gel will stick to one of them. Remove the gel from the plate, and place in a gel box. If the gel is on the shorter, notched plate, use the gel knife to cut off and remove the thick bottom of the gel. Then use the knife to pry one corner of the gel from the plate, and allow the gel to peel away from the plate into the stain. If the gel is on the longer plate, hold the cassette plate over the gel box containing the stain. Gently push the knife

through the slot in the cassette, and let the gel peel away from the plate. Cut the foot of the gel off before drying the gel.

6. Put lids on the boxes, and place on a shaker for 15-30 minutes.
7. For the catechol-stained gels, after 15 minutes, pour out the loading buffer and replace with 30 ml Catechol stain. Let the gel incubate for an additional 15-30 minutes on the shaker.

Step 4: Destaining the gel

1. Pour the stain into the appropriate waste bottle (Used IEF gel stain or 5 mM catechol). Be careful to not pour the gel into the bottle. The Coomasie+Crocein stain can be reused.
2. Rinse the gel in the box with water to remove excess stain. When you dump this water, the stains are now dilute enough that you can pour them down the sink.

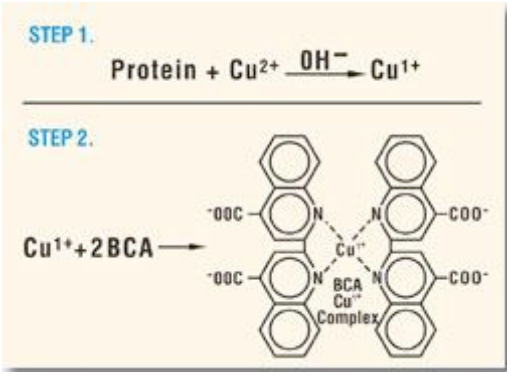
For gels stained with catechol, simply fill the box with 10 mM sodium phosphate, pH 7. The gel can be stored for a week in this buffer. Scan the gel using the scanner or photograph with the digital camera. Finally, dry the gel according to the protocol in appendix ##.
For gels stained with protein stain:

1. Fill the bottom of the box with 20-50 mL of protein gel destain (enough to sufficiently cover the gel). Crumple 1-2 Kimwipes, and place them in the corner of the box. The KimWipes will trap the dye while the gel is destaining on the shaker.
2. Microwave each box individually for 30 seconds on the high setting.
3. Destain for ~1 hour, or as long is necessary to visualize the bands. If necessary, old gel destain can be replaced with fresh gel destain to speed up this process. Do not leave the gel in destain overnight. Pour used gel destain in the appropriate waste bottle. This cannot be reused again. The destained gel can be stored in water.
4. Scan the gel using the scanner or photograph with the digital camera.
5. Dry the gel according to the protocol in appendix 2.

APPENDIX 4: PROTEIN QUANTIFICATION WITH BICINCHONINIC ACID

To measure protein concentration, you will use a bicinchoninic acid (BCA) assay. Your protein sample is mixed with Cu^{2+} (cupric) and BCA under alkaline conditions. The proteins chelate Cu^{2+} and form a blue colored complex (λ_{max} =540 nm). This is followed by reduction of Cu^{2+} to Cu^{1+} (cuprous) by peptide bonds, cysteine, tryptophan, and tyrosine (STEP 1 in the figure). Because the amount of reduction has been demonstrated to be proportional to protein concentration, this step alone (known as the Biuret assay) can be used to determine protein concentrations in the 5 mg/ml to 160 mg/ml range. This first step is extremely temperature and time dependent.

The BCA then reacts with the Cu^{1+}, with two molecules of BCA chelating one ion (STEP 2 in the figure). This product is purple while free BCA is apple-green. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing Cu^{1+} concentrations. The second step makes the assay over 100 times more sensitive then a Biuret assay, allowing for measurement of protein concentrations in the range of 20 μg/ml to 2 mg/ml.



In order to determine the protein concentration of an unknown sample, a standard calibration curve is generated using bovine serum albumin (BSA). A series of known concentrations of BSA are prepared and assayed alongside the unknown protein samples. This is crucial since the measured absorbances are highly dependent on timing and temperature.

You will be using the Pierce BCA Protein Assay kit.

Step 1: Making the dilution series

Make the six standards listed below. You will be given a stock solution with 2000 μg/ml BSA

Dilution #	[BSA] (μg/ml)	Buffer[1] (μl)	BSA (μl) (stock or dilution)
1	1000	100	100 of BSA stock
2	500	100	100 of dilution 2
3	250	100	100 of dilution 3
4	125	100	100 of dilution 4
5	62.5	100	100 of dilution 5
6	31.25	100	100 of dilution 6
7	0	200	-

Step 2: Setting up the reaction

1. Make dilutions of your samples. For each sample, you should make 3 diltions: a 1:0 (all sample) dilution, a 1/5 dilution (1 part sample, 4 part buffer), and a 1/25 dilution (1 part sample and 24 parts buffer). This will ensure that the protein concentration is in the proper range for at least one sample.
2. Determine how much Working Reagent (WR) you will need. One ml WR is needed for each standard and sample diltion. Only one group should make the WR per day; check with those around you before making it. Always make ≈5 ml extra.
3. Prepare WR by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B. For example, for 20 ml of WR, combine 400 μl Reagent B and 19.6 ml reagent A. Mix very well.
4. You will be preparing your reactions directly in cuvettes. Line up one cuvette per reaction. Make sure to indicate the order of your standards/unknowns in your notebook since you cannot label the cuvettes.
5. Add 50 μl of the appropriate sample into each cuvette.
6. Add 1 ml WR to each cuvette.
7. Seal with parafilm and mix well by inverting 6-8 times. You can use a single piece of parafilm for mixing all the cuvettes if you are careful to utilize an unused portion of the parafilm for each one. Alternately, use little squares of parafilm.
8. Store at 37°C (use the plate incubator) for 30 min.
9. Cool all cuvettes to RT (10 min).

Step 3: Measuring absorbance and calculating concentration

Color development is time-dependent, so work quickly once you begin this step.

1. Blank the spectrophotometer with ddH_2O
2. Measure absorbance at 562 nm (A562) for each cuvette.

[1] Use HIS-Elution buffer for the ZsYellow unknown samples and DEAE Loading Buffer for the tyrosinase samples.

3. Using Excel or another data analysis program, plot A562 as a function of protein concentration for your BSA samples. Fit the points to a line, and write down the fit parameters and R^2 value. Include the fit data in your lab notebook.
4. Use the equation generated above to determine the protein concentration of your various dilutions.
5. Picking the dilutions, that fall in the linear range, determine the concentration of your samples.

APPENDIX 5: SPECTROFLUOROMETER

We will be using the RF-1501 Spectrofluorophotometer from Shimadzu. It can be used to obtain two types of fluorescence scans: an excitation scan (EX) and emission scan (EM). In an excitation scan, the excitation beam scans through a range of wavelengths while the emission wavelength is held at a specific wavelength. In an emission scan, the sample is excited at a specific wavelength while emission is measured over a range of wavelengths. Instructions for obtaining scans and transferring them to a computer are given below. Turn the spectrofluorometer on 10-15 minutes before you will need to use it (they need time to initialize and warm-up), leave it on throughout the class time, and turn it off when you leave for the day unless another student has expressed interest in using the machine. The RETURN key allows you to move to the previous menu.

Scanning samples

1. From the *main menu*, select 1 for spectra.
2. Select 1 to change the spectra type to EX (excitation) or EM (emission).
3. For an excitation scan:
    a. Set your emission wavelength by selecting EMλGOTO, entering your wavelength using the numeric key pad, and hitting ENTER.
    b. Set your excitation range by selecting 2 (EX SCANNING RANGE), entering the starting wavelength using the numeric key pad, hitting ENTER, entering the ending wavelength, and hitting ENTER again.
4. For an emission scan:
    a. Set your excitation wavelength by hitting the EXλGOTO, entering your wavelength using the numeric keypad, and hitting ENTER.
    b. Set your emission range by selecting 3 (EM RANGE), entering the starting wavelength using the numeric keypad, hitting ENTER, entering the ending wavelength, and hitting ENTER again.
5. Select 4 to set your scan speed. Use FAST to test samples for fluorescence and SLOW to collect data for analysis.
6. Select START/STOP to begin the scan.

Determining the peak wavelength

The following procedure is ideal for a quick analysis. For a more detailed analysis, transfer the data to the computer and analyze using Excel. The spectrophotometer will give you numerous peaks, some of which may be artifacts.

1. After the scan is complete, select F3 for DATA ANALYSIS.
2. Select 1 with the numeric keypad for PEAK PICK.
3. To return to the previous menu, select RETURN.

Transferring the scan to the computer

Step 1: After the scan is complete, save as file 1:

1. Select F4, FILE.
2. Select F2, SAVE.
3. Select 1 to save the file in slot 1, and hit ENTER.
4. Hit ENTER if the spectrofluorometer asks you if you want to write over file 1.
5. Name the file a letter and make sure it is different than the letter currently used; you can select a letter using the arrows and then hitting F1, END.

Step 2: Transfer saved filed to the computer

1. On the computer, create a new folder in the SCANS folder with the name *your last name_date*. For example: elrad_0403.
2. Open the hyperterminal program by double clicking on the umbrella icon.
3. Under the *transfer menu*, select *capture text*.
4. Select *browse* to choose your folder and enter your file name. Record this name with all relevant sample information in your lab notebook.
5. Hit ENTER on the computer.
6. Push START on the spectrofluorometer and select F3, TRANSFER FILE.
7. On the spectrofluorometer, Select 1 to transfer file 1 and hit ENTER.
8. Enter 12 on the spectrofluorometer when the spectrofluorometer asks you to input the destination file.

You will see the data stream across the computer screen.

9. On the computer, select *Capture text*, *stop* under the *transfer menu*.

Step 3: Opening the file on the computer using Excel

1. To graph your data in Excel, open your file in Excel, choose deliminate, and select the space bar as the deliminator. When printing scans for your notebook, the title of your graph should contain the file name and a description of your sample (including sample name, dilution, scan type, and excitation or emission wavelength). Make sure your axes are labeled and that the labels include units where appropriate.
2. Once you have analyzed your data, be sure to save your file as an Excel workbook and not a text file.

APPENDIX 6: SPECTROPHOTOMETER

We will be using the BioSpec-mini UV-Visible Spectrophotometer from Shimadzu. The BioSpec-mini can be used to measure absorbance at a single wavelength or to scan absorbance over a range of wavelengths. Scans can be saved, transferred to a computer, and opened in Excel for analysis. In addition, the BioSpec-mini has a nucleic acid analysis feature that can be used to determine the concentration of DNA in a sample. In addition to the BioSpec-mini, we have two GeneSys20 Spectrophotometers from Thermo Scientific. These can only be used to measure absorbance at a single wavelength. Instructions for these instruments are included in this appendix. Turn a spectrophotometer on 10-15 minutes before you will need to use it (they need time to initialize and warm-up), leave it on throughout the class time, and turn it off when you leave for the day unless another student has expressed interest in using the machine. The RETURN key allows you to move to the previous menu.

BioSpec-Mini: Measuring at a single wavelength

1. Set the wavelength by hitting GOTO WL, entering your wavelength, and hitting ENTER. You will see the wavelength change in the upper right corner of the screen.
2. Blank the spectrophotometer by inserting your blank (a cuvette with the solution your samples are in) and hitting AUTO ZERO.
3. Place the sample in the cuvette and read the absorbance in the upper right hand corner of the screen.

BioSpec Mini: Scanning and analyzing scans

Step 1: Scanning

1. From the *mode menu*, select 3 for spectrum.
2. Select 1 to change the *measuring mode*, and use the up and down arrows to select ABS. Hit ENTER when ABS is highlighted.
3. Select 2 to change the wavelength range. Using the numeric keypad, enter the starting wavelength, hit ENTER, enter the ending wavelength, and hit ENTER again. The starting wavelength must be larger than the ending wavelength.
4. To change the scan speed, select 4 and use the arrows to change the speed.
5. Place your blank sample cuvette in the holder and hit F1 for *BaseCorr* (baseline correction). Your blanking cuvette should contain the solution your sample is in.
6. Place your sample in the holder and hit START/STOP.

Step 2: Transferring the scan to the computer

1. On the computer, create a new folder in the SCANS folder with the name *your last name_date*. For example: elrad_0403.

2. Open the hyperterminal program by double clicking on the umbrella icon.
3. Under the *transfer menu*, select *capture text*.
4. Select *browse* to choose your folder and enter your file name. Record this name with all relevant sample information in your lab notebook.
5. Hit ENTER on the computer.
6. On the BioSpec-mini, hit F3 for *ExtTrans* (external transfer).

You will see the data stream across the computer screen.

Step 3: Opening the file on the computer using Excel

3. To graph your data in Excel, open your file in Excel, choose deliminate, and select the space bar as the deliminator. When printing scans for your notebook, the title of your graph should contain the file name and a description of your sample. Make sure your axes are labeled and that the labels include units where appropriate.
4. Once you have analyzed your data, be sure to save your file as an Excel workbook and not a text file.

APPENDIX 7: RECIPES

All solutions are brought to their final volumes using ddH_2O. For solutions made at a particular pH, water is added until the volume is just short of the desired final volume, the pH is adjusted with NaOH or HCl, and water is added to reach the final volume. If a buffer will be used in the LP system, it is filtered through a 0.22 μm filter. Buffers are stored at room temperature unless otherwise indicated. Growth media is autoclaved for 20 min.

Buffers and Salts for Tyrosinase Experiment

1 M dibasic sodium phosphate
Total Volume500 mL

Na_2HPO_4 (FW 141.96)	71 g

1 M monobasic sodium phosphate
Total volume 500 mL

NaH_2PO_4-H_2O (FW 137.99)	69 g

1 M sodium phosphate stock, pH 7
Total volume 100 mL

1 M dibasic sodium phosphate	57.8 mL
1 M monobasic sodium phosphate	42.2 mL

Adjust to pH 7 with NaOH.

DEAE loading buffer - 10 mM sodium phosphate, pH 7
Total volume 500 mL

1 M sodium phosphate, pH 7	5 mL

Tyrosinase lysis buffer - 10 mM sodium phosphate, pH 7, plus protease inhibitors
Total volume 20 mL

10 mM DEAE loading buffer, pH 7	18 mL
Protease inhibitor cocktail, 10x stock	2 mL

Protease inhibitor cocktail stock is made by re-suspending Sigma P2714 in 10 mL of water.

DEAE elution buffer - 10 mM sodium phosphate, pH 7, 25 mM sodium chloride
Total volume 500 mL

1 M sodium phosphate, pH 7	5 mL
Sodium chloride (FW 58.44)	0.73 g

Catechol stock - 50 mM catechol, 10 mM sodium phosphate, pH 7
Total volume 100 mL

Catechol (FW 110.1)	0.55 g
DEAE loading buffer	100 mL

Catechol stain - 5 mM catechol, 10 mM sodium phosphate, pH 7
Total Volume 50 mL

Catechol stock	5 mL
DEAE loading buffer	45 mL

Buffers for Protein Gels (IEF and SDS-PAGE)

10x SDS-PAGE running buffer - 0.25 M Tris, 2 M Glycine, 1%SDS
Total volume 500 mL

Tris base (FW 121.14)	15.1 g
Glycine (FW 195.18)	72.1 g
Sodium dodecyl sulfate (FW 288.38)	5 g

10x IEF cathode buffer - 400 mM lysine
Total volume 100 mL. Store at 4°C.

Lysine base (FW 146.2)	5.8 g

50x IEF anode buffer - 350 mM phosphoric acid
Total volume 100 mL

85% phosphoric acid	2.4 mL

5x SDS-PAGE Sample Buffer - 157 mM Tris pH6.8, 62.5% glycerol, 5% SDS, 0.025% bromophenol blue
Total volume 100 mL

Glycerol	62.5 mL
1 M Tris, pH 6.8	15.7 mL
Sodium dodecyl sulfate (FW 288.38)	5 g
Bromophenol blue	0.025 g

Before using, add 62.5 µl β-mercaptoethanol (β-Me) to 500 µl 5X sample buffer. Buffer with β-Me must be made fresh daily.

2x IEF sample buffer - 80 mM lysine, 30% glycerol
Total volume 10 mL. Store at 4°C.

10x IEF Cathode Buffer	2 mL
Glycerol	3 mL

CIEF Gel Stain - 27% Isopropanol, 10% acetic acid, 0.04% Coomassie Blue, 0.05% Crocein Scarlet
Total volume 500 mL

Isopropanol	135 mL
Acetic acid	50 mL
Coomassie Brilliant Blue R250	0.2 g
Crocein Scarlet	0.25 g

Gel Destain - 40% methanol, 10% acetic acid
Total volume 500 mL

Methanol	200 mL
Water	250 mL
Glacial acetic acid	50 mL

Media for *E. coli*

LB Broth, Total volume (1 L)

Sodium Chloride (NaCl)	10 g
Tryptone	10 g
Yeast extract	5 g

Adjust to pH 7.0 with NaOH.

LB Agar plates , 40 plates

LB broth	1 L
Bacto Agar	20 g

After autoclaving, cool to 50°C and pour into Petri plates (~25 ml per 100-mm plate).

LB-Amp plates, 40 plates

LB broth	1L
Bacto Agar	20 g

After autoclaving, cool to 50°C, add 1 ml 100 mg/ml filter-sterilized ampicillin, and pour into Petri plates (~25 ml per 100-mm plate).

NZY Broth, Total volume 1 L

NZ amine (casein hydrolysate)	10 g
Yeast extract	5 g
Sodium chloride (FW 58.44)	5 g

Adjust to pH 7.5 using NaOH.

NZY+ Broth 10 mL

NZY Broth	10 ml
1 M magnesium chloride, filter sterilized	125 µl
1 M magnesium sulfate, filter sterilized	125 µl
20% (w/v) glucose, filter sterilized	200 µl

Filter sterilize NZY+ before using.

Buffers for His-Purification

HIS lysis buffer - 50 mM NaH_2PO_4, 300 mM sodium chloride, 10 mM imidazole
Final volume 1 L

$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	6.90 g
Sodium chloride (FW 58.44)	17.54 g
Imidazole (FW 68.08)	0.68 g

Adjust pH to 8.0 using NaOH

HIS wash buffer - 50 mM NaH_2PO_4, 300 mM sodiumchloride, 20 mM imidazole
Final volume, 1 L

$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	6.90 g
Sodium chloride (FW 58.44)	17.54 g

Imidazole (FW 68.08)	1.36 g
Adjust pH to 8.0 using NaOH	

HIS elution buffer - 50 mM NaH_2PO_4, 300 mM sodium chloride, 250 mM imidazole
Final volume 1 L

$NaH_2PO_4 \cdot H_2O$ (FW 137.99)	6.90 g
Sodium chloride (FW 58.44)	17.54 g
Imidazole (FW 68.08)	17 g
Adjust pH to 8.0 using NaOH	