Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

SCST Genetic Technology Superworkshop
Ames, IA
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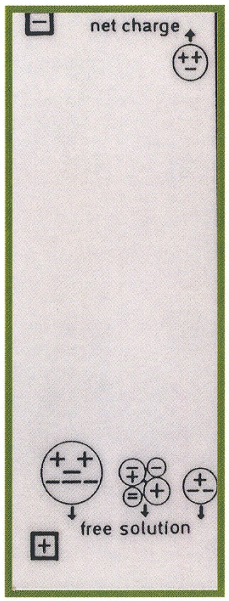
Theory of how it works

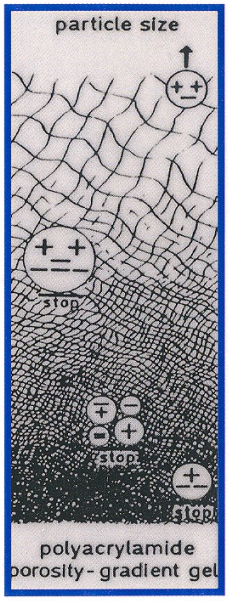
- Electrophoresis is a method where charged molecules in solution, mainly proteins and nucleic acids, migrate in response to an electrical field. The rate of migration or mobility through the electrical field depends on the strength of the charge, on the net charge of the protein/nucleic acid, the size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium through which the molecules are moving.

Predominant Factors in Electrophoretic Separation

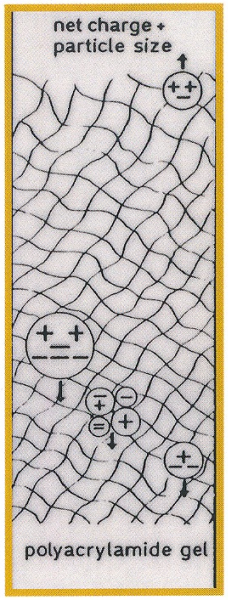
(Main parameters in electrophoretic separations. Proteins of different charge and size are compared)



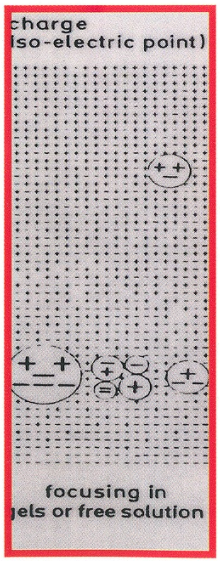
Electrophoresis *without or with little* molecular sieving (carrier-free, paper, starch agarose).



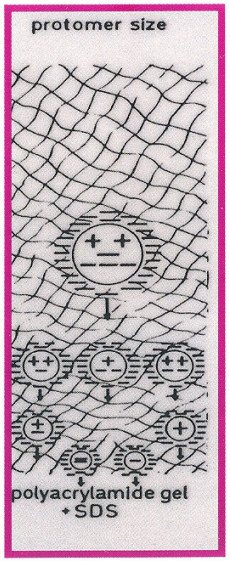
Porosity gradient: created by decreasing pore size of PAA (polyacrylamide). Proteins are finally trapped.



PAGE: both molecular sieving and charge separation affecting the migration rate.

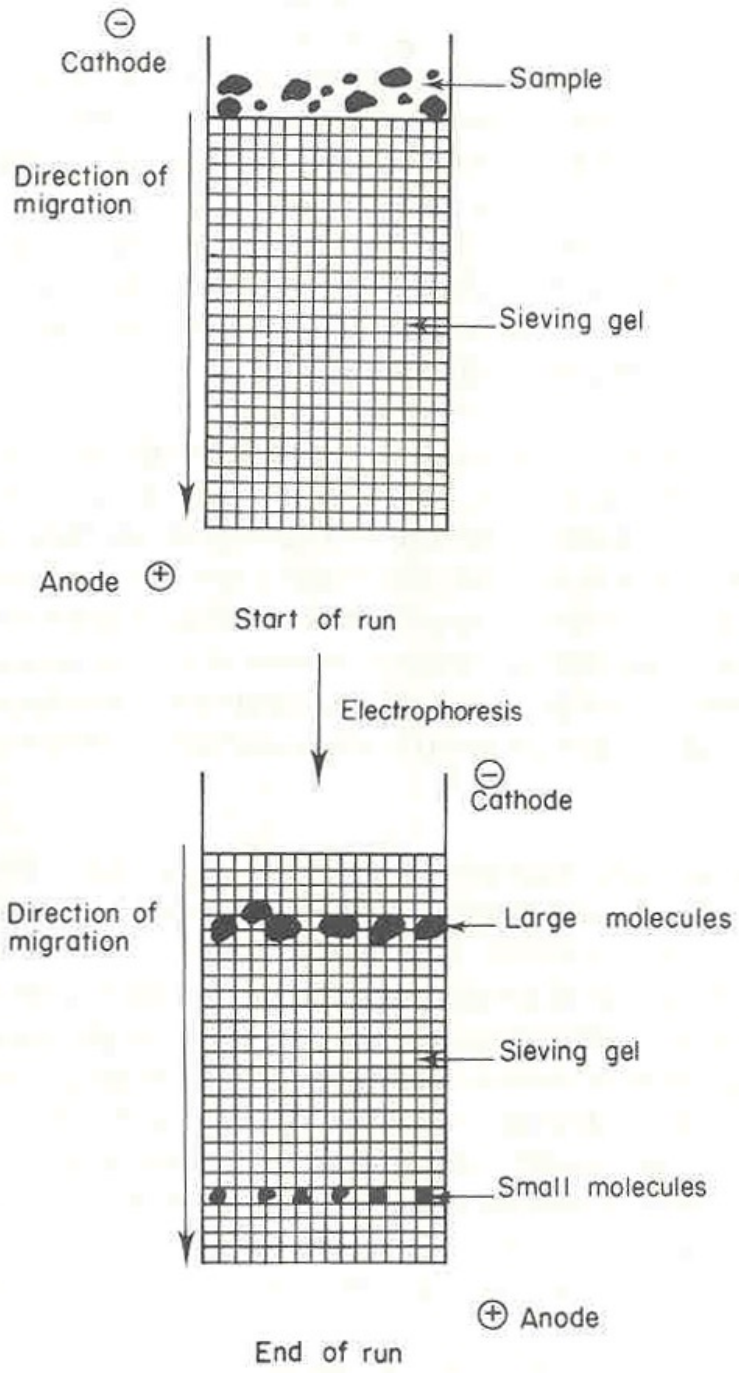


Focusing: a pH gradient is created by small molecules of slightly different charge. Proteins focus at their isoelectric points.



SDS-PAGE: Sodium Dodecrylsulfate, highly negatively charged, unfolding proteins to a random coil and giving them a uniform load.

Source unknown



Source unknown

Proteins are linear polymers of amino acids – found in all living systems.

Only 20 amino acids are commonly found in plant proteins, and these are combined in countless ways to form an enormous variety of different protein molecules.

Seed Proteins (classification)

- Albumins – enzyme proteins, metabolically active
- Globulins – mainly storage proteins, predominately in legumes and metabolically active
- Glutelins – storage proteins, mainly in cereals. Glutenin fraction of wheat (not alcohol soluble)
- Prolamines – storage proteins, only in cereals. Gliadin fraction (alcohol soluble)

Uses of Polyacrylamide Gel Electrophoresis (PAGE)

- Quality control
- Replace field growouts
- Check out mixtures
- Check “rogues” in Foundation/Breeder seed fields
- PVP applications
- PVP enforcement

- Criminal court cases
- Backup test to other varietal purity tests
  - Fluorescence checks
  - Hilum checks
  - Phenol tests
  - KOH for white vs. red wheat
  - Etc.
- Forgot to label bins

Why Use PAGE Protein Electrophoresis?

- Chemically inert
- Stable over a wide range of pH, temperature
- Transparent
- Strong gel matrix
- Pores more controlled and uniform sized
- Sharper and better band resolution
- Can load more extract
- Gels easily preserved

- Range of pore size much broader, size of pores controlled by varying the ratio of bis to acrylamide (3% to 30%)
- Can separate molecules that differ in size by as little as 2% of MW
- Can form gradient gels (ex. – 10-20% gel)
- Synthetic polymer

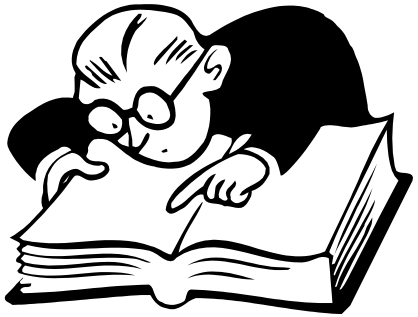
Disadvantages of PAGE

- Gel preparation more difficult
- When polyacrylamide is unpolymerized it acts as a cumulative neurotoxin

Types of PAGE Gels

Many types exist – can be customized

- Acid Page – Al Lactate, Acetic acid-based, Urea
- Basic Page – Tris-based
- Native Page
- SDS Gradient Page
- Continuous/Discontinuous



Break it down another way:

1. Native PAGE
    a. Continuous
    b. Discontinuous
2. Denaturing PAGE
    a. Continuous
    b. Discontinuous
3. SDS PAGE – continuous, discontinuous
    a. Straight percent (%) gel (i.e. – 7% gel)
    b. Gradient gel (5 – 20% gel)

Native PAGE - continuous

- Separates whole proteins extracted with various extraction buffers (alcohols, water, acids, bases, etc.)
- Method uses the same buffer as used in the electrode buffer
- Gel is a straight percentage throughout
- Separates on the basis of size (MW) and electrical charge.

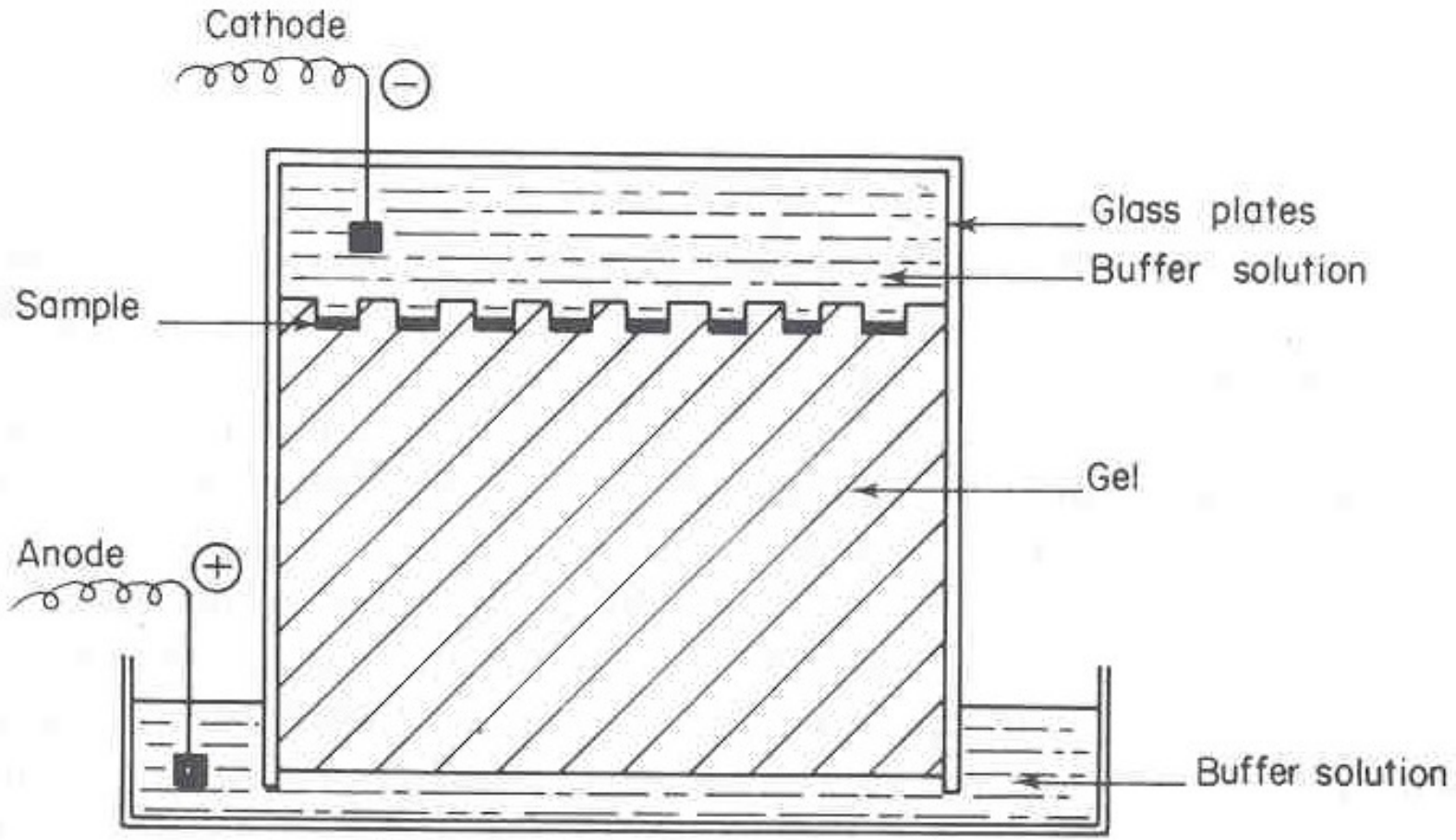


Fig. 3.3c. *Apparatus for vertical slab gel electrophoresis*

Source unknown

Native PAGE - discontinuous

- Two gel system
  - Stacking gel (4%)
  - Resolving gel (can range from 5 to 15%)
- Resolving gel poured first, then the stacking gel.
- Allows proteins to have higher band resolution and sharpness.

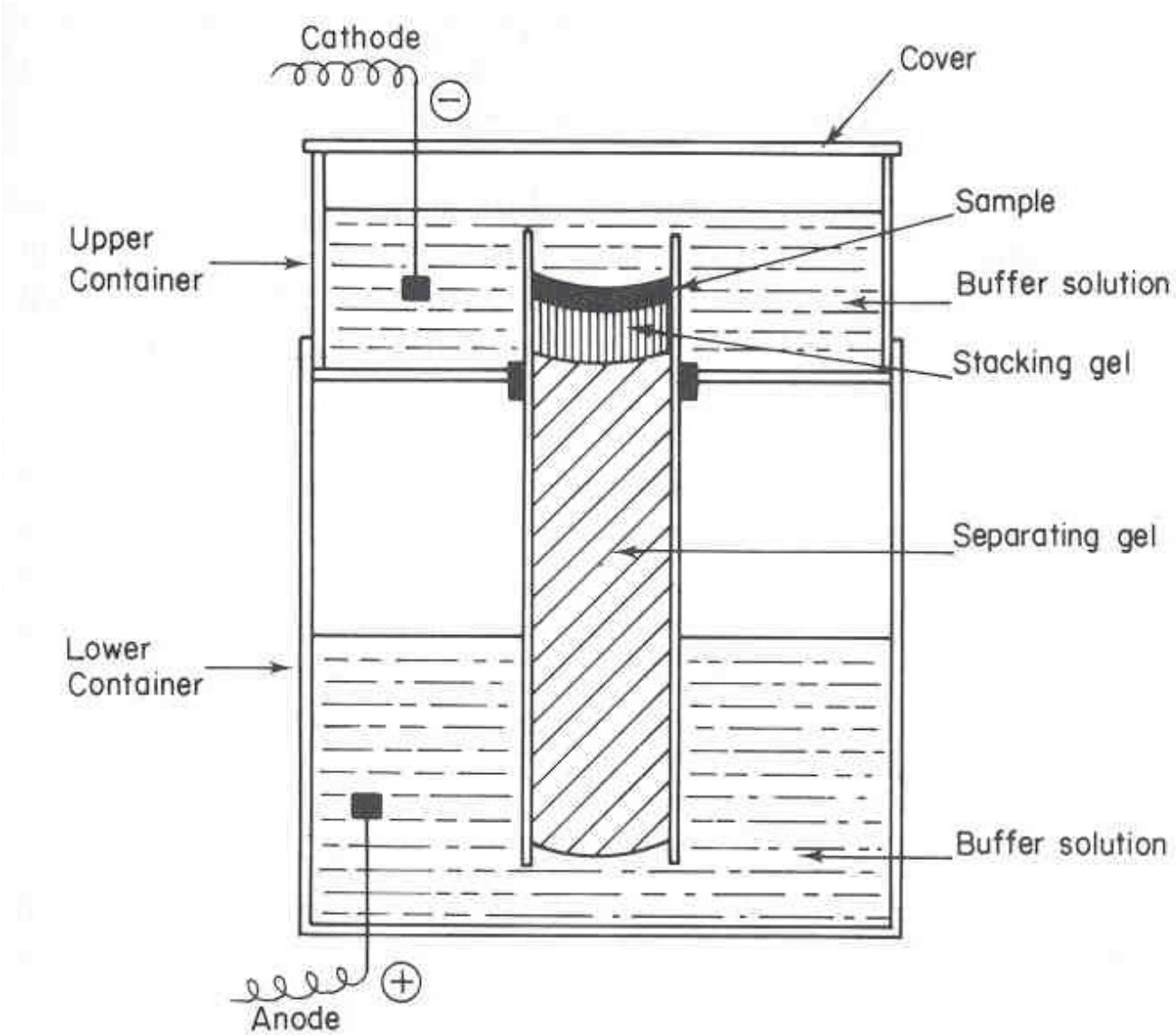


Fig. 3.3d. *Apparatus for disc electrophoresis*

Source unknown

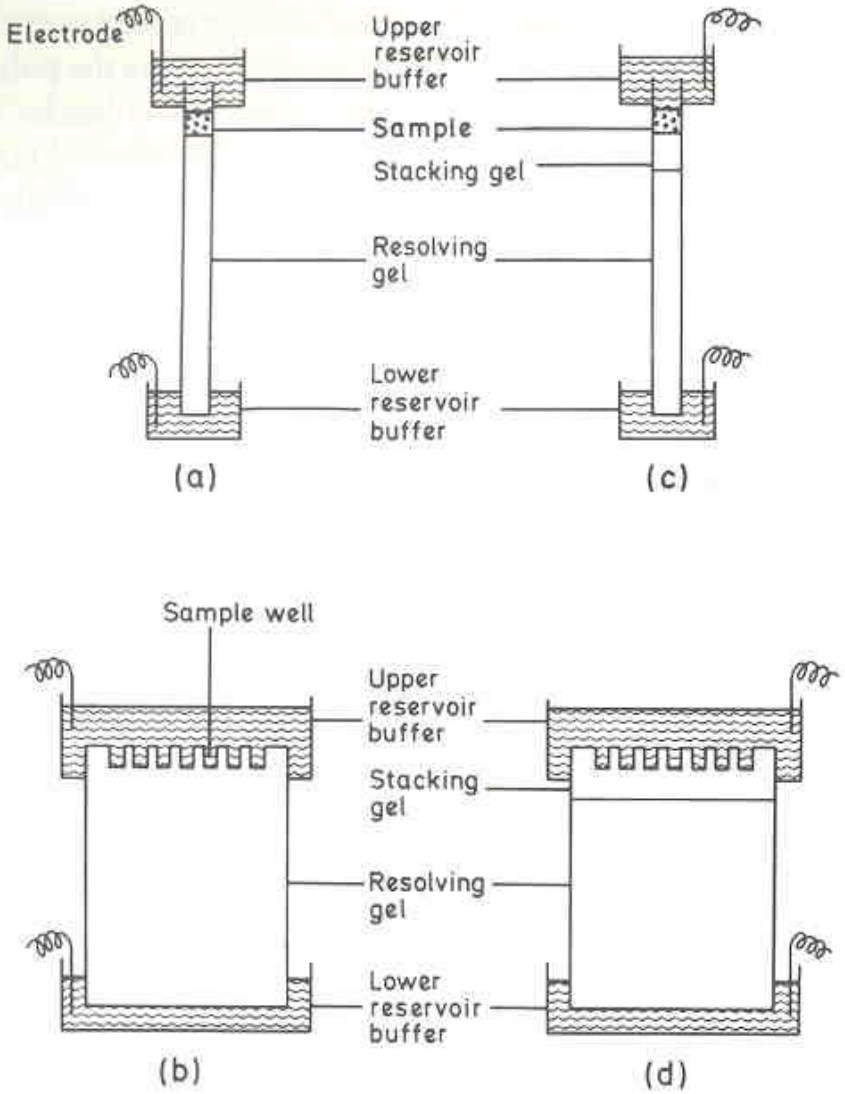


Figure 3. Use of continuous and discontinuous buffer systems with rod and slab gels. (a) continuous buffer system used in conjunction with a rod gel; the sample is loaded directly onto the resolving gel. (b) continuous buffer system used in conjunction with a slab gel; samples are loaded into wells formed directly in the resolving gel. (c) and (d), discontinuous buffer system used in conjunction with rod and slab gels, respectively; samples are loaded directly onto the stacking gel or into wells formed in the stacking gel, respectively.

Source unknown

SDS PAGE - discontinuous

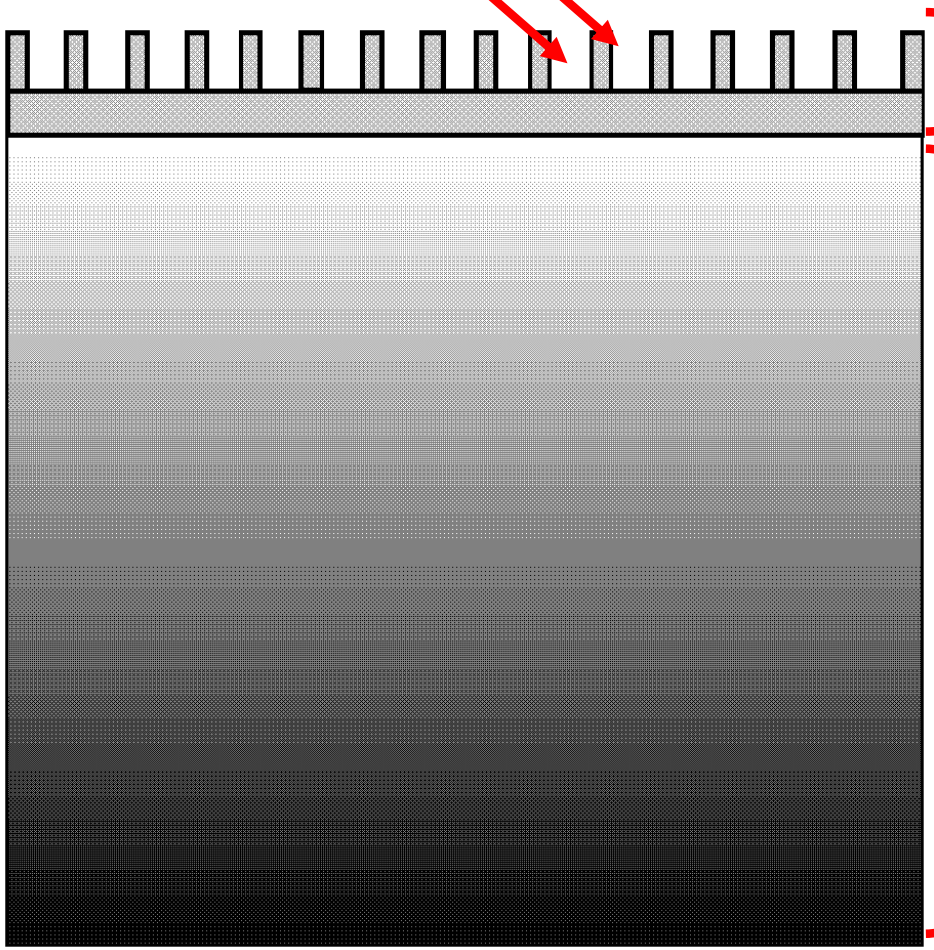
- Has the two gels (stacking and resolving)
- Like previous type but uses sodium dodecylsulfate (SDS) to dissociate proteins and remove charge effects.
- Allows for separation on basis of molecular weight of proteins.
- SDS is used in the extraction buffer and the in the gel running buffer.

SDS Gradient PAGE - discontinuous

- Has a stacking gel like others
- Resolving gel is a gradient (i.e. – going from 5%-20%, or from larger pores to smaller pores as you move down the gel).
- Use in combination:
  - SDS
  - Dithiothreitol (DTT) or 2-mercaptoethanol as reducing agents
  - Heat at 100C for minutes

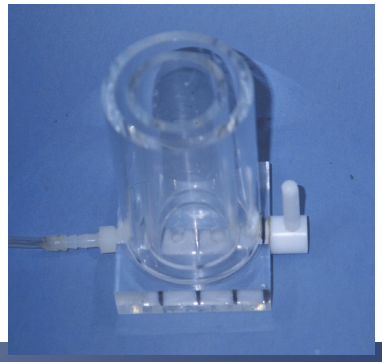
Responsible for denaturation of proteins into polypeptide units – separates strictly on size (MW).

Sample wells



Stacking gel. (Has large pore size ~4%)

Resolving gel. Can be a single pore size or a gradient gel (portrayed here)

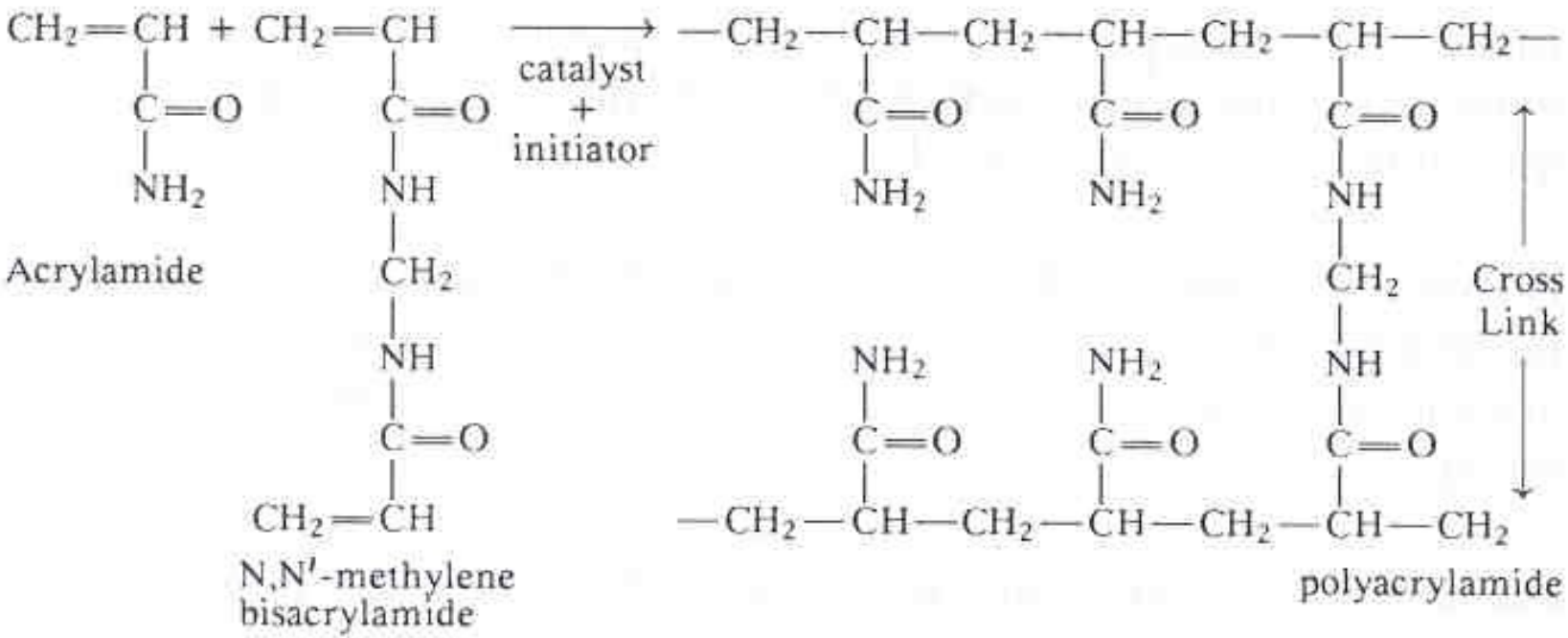


Gradient maker

How is the gel formed?

- Gels are a 3-dimensional polymeric network (think of jello)
- Gels consist of a network of polymer molecules surrounded and penetrated by buffer
- Spaces between the gel molecules are the pores of the gel!

- Polyacrylamide gels are prepared by cross-linking acyrlamide with N,N' methylenebisacrylamide (bis-acrylamide).
- The reaction requires an initiator and catalyst (cross-linking reagent).
  - Commonly used
    - Ammonium persulfate (initiator)
    - TEMED (N,N,N',N'-tetramethylethylenediamine) cross-linker

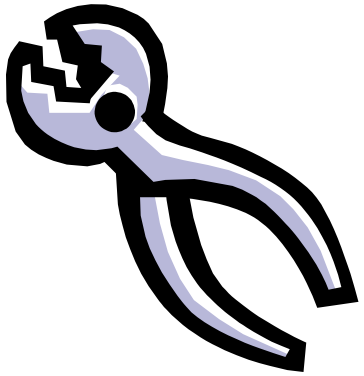


- Polymerization reactions are very sensitive to certain chemical impurities that may be present in the components of the gel mixture.
- Important to use high quality reagents.
- Both acrylamide and bis-acrylamide should be white crystalline substances.

- **When making polyacrylamide gels, the ratio of cross-linking agent to acrylamide is a critical factor as it determines the pore size of the gel and influences it's mechanical properties!**
- **%T represents the total concentration of monomer (acrylamide + Bis) expressed in grams per 100 ml (weight per volume %)**
- **%C is the percentage (by weight) of total monomer T which is due to the cross-linking agent (Bis)**
- **The concentration of acrylamide used determines the average polymer chain length while the Bis concentration determines the extent of cross-link formation. Important in determining such physical properties of the gel as density, elasticity, mechanical strength, and pore size.**

Protein Extraction

- Grinding of seeds (bulk or single seed)
  - Mortar and pestle
  - Coffee grinder
  - Udy mill
  - Pliers method
  - Etc.



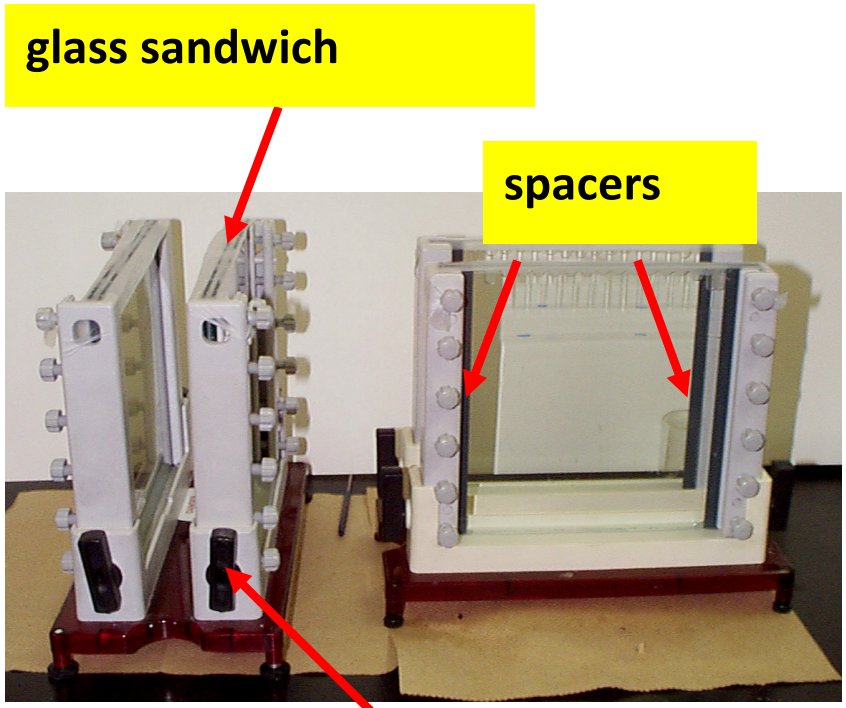
Protein Extraction

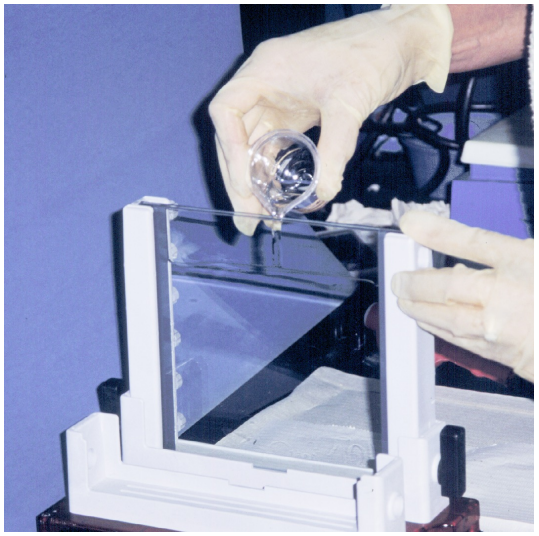
- Extraction buffers
  - Ethylene glycol
  - Ethanol
  - 2-Chloroethanol
  - Water
  - SDS-based extraction
  - SDS/2-mercaptoethanol/heat
  - Etc.
- Centrifuge extract


VORTEX MIXER
KMC-1300V
AUTO
OFF
MANU
VISION SCIENTIFIC CO., LTD

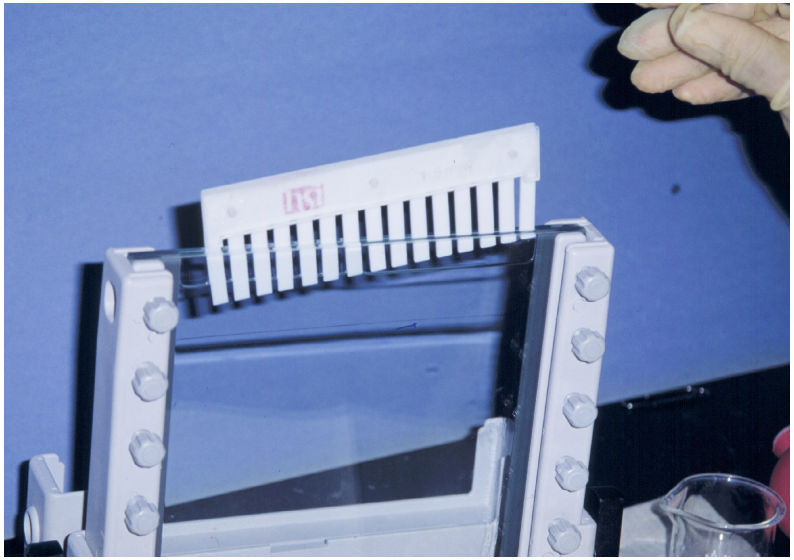
Gel Preparation

- Set up gel casting apparatus
- Mixing of gel ingredients (follow recipe/directions and adjust to correct pH)
- Pouring the gel
  - Separating gel
  - Stacking gel
  - Comb placement/removal
- Polymerization

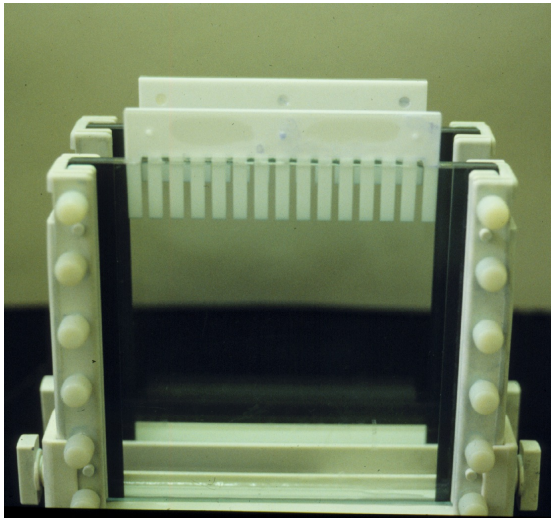




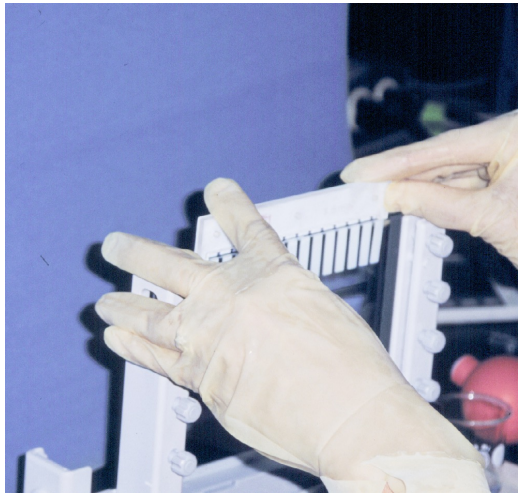
Pouring the gel



Inserting the comb to form wells



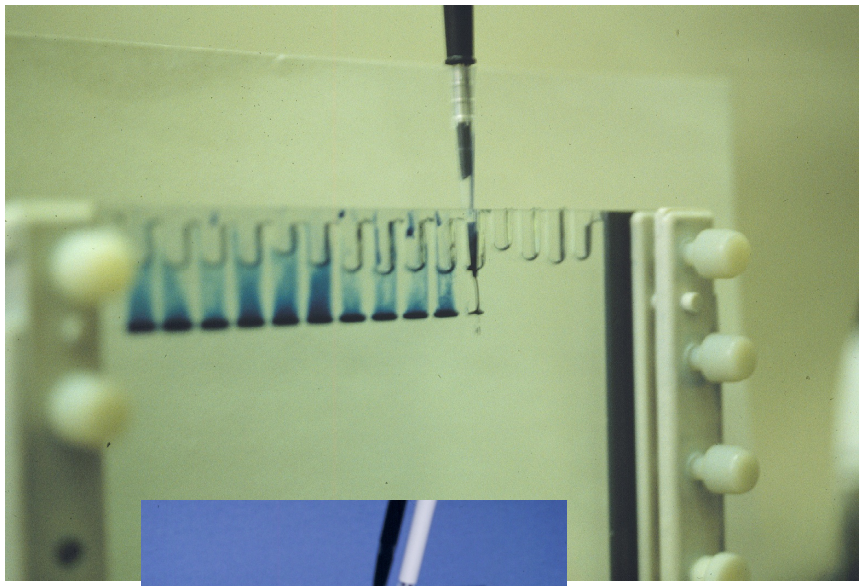
Double gel setting up



Removing the comb

Loading the gel

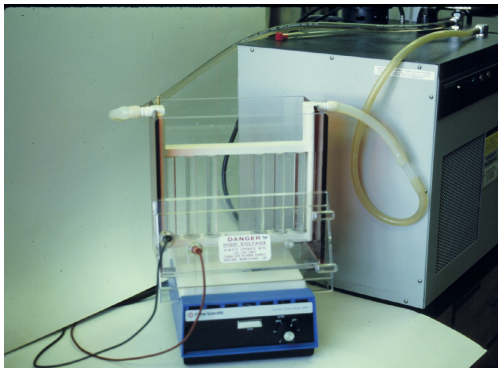
- Fill wells with distilled water or tank buffer as a rinse
- Pour off rinse and ½ fill with tank buffer
- Place extract into sample well using a digital pipette with gel loading tips. Sample size loaded will vary on species and gel type.

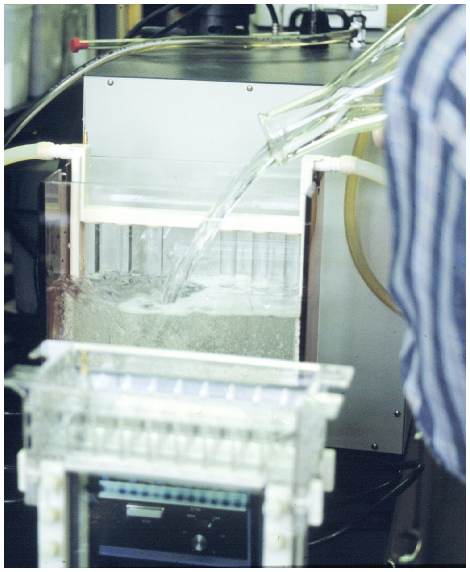


Running the Gel

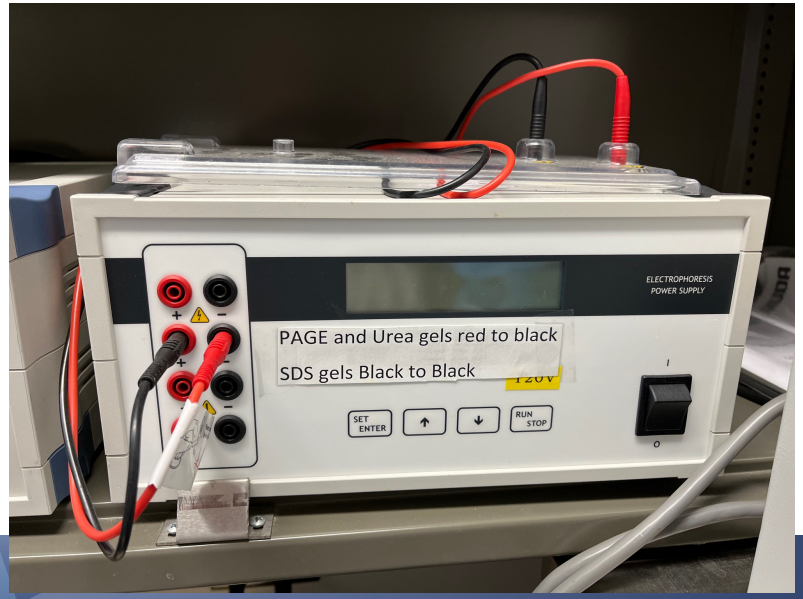
- If using a recirculating water bath to keep gel cool, start it early.
- Attach the upper buffer reservoir to the gel plates and fill with buffer solution to recommended fill levels.
- Pour the remaining buffer solution into the lower reservoir chamber along with a stir bar.
- Place the bath cooling columns into the lower tank and and lower the gel plate(s) into the chamber
- Place the lid on the upper buffer chamber and connect to the power supply correctly.
- Prior to turning on the power source, zero out the settings.
- Turn on power source and set to desired settings. Usually run at constant current.
- Run times will range from 2 hours to 16 hours, depending on your setup.

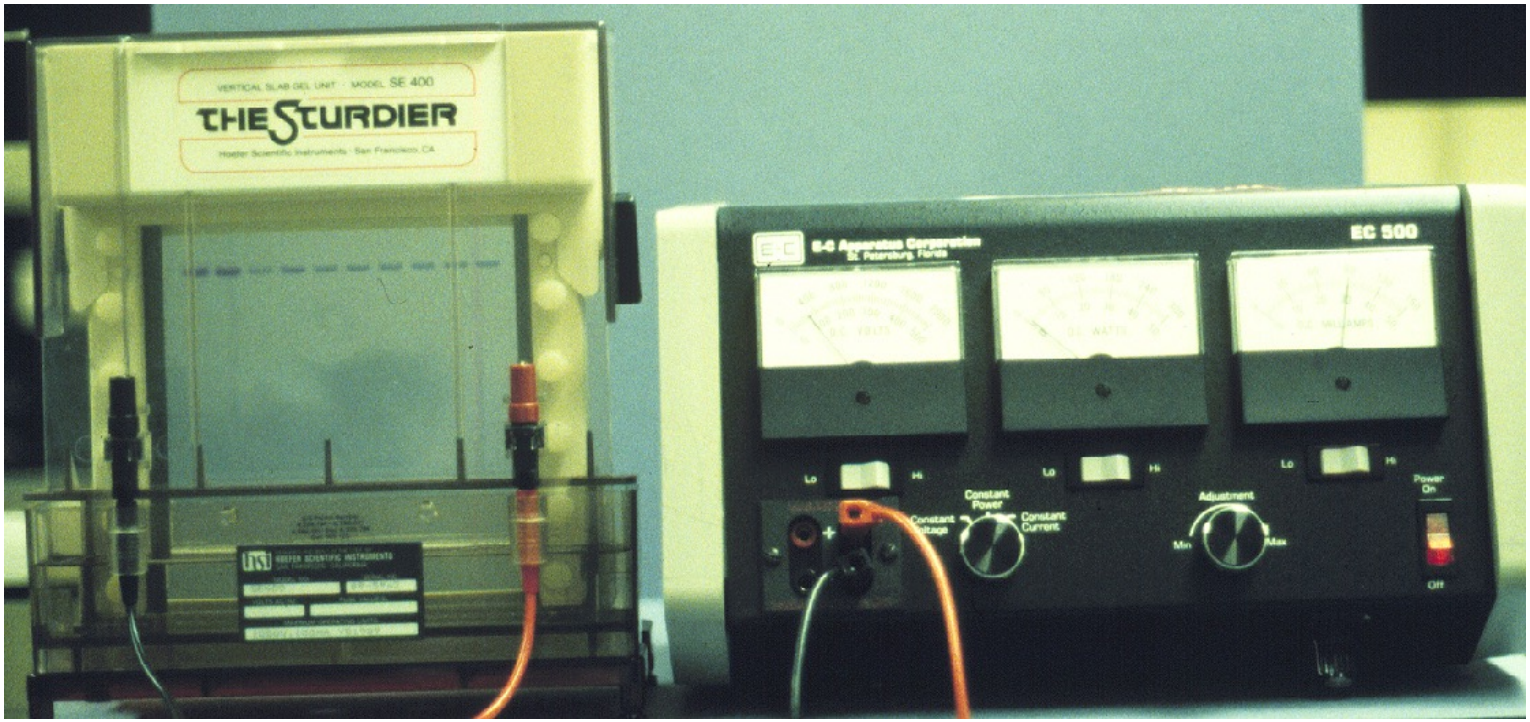
Note: Temperature regulation is very important during the run time.






HOEFER SCIENTIFIC INSTRUMENTS
SAN FRANCISCO
CURRENT RANGE (MA)
METER READ
ON—OFF
PS 2500 DC POWER SUPPLY
MODE CONSTANT
PROGRAM ADJUST
MAIN ADJUST
VOLTAGE RANGE


ELECTROPHORESIS
POWER SUPPLY
PAGE and Urea gels red to black
SDS gels Black to Black
120V
SET
ENTER
RUN
STOP



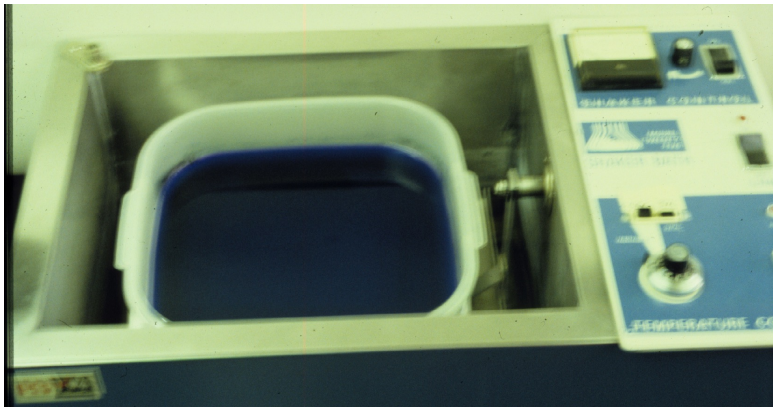
Single gel apparatus – no temperature regulation

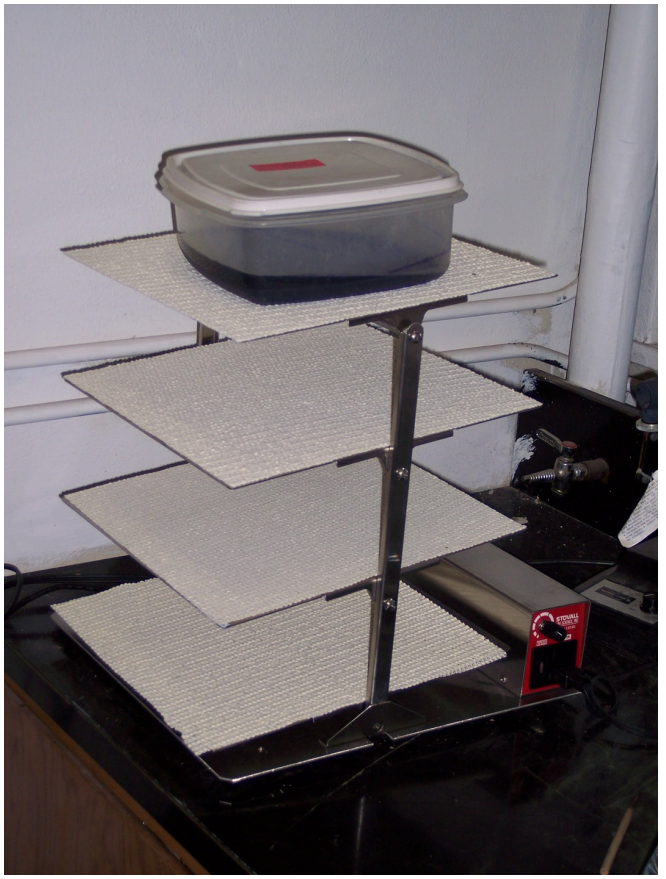
- After running – disassemble gel apparatus carefully.
- Items that are handy to use in getting gel off of glass plate:
  - Squirt bottle of water ✓
  - Spatula – long handled
  - Plastic mesh screen – large enough to lift gel on it. ✓
- Critical to use good glass plates without major scratches or chips, etc. Should have been cleaned with ethanol prior to casting gel.

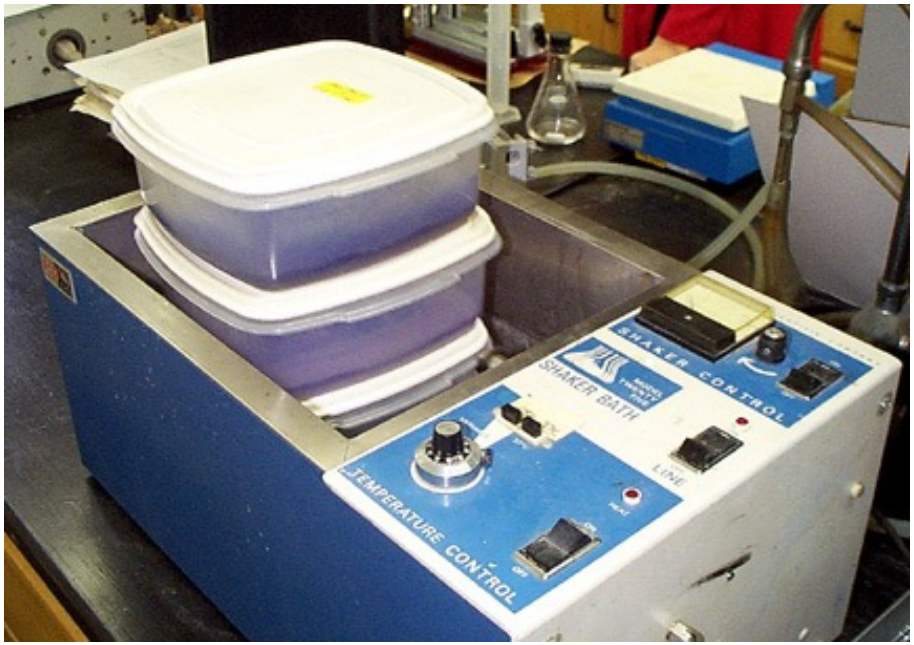
Staining of Gels (Proteins)

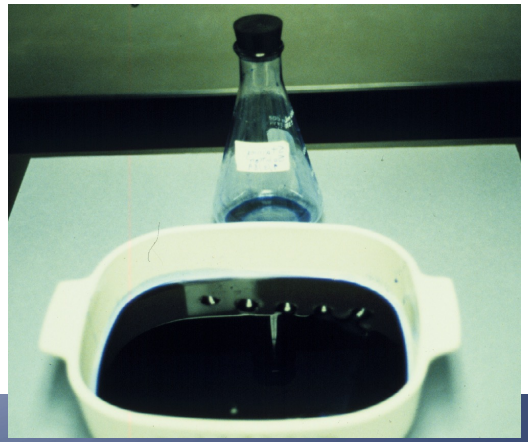
- Proteins are commonly visualized by staining them with dyes or metals.
- Each stain has different characteristics as to sensitivity and type of protein stained best.

- Popular Stains
  - General Protein Stains
    - **Coomassie Blue R**
    - **Coomassie Blue G**
    - **Silver Staining**
    - **Copper Staining**
  - Specific enzyme stains



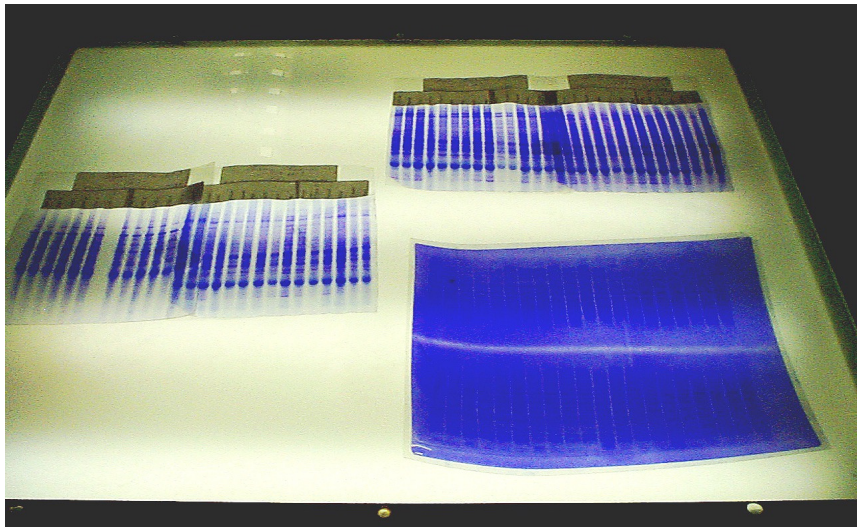



SHAKER CONTROL
SHAKER BATH
TEMPERATURE CONTROL

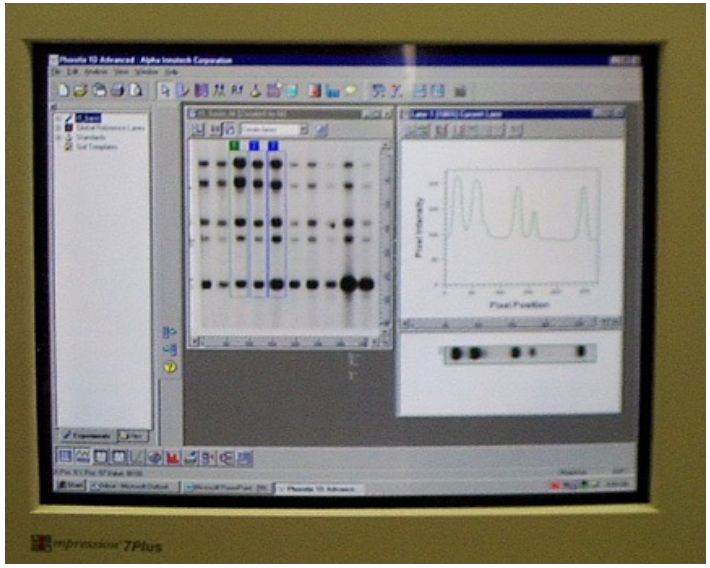


Gel Evaluation and Documentation

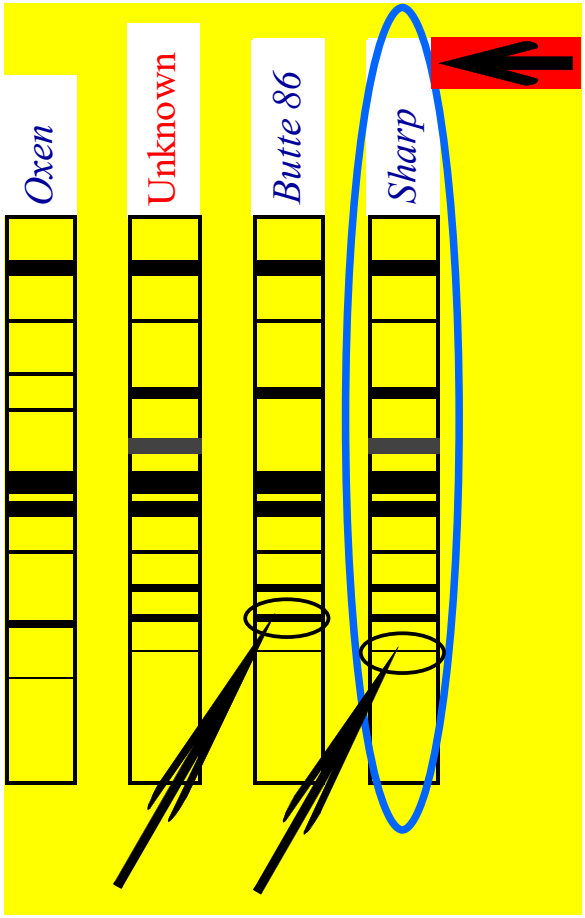
1. Visual
   - Experience needed
2. Computer Scanned
3. Photographed



Both 2 and 3 can be brought into a computer gel analysis program and have automated band detection and lane comparison.



Electrophoresis testing at SDSU utilizes the proteins in a seed to obtain a "fingerprint" pattern (unique for each variety) and compare it to a check sample (known variety) to verify or identify the variety of the sample. Below is an simplified example and explanation using HRS wheat variety names.



This is known as a "lane" or "well". Within each lane there are protein bands which have separated (due to an electrical current being applied). The result is a "fingerprint" pattern that develops for each variety.

Bands in lanes are compared or matched (using band size, location, and intensity) to determine or verify varietal identification of the unknown sample submitted. In this example you can see that our unknown matches the variety "Sharp" (showing differences from other spring wheat varieties listed).

XXXXX– Spelt

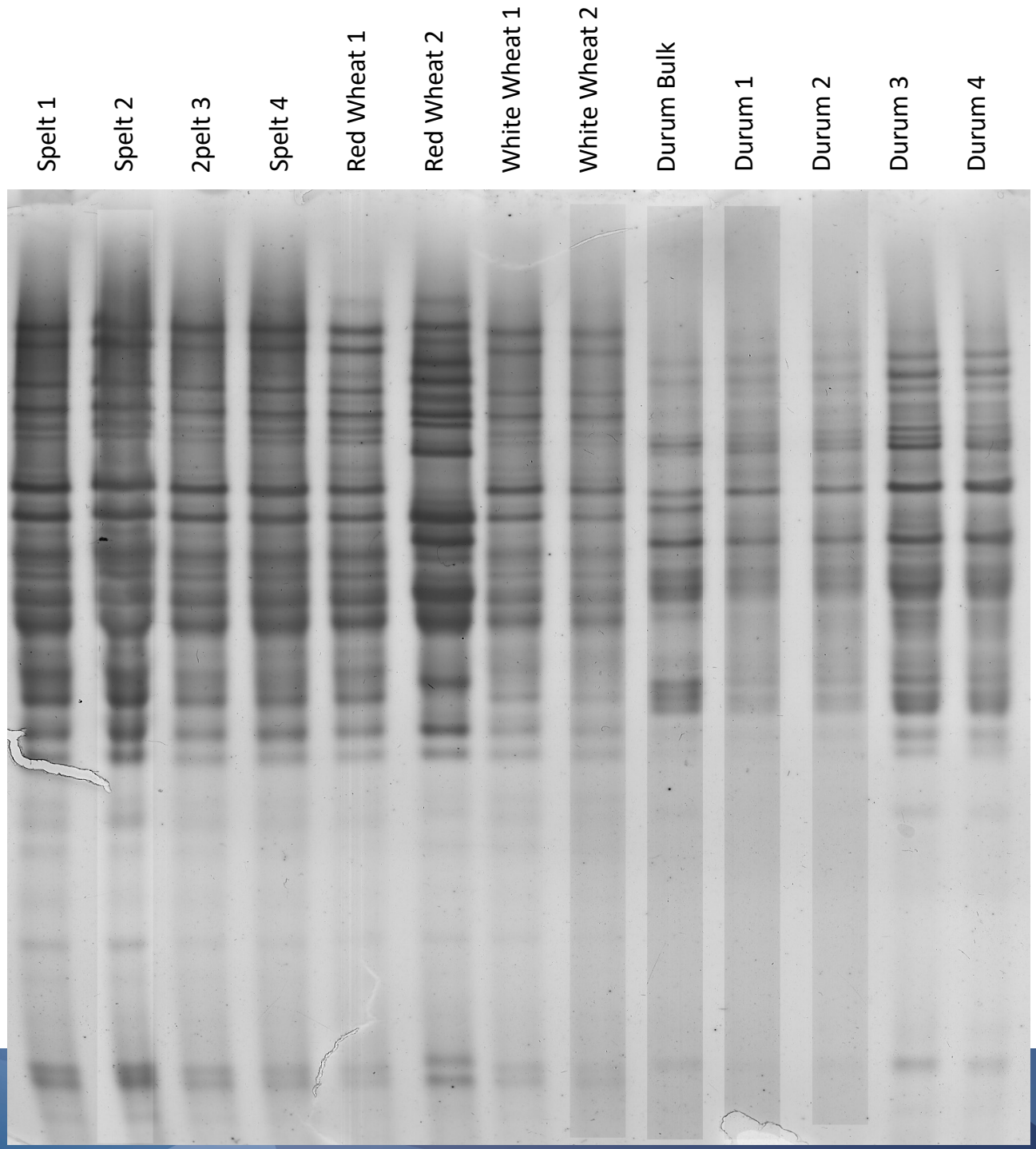
SDSU STL PAGE
Sample SD104
(STL #2803)
Gel #04-038B
06/03/04

Notes:

1) One of the suspect "red wheat" samples (#1) and both of the suspect "white wheat" samples appear to have the same banding pattern as the "spelt" samples.

2) Durum samples all have a different banding pattern than either the spelt or wheat.

3) All samples are single seed extractions, except where noted as "bulk".



Montana Brome

SDSU Seed Testing Lab
Gel #02-103B
09/24/02

A: “03-814, ?, Bromus marginatus, catharticus, stamineus”.

B: “Bromus stamineus from Oregon “Gala Brome”.

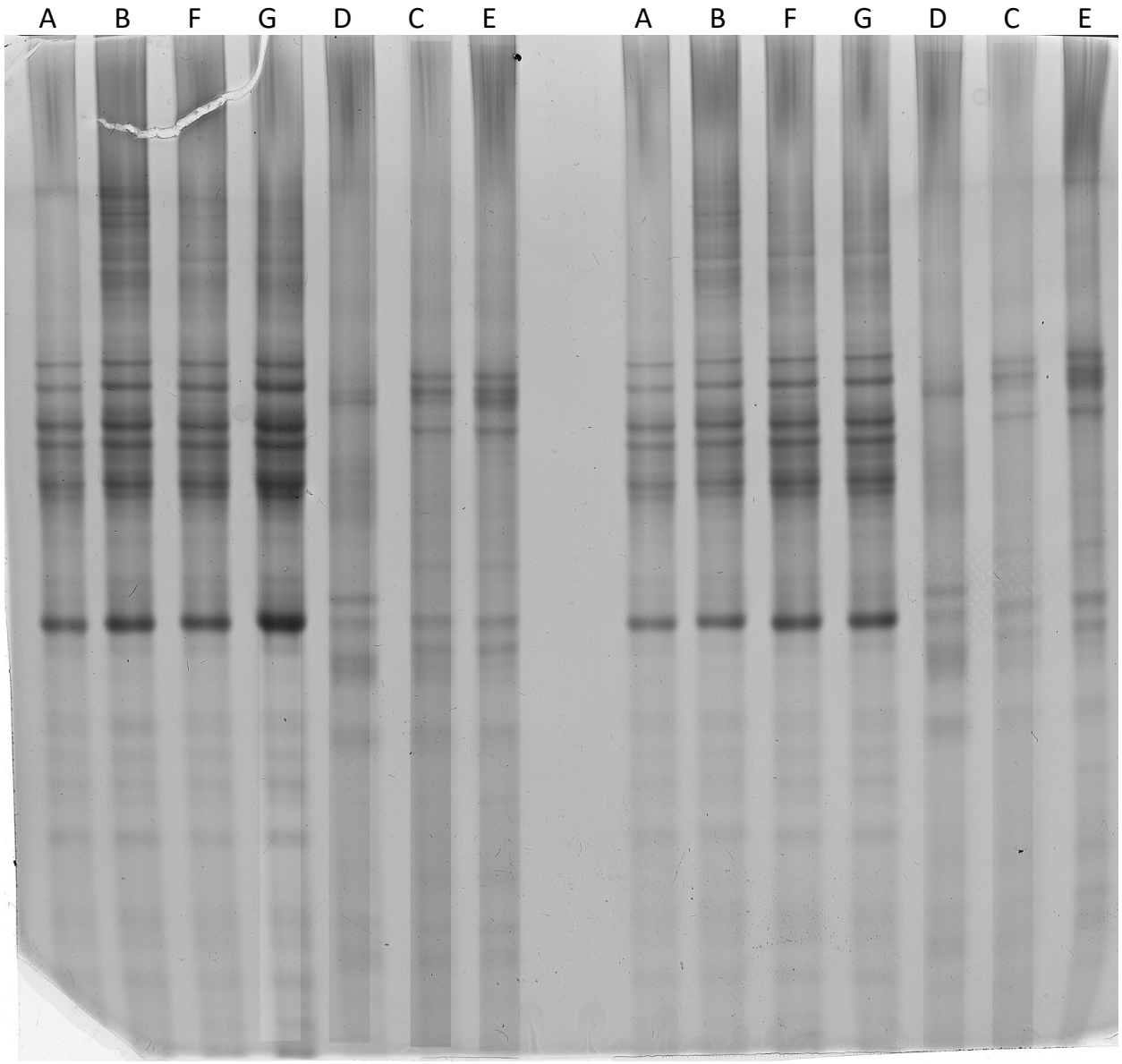
F: “03-813, ?, Bromus marginatus, catharticus, stamineus”

G: “03-780, ?, Bromus marginatus, stamineus, catharticus”.

D: “Garnet, Cert. Wyo.”

C: “Bromar, Cert. Wyo.”

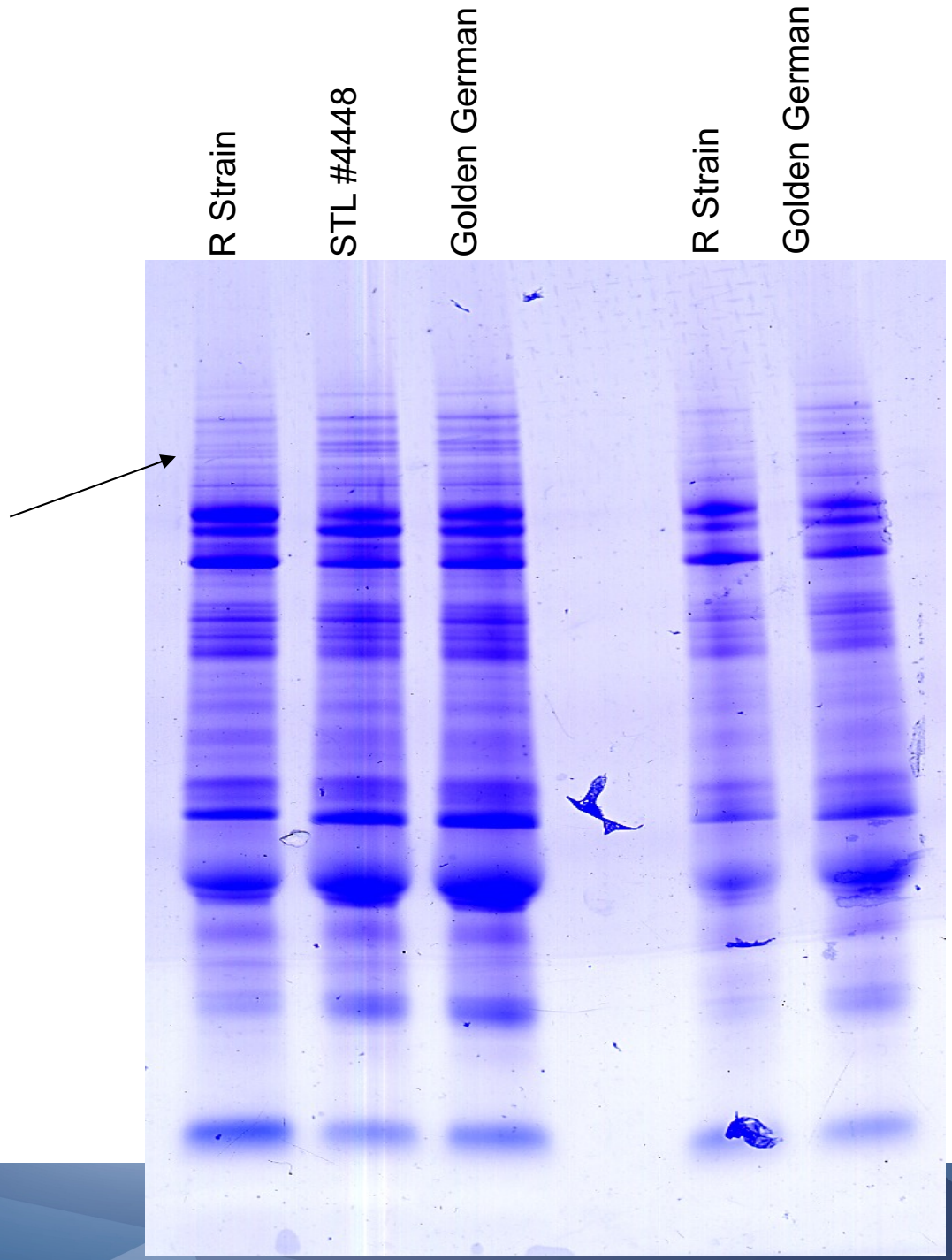
E: “Bromus sitchensis from Oregon. “Alaska brome”.



Foxtail Millet

SDSU Seed Testing Lab
SDS Gel #01-052
05/07/01

Note:
Primary area of difference is indicated by arrow.



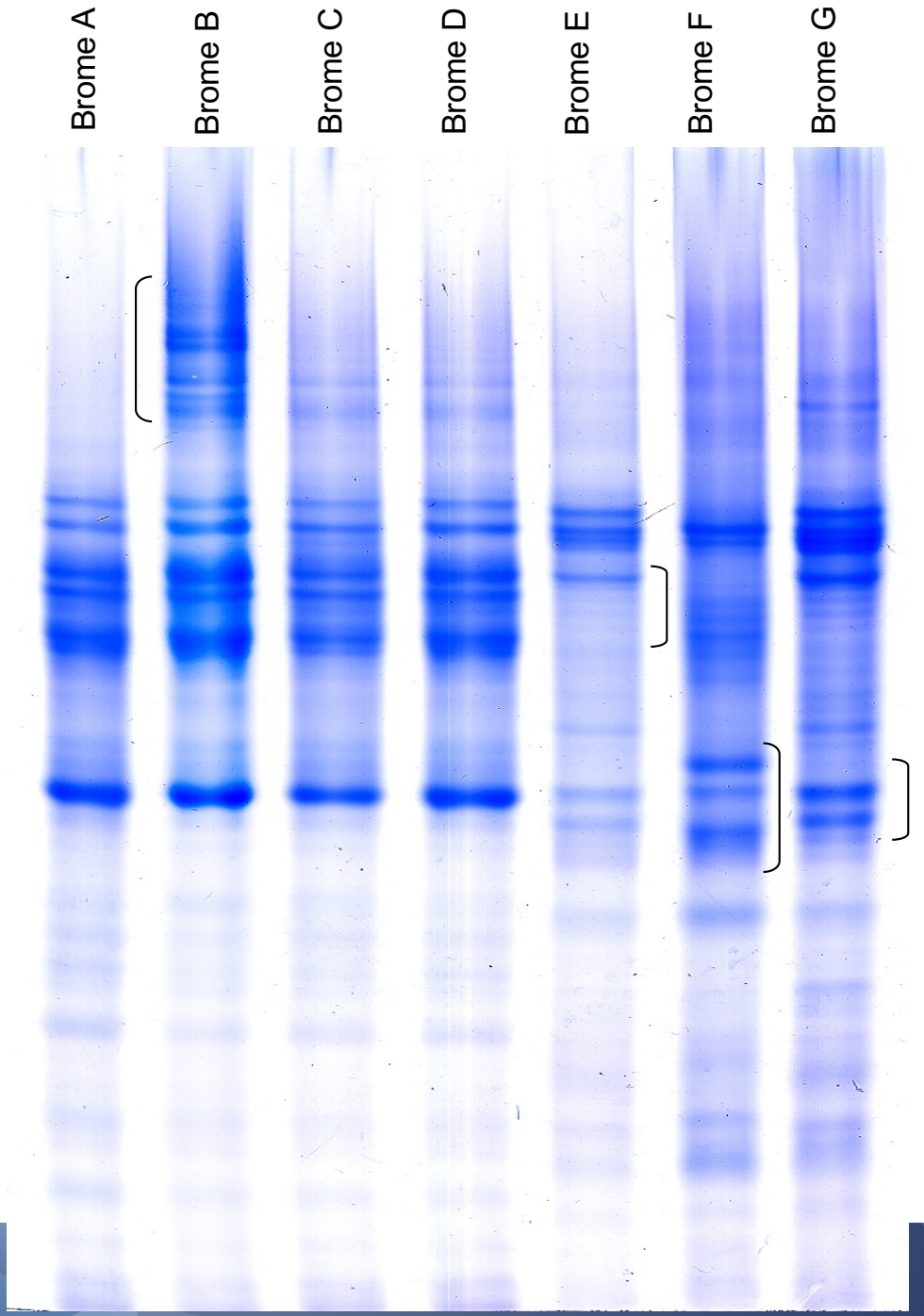
Brome

SDSU Seed Testing Lab
PAGE Gel #02-097
09/09/02

Note:
Submitted samples included Bromus stamineus (Mountain Brome), Bromus sitchensis, plus 3 from the same or sister lots. (It is unknown which is which.)

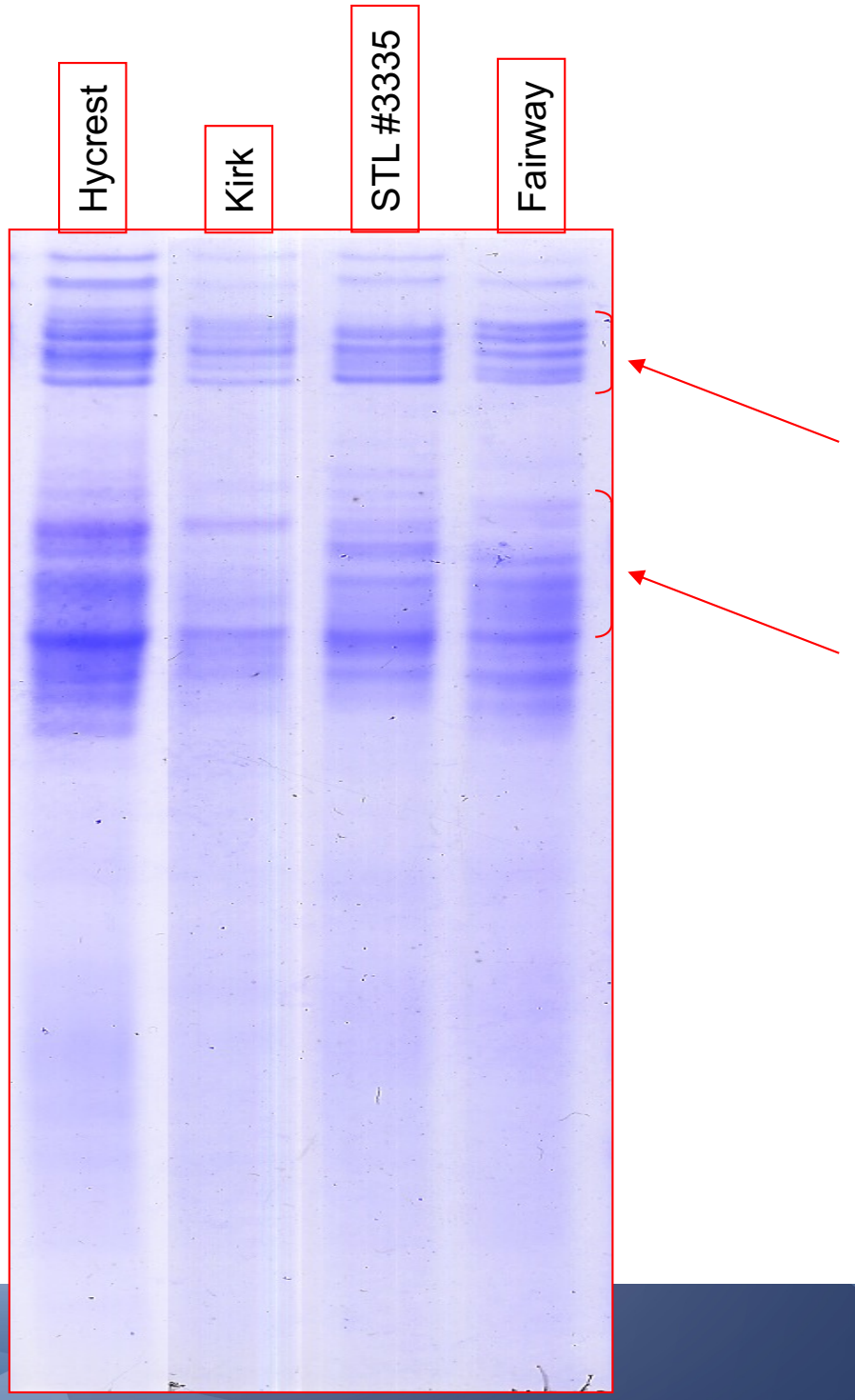
It looks like A, B, E, F & G area all different. C & D appear to match each other.

See bands for primary areas of differences.



Crested Wheatgrass

SDSU Seed Testing Lab
Urea Gel # 00-034
03/16/00



Note:
Primary areas of differences are indicated
by bracket and arrows.

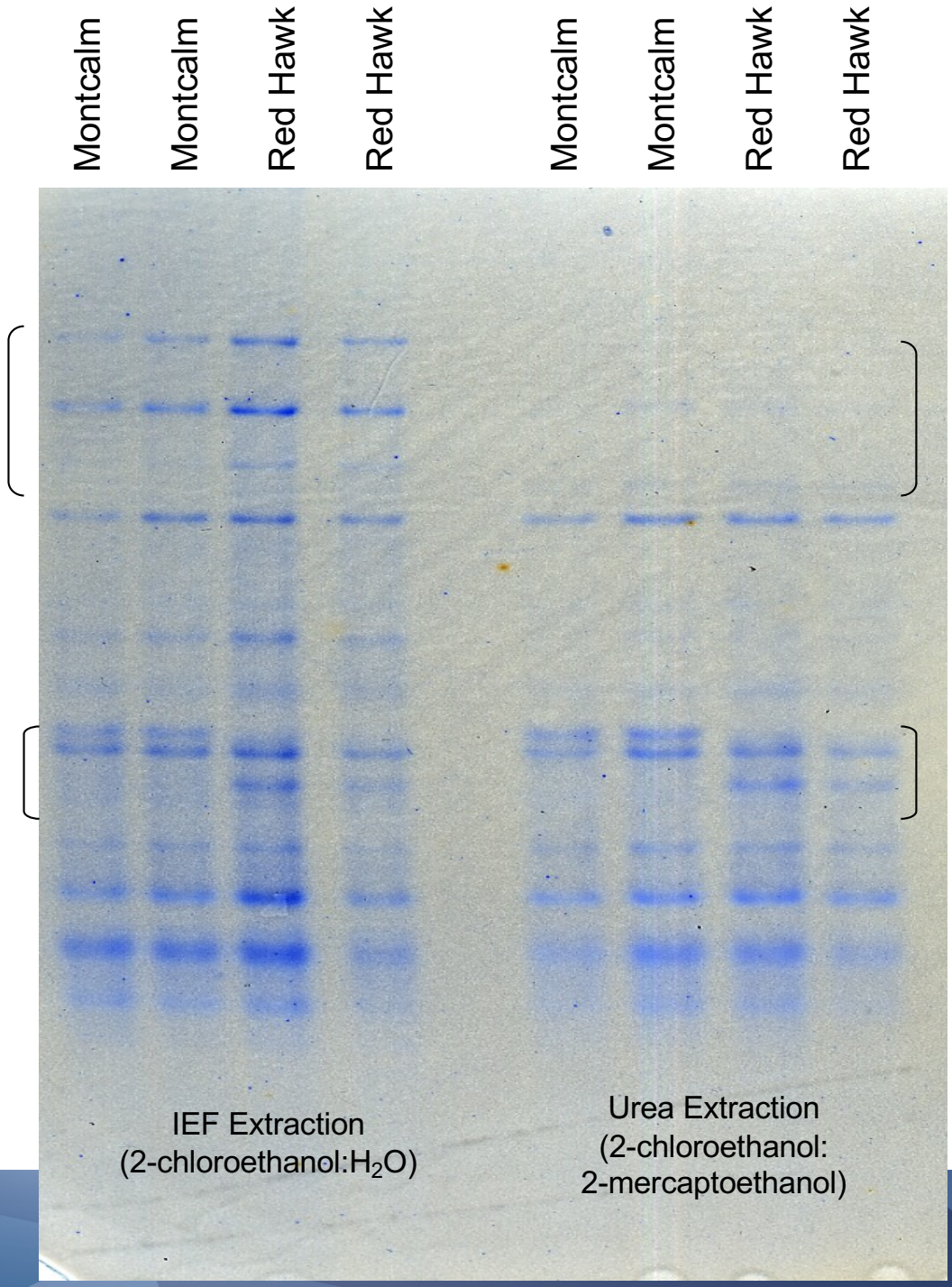
Kidney Beans

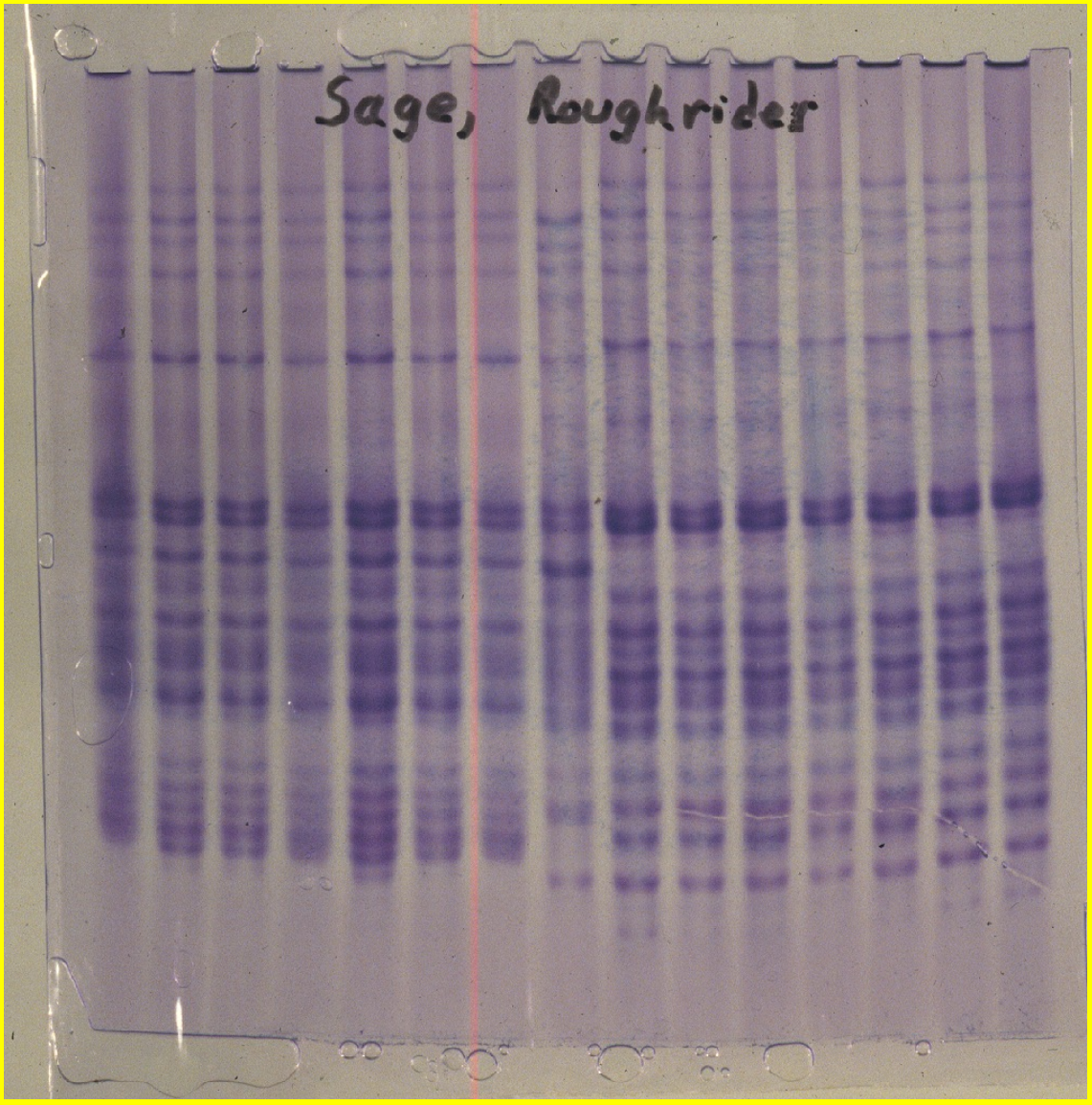
SDSU Seed Testing Lab
PAGE Gel #3-057
06/10/03

Note:

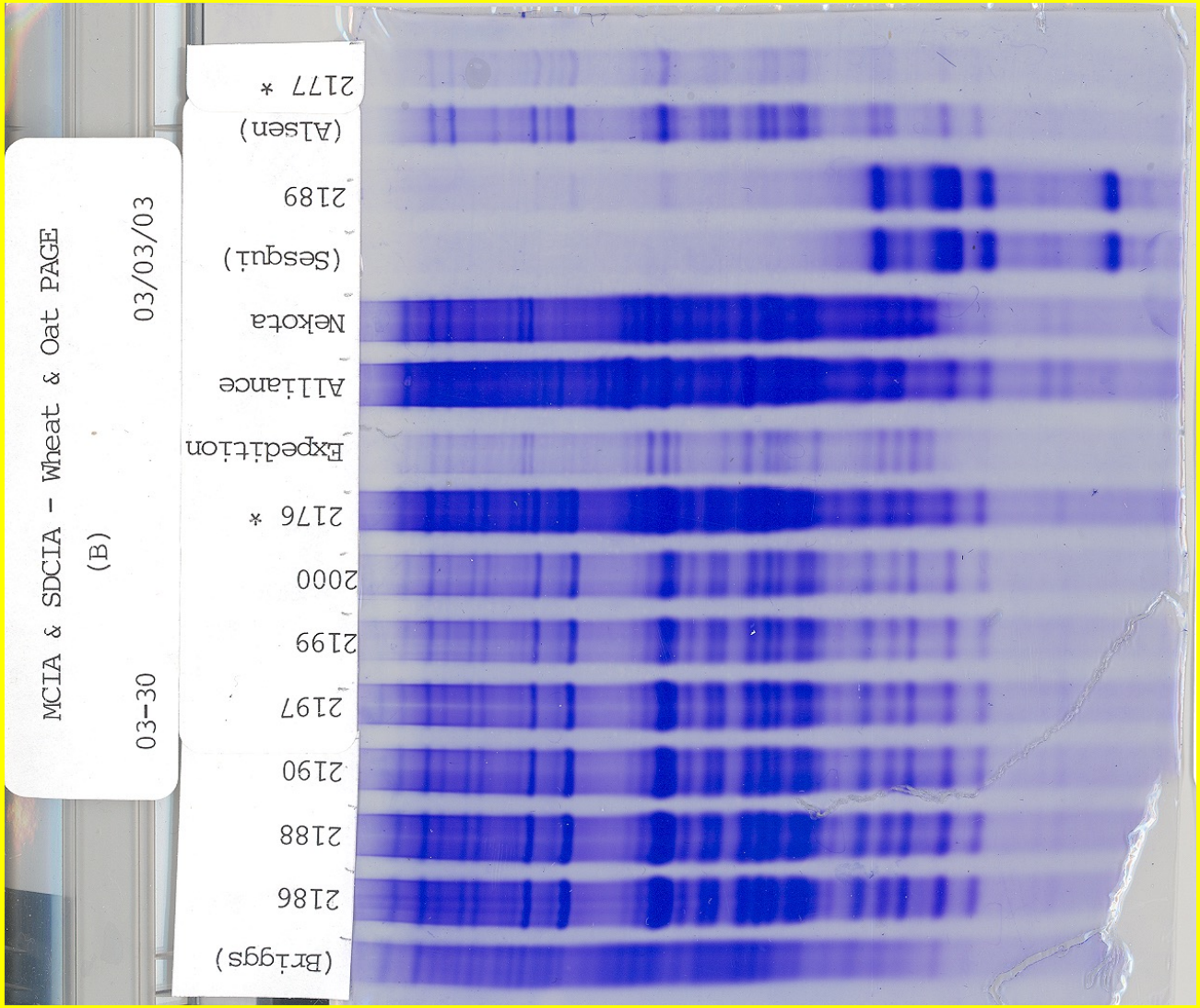
2 different types of extract were used on a PAGE gel: IEF and Urea method extractions. Both showed the same results.

See bands for primary areas of differences.

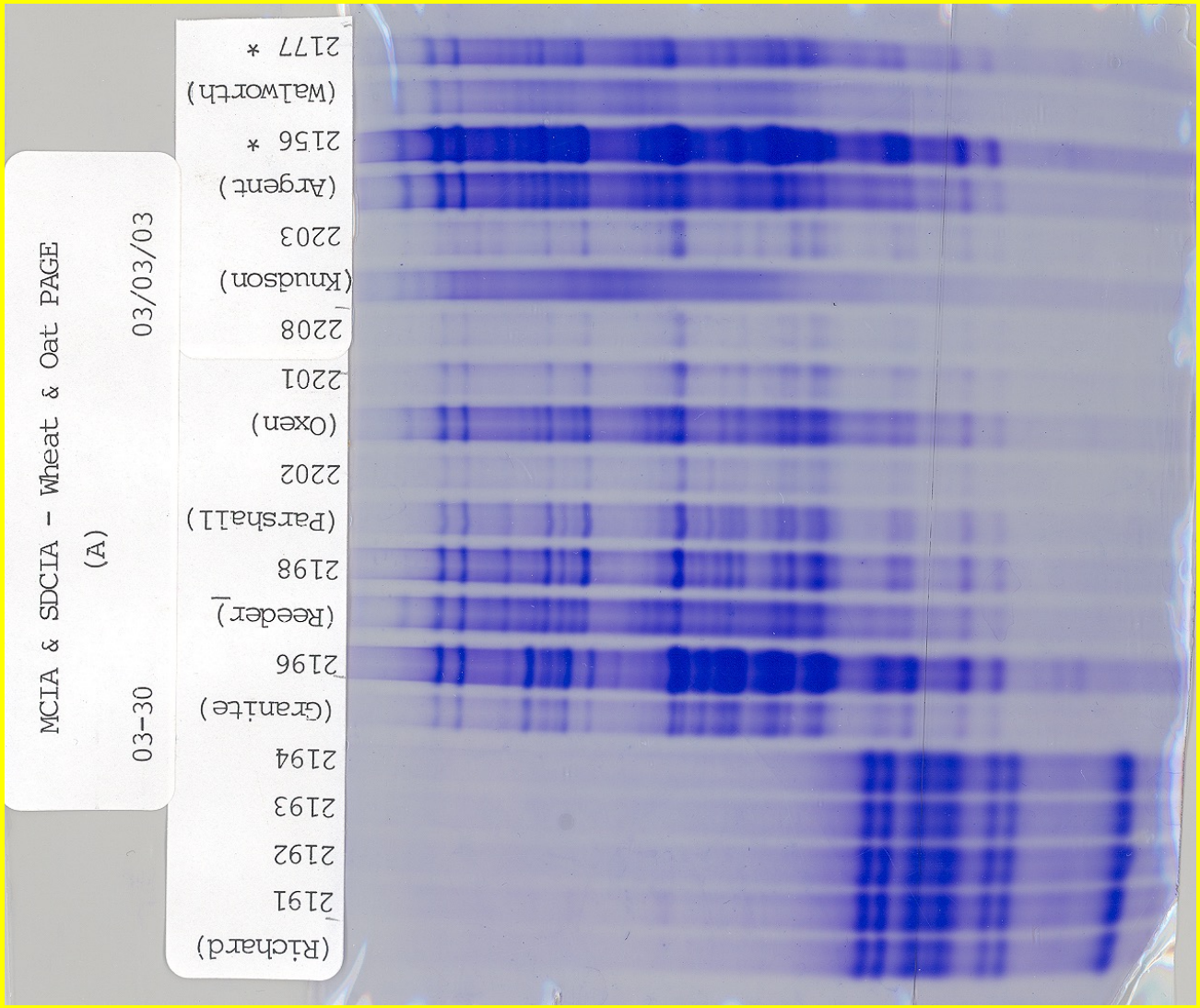




Stored in zip lock bag with water. Will store for a couple of months.



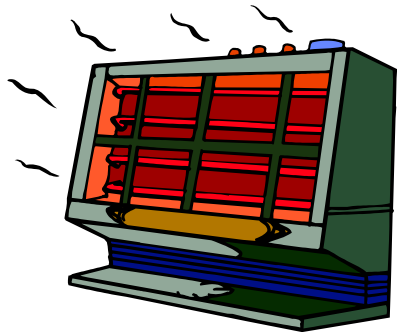
Gel dried between two sheets of cellophane. Will store for years.

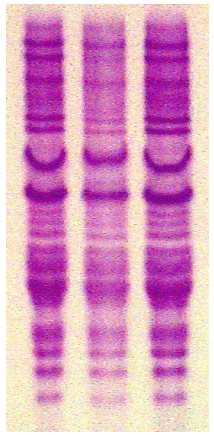


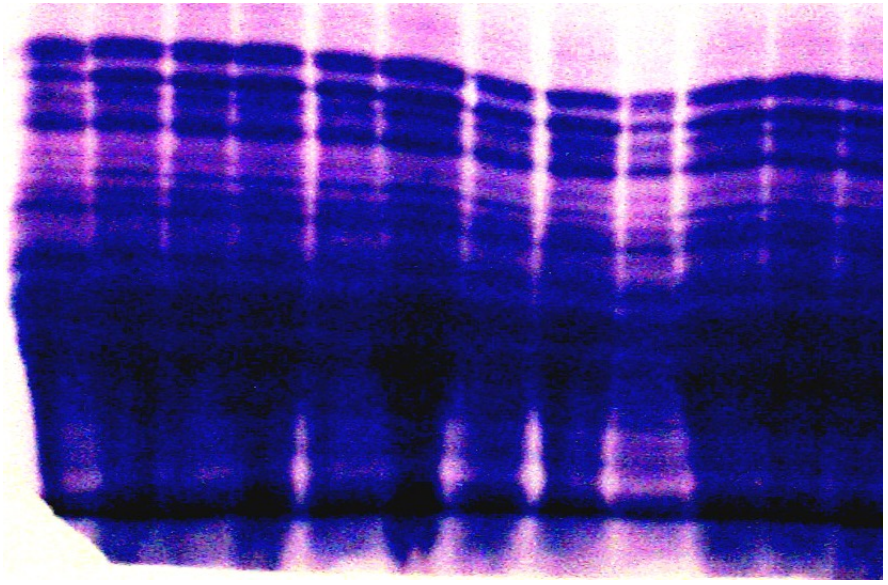
Gel dried between two sheets of cellophane. Will store for years.

Problems observed on gels or during the running of gels, or afterwards

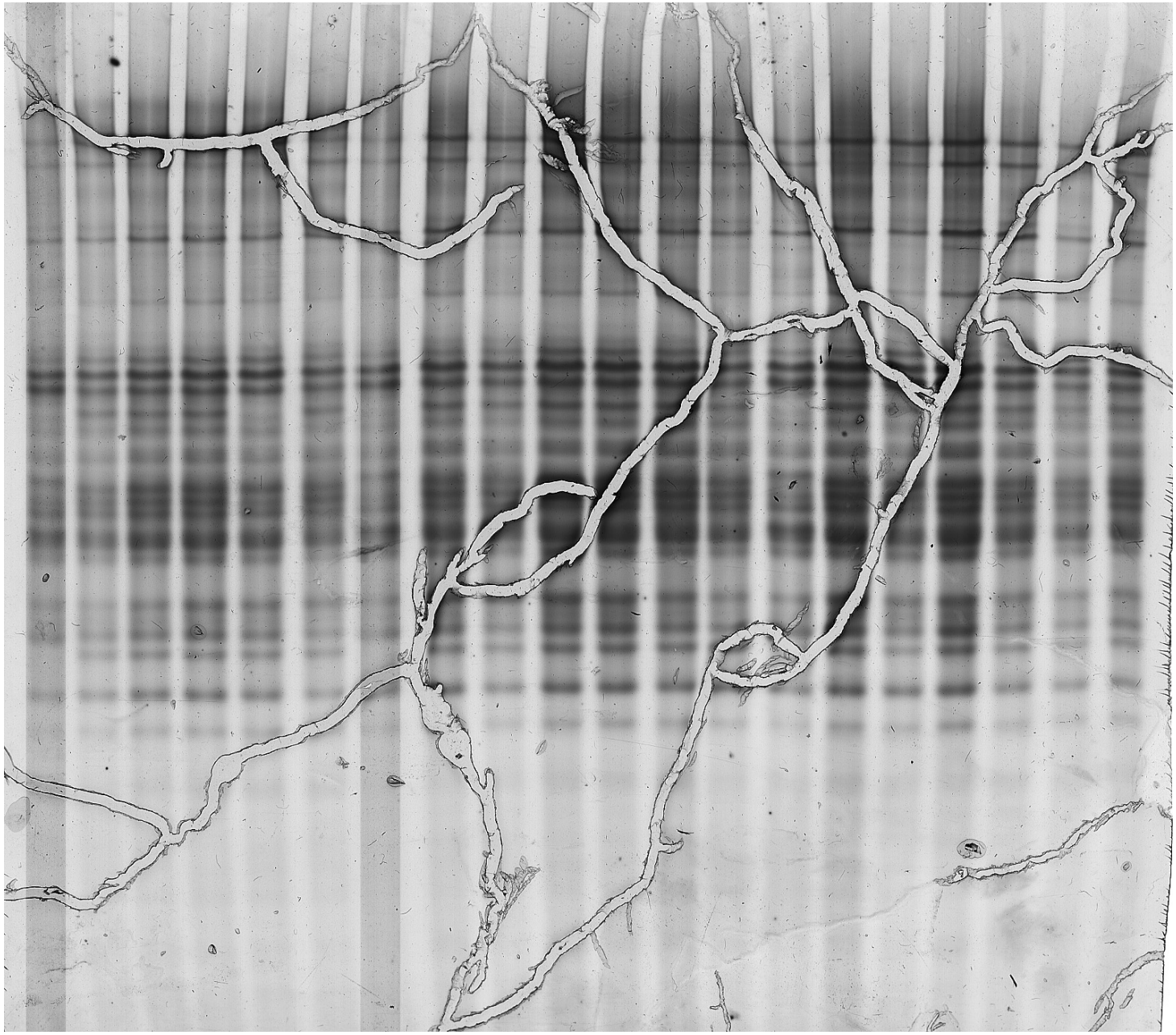
- Heating!!!
  - Band smiling
- Poor banding/staining
  - Check stain
  - Dead/dying seed
  - Low protein year (wheat)
  - Run-time too long/short
- No banding/staining
  - Power leads improperly connected/swapped around
  - Forgot to load wells
- It was a Monday!! (in the middle of the week)





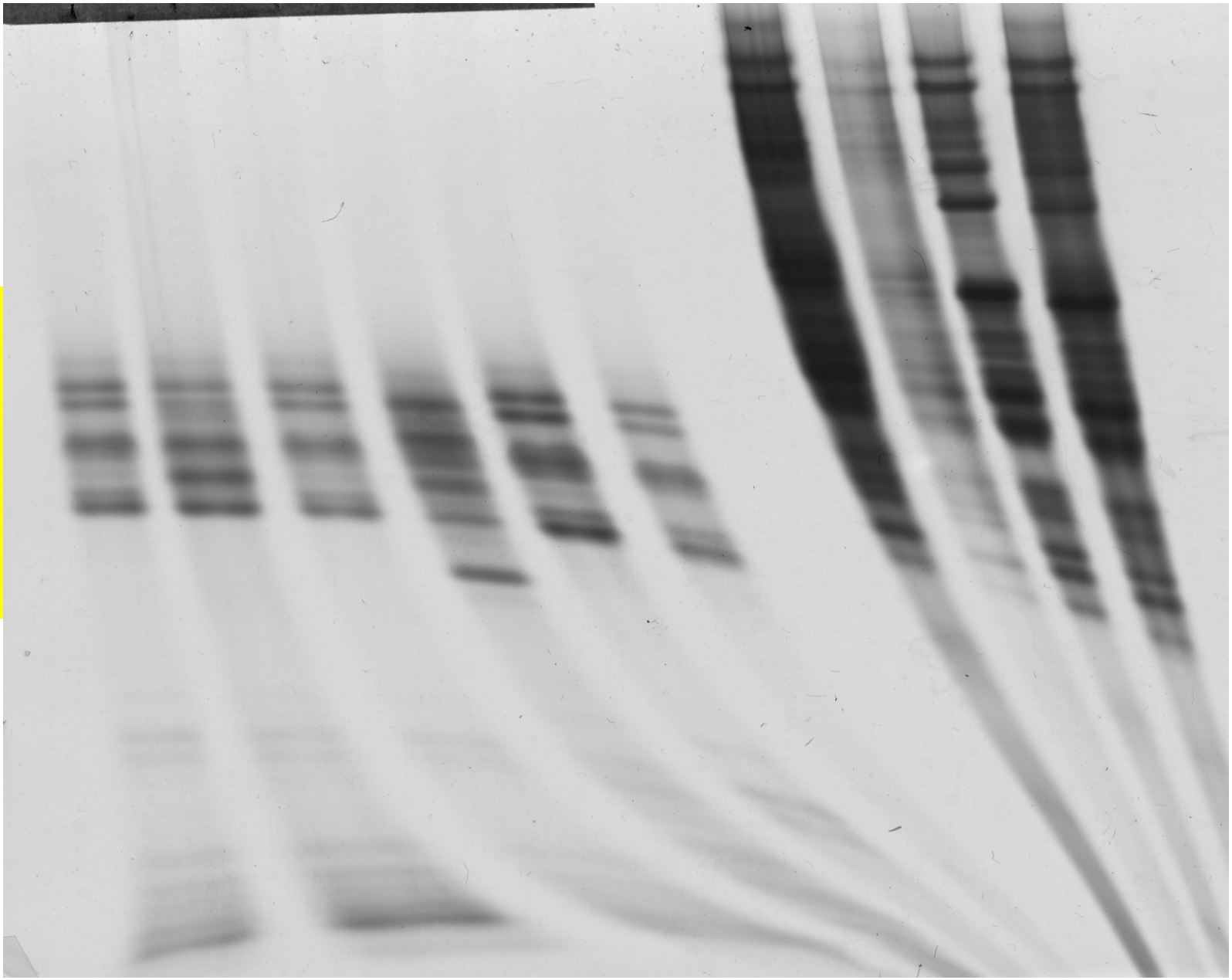


Cracked gel



Heating?

Dirty platinum wire?



Missing gel piece.