Methods for Assessing Genetic Purity

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Methods Available

- Nucleic Acid Based Screening
  - SNP (single nucleotide polymorphism)
  - iLLumina Sequencing
- Protein Based Screening
  - IEF (isoelectric focusing)
  - Isozyme screening (starch gel electros)
  - PAGE (polyacrylamide gel elec.) of Protein

SNP Genotyping

- Can be used to determine genetic purity
- Can detect one base pair difference in DNA
- Usually accomplished via TaqMan assays
  - Applied Biosystems approach
  - Would require ABI Prism Seq. Detector as well as Thermal Cyclers
  - Results would be computer generated numerical results

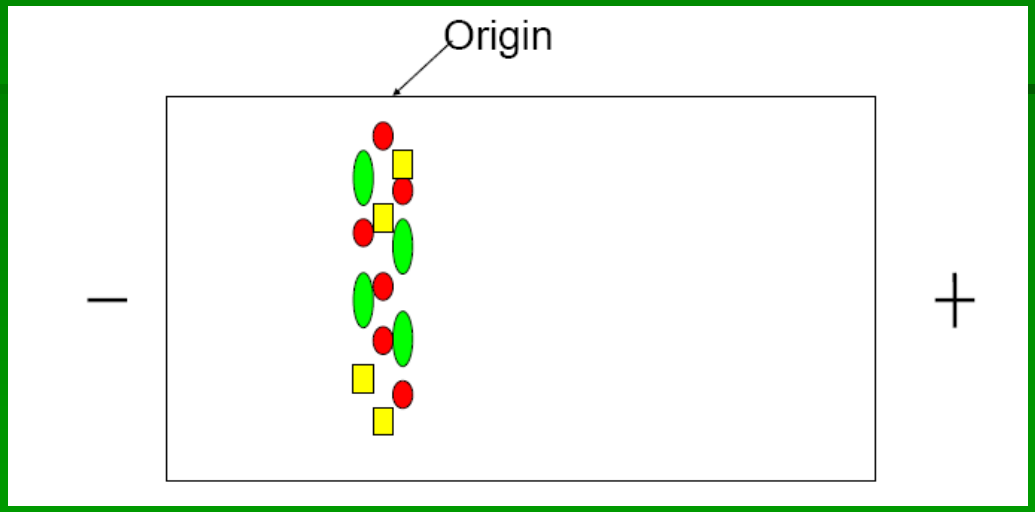
SNPs Continued

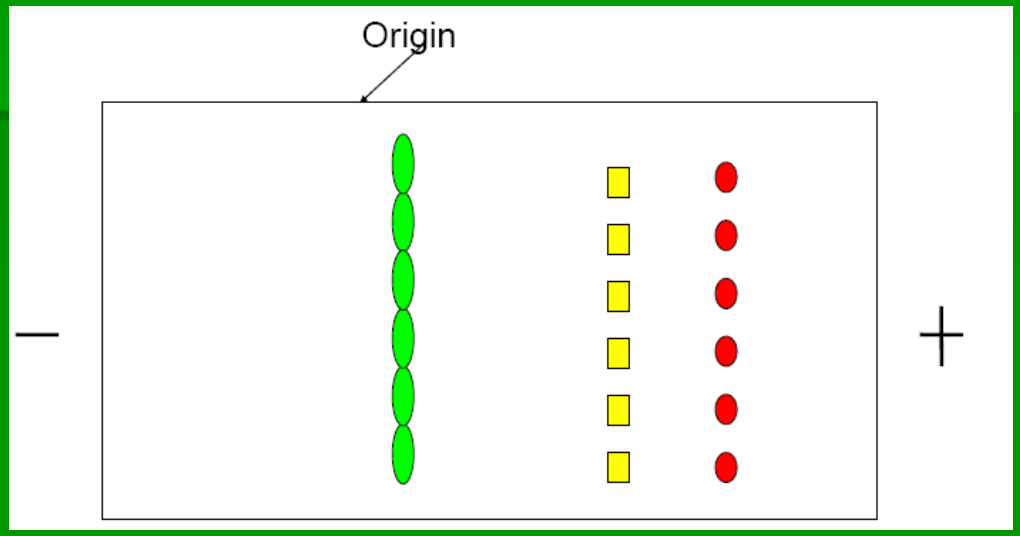
- Would require specific genomic sequences of proprietary parents to generate specific primers
  - Primers would need to be developed unless Lebanon is already using them
- Requires high level of knowledge and extensive optimization
  - Training necessary
- Initial costs are very high
  - Equipment nearing $150,000 and commodities are from a single manufacturer
- Advantage: Methdod of the future

Isozyme Screening

- Starch Gel Elect. to separate isozymes based on size and charge
  - Staining step to reveal bands of activity
- Allows for a direct observation of genotype for each locus
- Methods have been perfected over many years
- Starch gels currently used by BGS, BDI, Pioneer, Syngenta, IN Crop Improv.

Starch Gel Electros



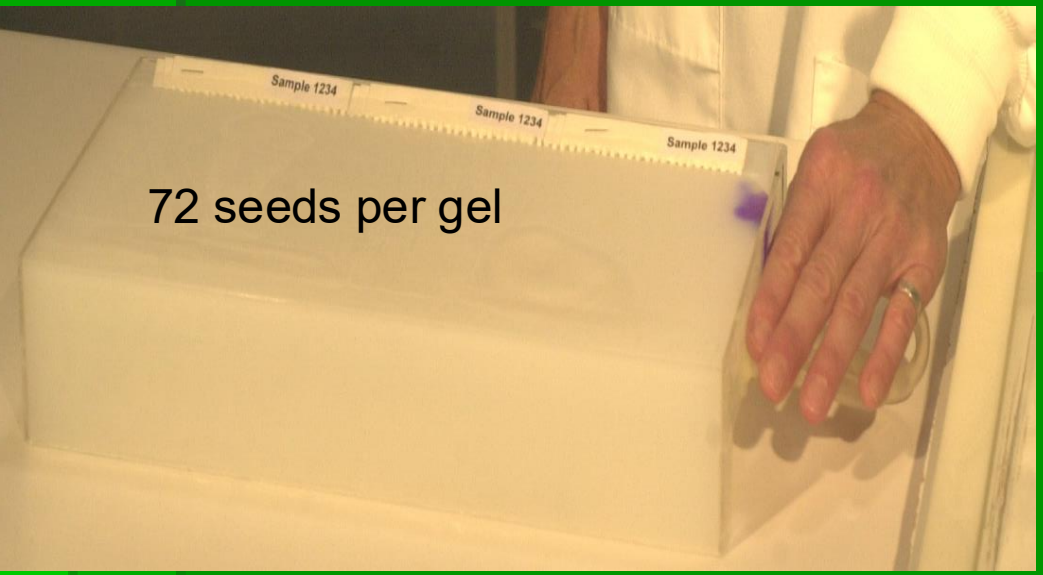


Starch Gel Procedures

- Cooking Starch via microwave or kettle
- Casting Gel in mold, curing at 4°C (total ~1.5 hours
- Extracting protein from coleoptiles
- Loading wicks onto gel and running ~4 hours
- Removing gel, slice into 8 slices for staining
- Place slices into stain boxes and add (stain + substrate + cofactor) mix
  - Total slicing/staining process ~1.5 hours
- Read gels, possibly photograph for reference
- Assign genotypes, describe off-types, selfs
  - Total reading/documenting ~0.5 hours for expert
- Total time to run a gel = ~8 hours
  - need to stagger gel runs to accommodate stain/sub addition

Optimizing Starch Gels

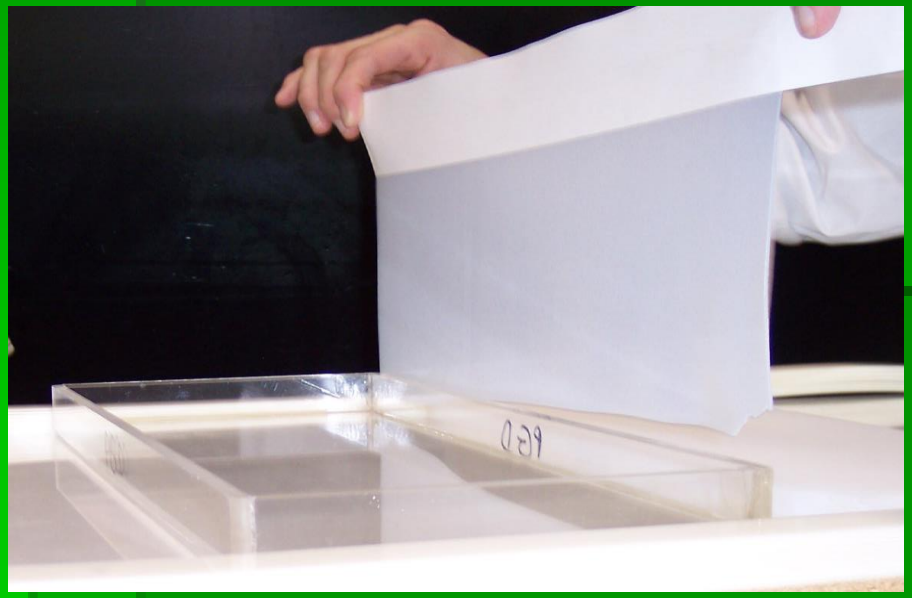
- Gel / Mold Considerations
  - Seeds per gel, one row or two
  - Rectangular gel with sponges or cast legs?
  - Sponges may not transfer current efficiently in large scale

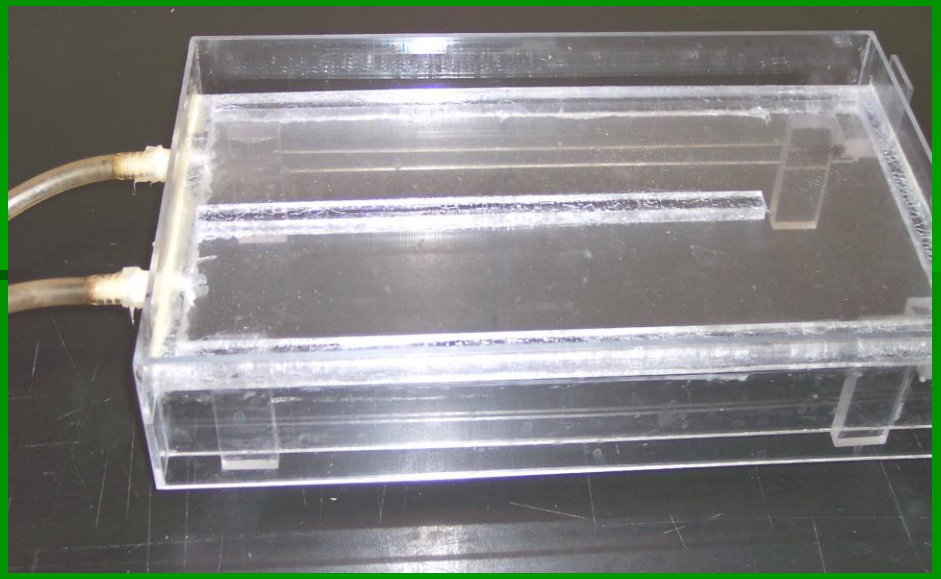


72 seeds per gel

Optimizing Starch Gels

- Use of blended starch, ease of handling
- Recipes vary for gels (BDI uses F or Tris gel)
- Microwaving, or bulk production (shelf life)
- Water cooled molds (BDI)





Optimizing Starch Gels

- Configuration of extraction plate, 48 wells?
  - Coleoptiles require less homogenization
  - Extraction set up needs to match wicks
- Wick combs can be premade (cut by hand)
  - Load sample onto wicks directly from ext. plate

Optimizing Starch Gels

- Running the Gel
  - Gels must be kept cold meaning cold water cooled in cold room or ice pack cooled in dairy case/cold room
  - May be able to fit 12 gels in large dairy case
    - would require routing elec. leads into case
  - High Voltage power supplies needed too achieve run-time of 3-4 hours
  - Faster run time = more heat

Optimizing Starch Gels

- Slicing Gels
  - Some groups use one slice for multiple stains (Syngenta and Pioneer)
    - Syngenta uses 4 slices for 15 loci
    - Pioneer is staining various regions of gel
    - BDI uses individual slices
  - 8 good slices is likely the maximum for one thick gel
  - Using multiple stains per gel requires advanced interpretation
  - Slicing equipment will need to be custom made
  - Filter paper used to transfer slices to trays

Optimizing Starch Gels

- Staining Gels
  - 30 gels x 8 stains = 240 stain boxes incubating at 37°C in dark
  - Substrate/cofactors mix can be premade in frozen aliquots
  - Liquid stain combinations are added to each gel slice

Optimizing Starch Gels

- Staining Reagents
  - Substrates and NAD are very expensive
  - ~50-200mg quantities need to be measured for mixes to add to gels
  - Sigma is only producer for IDH, PHI, PGM substrates
  - Unknown quality from other producers
  - Agar or Agarose overlays may reduce amount of substrate per gel
  - BDI, Pioneer, IN Crop do not use overlays

Optimizing Starch Gels

- Reading/Scoring Gels
  - Takes experience to decipher banding patterns
  - Will need to “decode” current data
    - May require samples being sent to BDI
  - Multiple stains per gel can be complicated
  - May need to run parents on gel every time
  - Light box required, UV for GLU locus
  - Photographs need to be taken initially

Advantages of Starch Gels

- Fairly inexpensive equipment, although custom made
- Commodities are relatively inexpensive
- Scoring system exists for corn
- Stain is only toxic/hazardous component
- Most chemicals can be purchased in large quantities or bulk
- Produces genotypical info for every locus tested, can identify ind. segregating loci

Disadvantages of Starch Gels

- Very labor intensive
  - May need 4 seasonal employees
  - Highly coordinated activity
- Gels have only 1 day shelf life
  - Must be cast night before
- Starch from 2 suppliers, lots can vary
- Some substrates only from Sigma
  - Costs rose 15-30% from last year
- All solutions must be made in the lab
- Coordinating 240 staining trays
- Possibly 3 Dairy cases for 30 gels

Equipment Costs for Starch Gels

- Fabrication of gel molds, buffer trays, slicers, mortars, pestles, staining trays, wick jigs = ??
- Large Microwave or stirring kettle = ~$1K
- Power Supplies 10 @ $2300 each = $23K
  - This is assuming 30 gels in one day
- Dairy Cases 3 @ $6500 each = $19.5K
- Ice Maker 1 @ $5000 = $5K
- 37°C Incubators 2 @ $8000 = $16K
- Fluorescent Light Box 2 @ $0.5K = $1K
- UV Light Box 1 @ $1500 = $1.5K
- Total = ~$67K
- General lab equipment = ~$23K

Starch Gel Consumables per Gel

- Assuming a 900mL Gel w/ scaled up chemicals, using no agarose overlays and no titration of substrate, cofactor, or stain
- Starch and Gel Buffers, only Starch Art = $11.80
- Staining Box Buffer = $0.30
- Substrates using Sigma alternatives = $25.55
- Cofactors using Sigma alternatives = $3.57
- Stains using Sigma alternatives = $5.52
- Not included: wicks, tape, paper towels, filter paper, chemical shipping costs or labor
- Total cost per 100 seeds (1 gel) = $46.74
- Using only Sigma chemicals = $62.67

Isoelectric Focusing

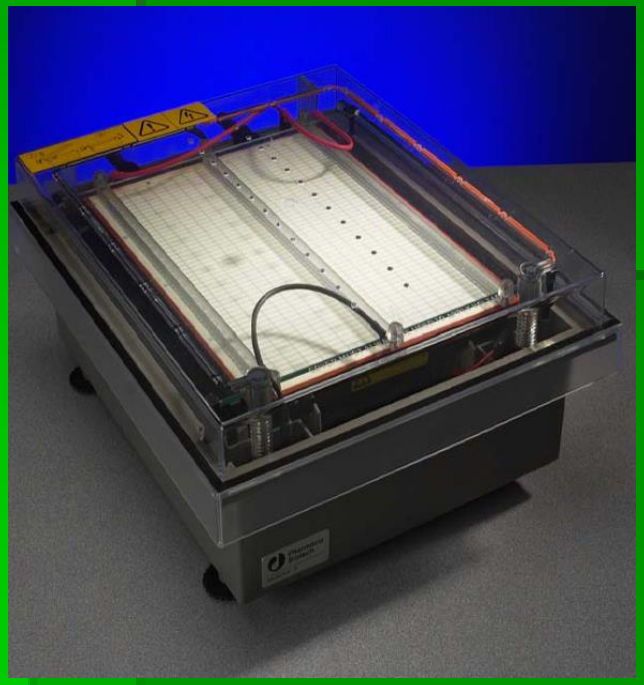
- Separates enzymes based on size, charge, and isoelectric point
  - One stain for each gel shows total protein present
  - Observe banding pattern to determine purity
- Uses manufactured gel rig, precast gels, and pre-made supplies
- Currently used by Mycogen and BDI

Isoelectric Focusing Procedures

- Crush/cut seeds to extract total protein overnight ~10 min.
- Arrange gel and electrode strips and load extract onto gel wicks ~15 min.
- Run gel ~2.25 hours
- Remove gel, fix proteins in gel ~15 min.
- Rinse gels with water twice, 2 hours
- Stain gel using silver stain ~15 min.
- Shake with stop solution ~10 min.
- Rinse with water ~10 min.
- Total time to run a gel = 5.5 hours

IEF Considerations

- Procedures, Equipment, and Commodities are standardized
- Consumables are pre-made

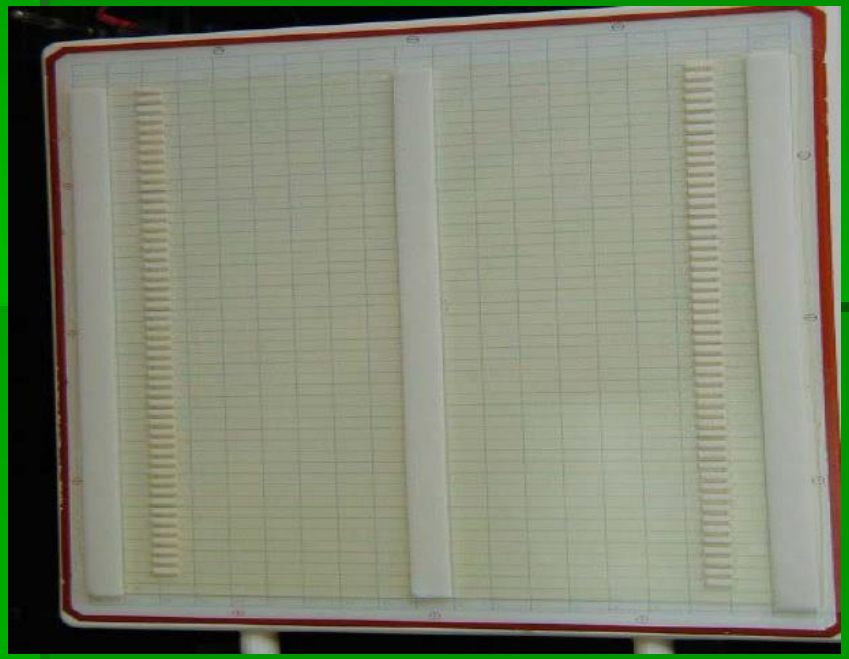


IEF Procedure

- Seed cutter/crusher used
- Total protein is extracted from crushed kernels that are soaked in water overnight

IEF Procedure

- Electrode Strips and sample templates are positioned on gel
- Protein extract is loaded onto chads on gel



IEF Procedure

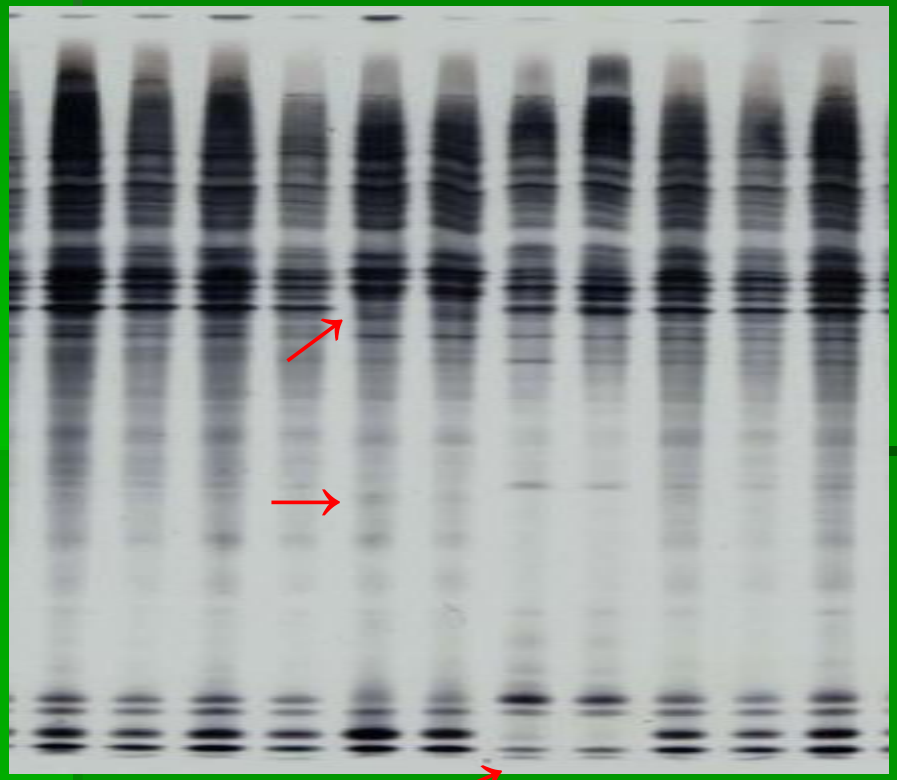
- Gel is run in three stages
  - Stage 1 50 min.
  - Stage 2 40 min.
  - Stage 3 40 min.
- Gel is removed, proteins fixed using TCA 20min.
- Two water washes of 1 hour each, 2 hours
- Gel is dried overnight at RT, or in incubator
- Gel is silver-stained for ~10 min.
- Staining reaction stopped in acetic acid for 10 min.
- Gel rinsed in water 10 min., and air-dried
- Total time required = 5.5 hours

IEF Procedure

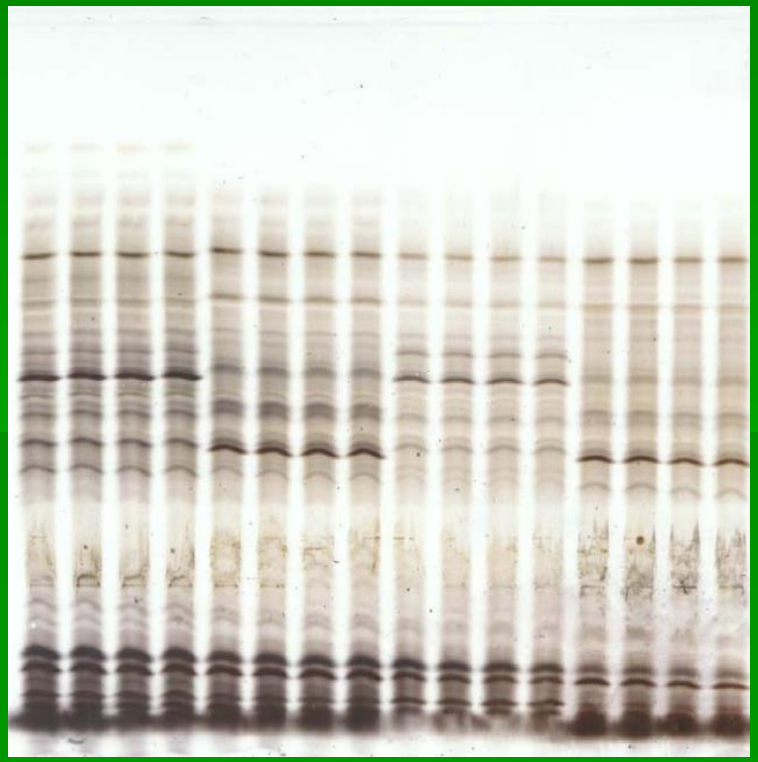
- Fixing, Staining, and Stopping involve toxic chemicals (formaldehyde)
- Must conduct incubation steps in hood
  - TCA must be kept in hood
  - Silver stain mix must be kept in hood
  - Acetic acid must be kept in hood
  - All solutions must be disposed of properly
  - Waste must have proper pH for disposal
  - Workers must wear proper clothing

IEF Results

- Gels show distinct, sharp banding patterns
- Able to distinguish selfs and offtypes
- Cannot assign genotype w/o further testing



Corn



Soybeans

Advantages of IEF

- Gels are pre-made, high quality, long shelf life
- Only fix, stain, and stop solutions need to be made in lab
- 30 gels = 30 gels to stain
- No substrate costs involved
- Less labor intensive than starch, shorter time required
- No germination required
- Stained gels can be stored indefinitely
- No custom-made equipment
- No large scale cooling
- Can be used on variety of crops, soybeans

Disdvantages of IEF

- No allele specific data is generated
  - Could use starch gels to identify segregating loci if needed
- No previous data sets to compare to
  - Would need to send samples out for verification purposes
- Multiple fume hoods needed
- Waste must be properly disposed of
- Workers must be careful with chemicals

Disdvantages of IEF

IEF Equipment Costs

- GE Multiphor II 10 @ $2.9K = $29K
- GE Mutitemp III Circulator 3 @ $3.3K = $9.9K
- Power Supply 5 @ $1.3K = $6.5K
- Orbital 3 tier shakers: Need 3 shakers per station
  - Possibly a total of 15 @ $1.5K = $23K
- Extra fume hoods 2 @ ~$6.5K = $13K
- Total for IEF = ~$82K

IEF Consumable Costs

- HyPure Agarose IEF Gels from Perkin Elmer are ~$40 each according to Mycogen
- Sample templates 2 @ $32.40 = $64.80 ??
- Cost of fixing, staining, stopping = $3.538
  - Chemicals are very inexpensive
- Total cost per gel ~ $105