Proteomics Workshop

Sample prep and complexity reduction

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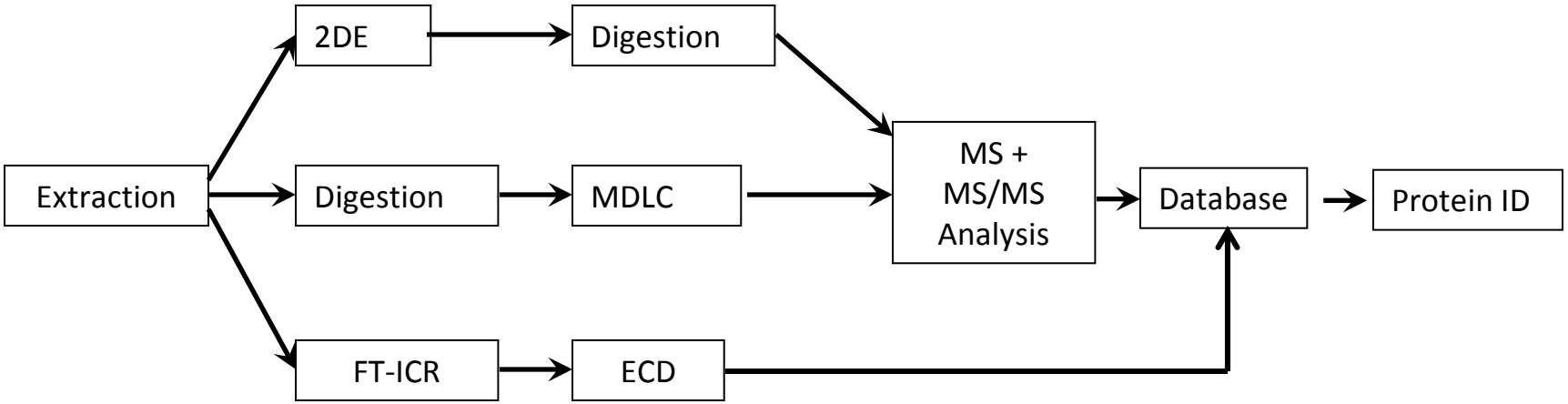
Overview:

- Proteomics workflows
  - Sample prep is key
- Samples are complex
  - require simplification
- Strategies for complexity reduction:
  - Pre-separation (tissue → cells → organelles)
  - Protein-centric approaches (2D electrophoresis)
  - Peptide-centric approaches (MuDPIT)
  - Mixed approaches (GeLC)

Proteomics Workflows:

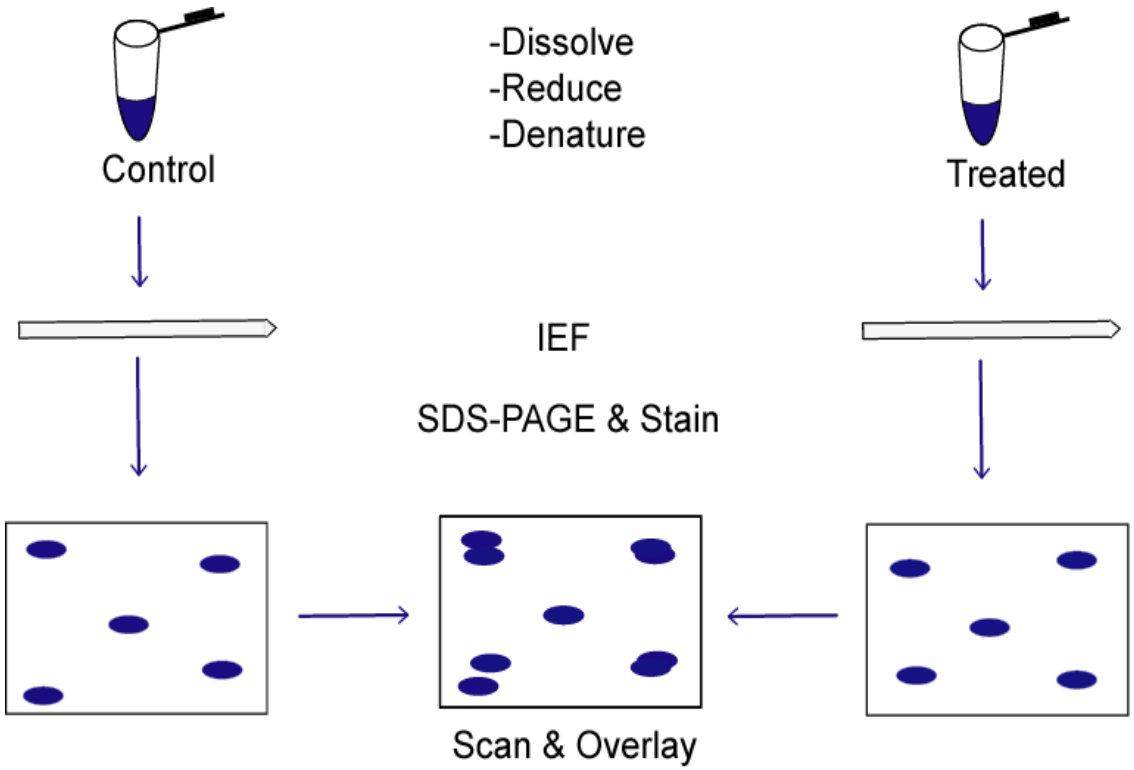


A linear process

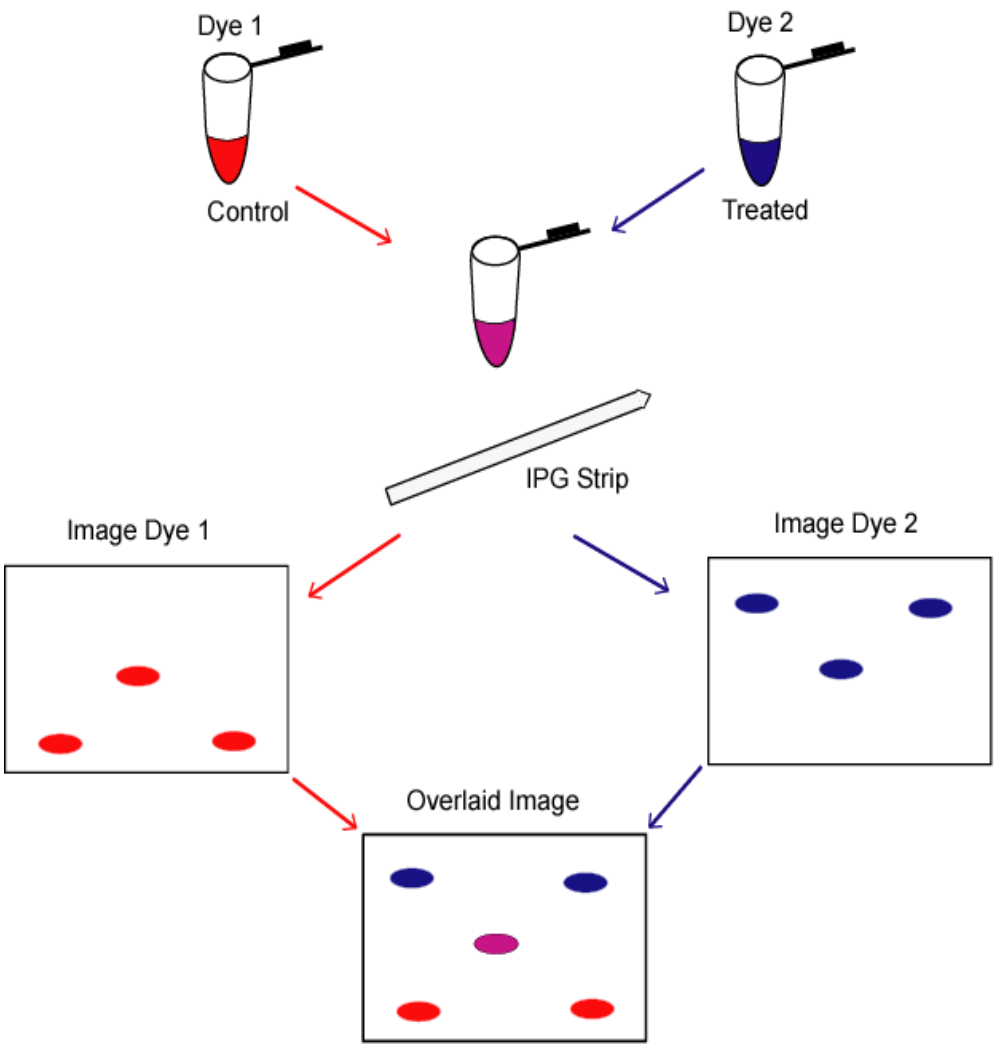
Proteomics or Extractomics?

- Extraction is the most important step
  - Errors introduced here propagate
- Often this is step receives little attention
  - Methods chosen from literature
  - Originally selected due to “good looking gel”
- Many are available but which is “best”?
- Can compare methods with DIGE
  - Difference Gel Electrophoresis

Standard 2D Electrophoresis



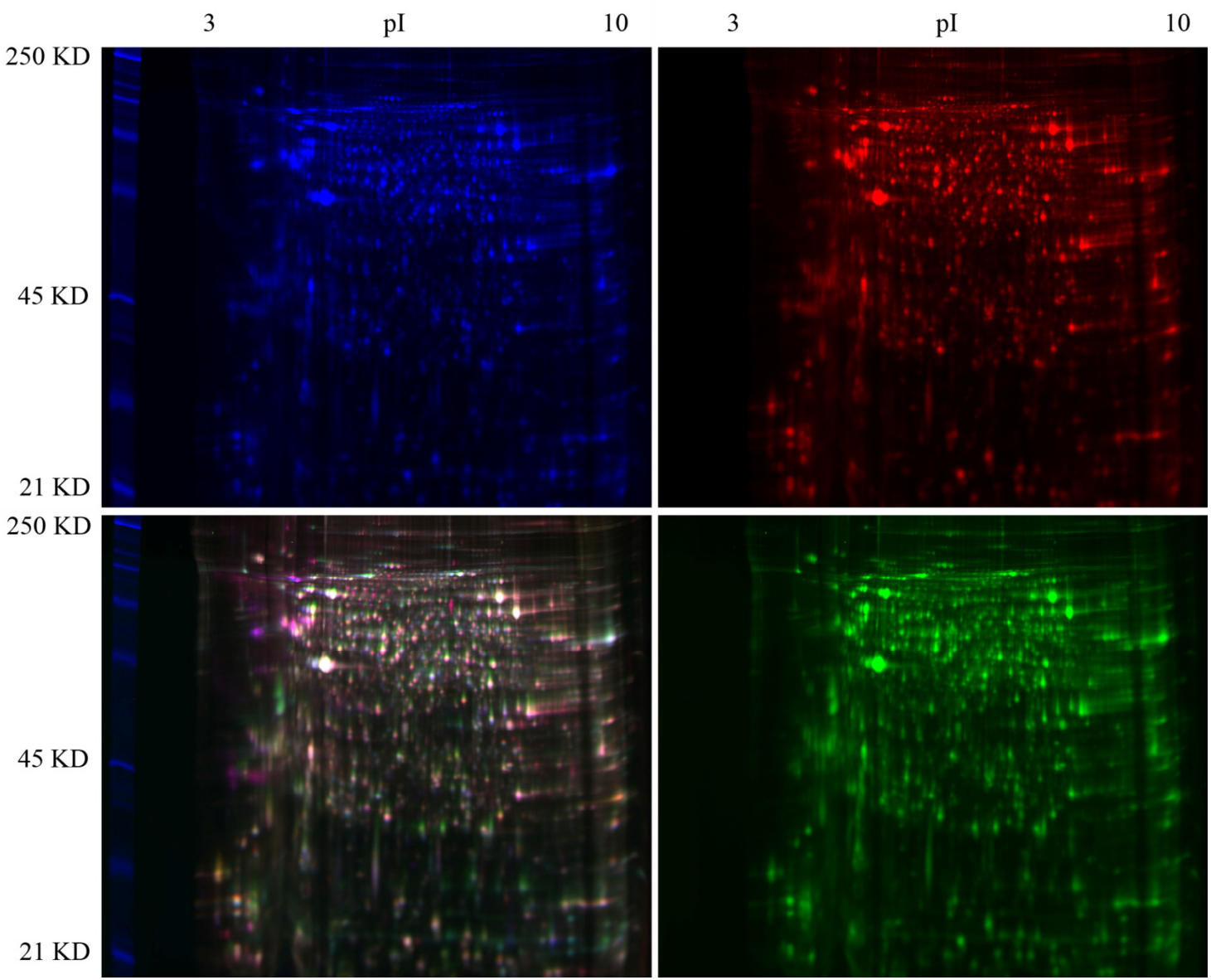
DIGE


Dye 1
Control
Dye 2
Treated
IPG Strip
Image Dye 1
Image Dye 2
Overlaid Image

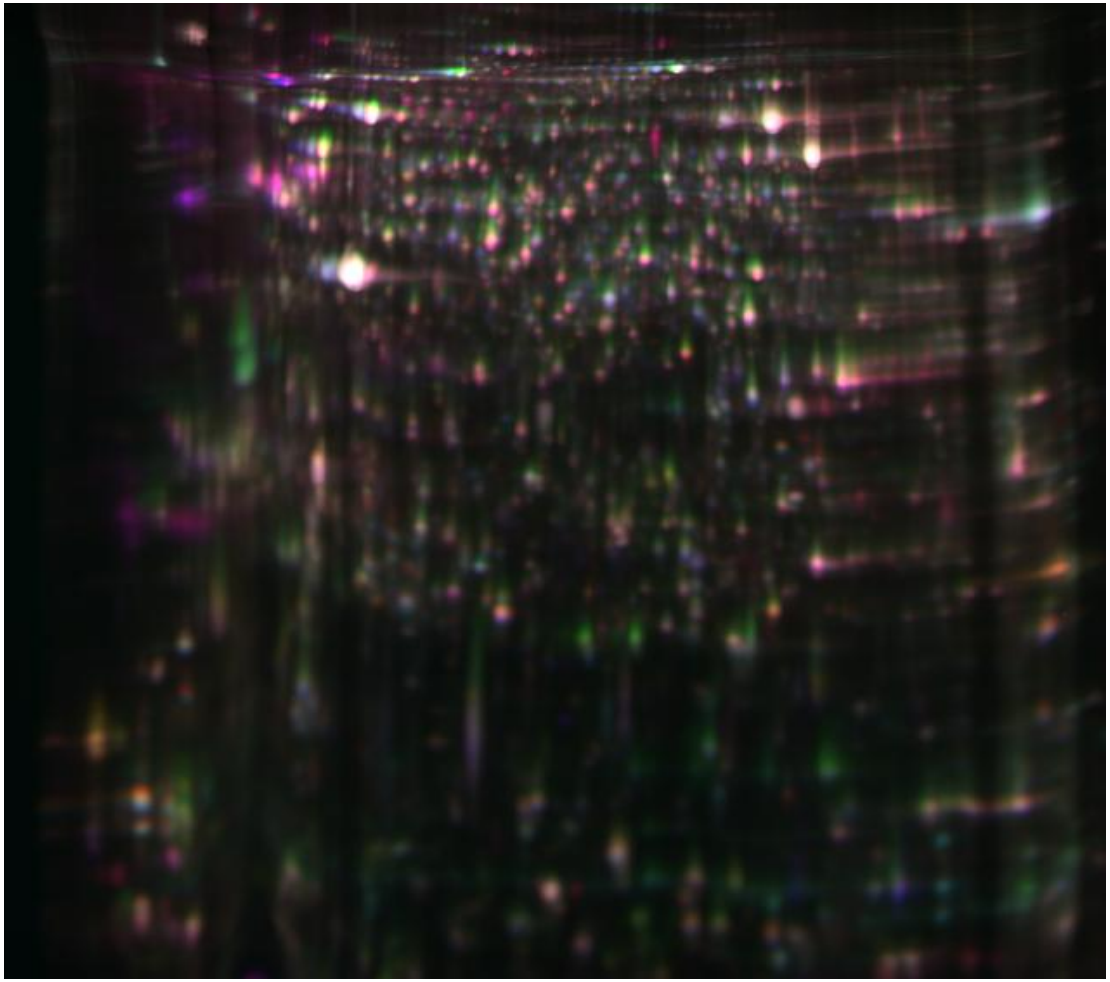
Experimental details

- Equal masses of biological material extracted
  - *Schizaphis graminum*
- TCA acetone extraction: Cy3
  - Isaacson, et al., Nat Protoc, 2006, **1**: 769-74.
- Phenol extraction: Cy5
  - Saravanan & Rose, Proteomics, 2004, **4**: 2522-32.
- Multi-detergent extraction: Cy2
  - Barrios-Llerena et al. Electrophoresis, 2007, **28**:1624-32.
- 50 μg protein from each extract → DIGE

Individual images plus an overlay



Enlargement of overlay



Comparison of Extractions

Extraction Type	Number of Spots	Mw Range	pI range
Phenol	1317 +/- 33	>200KD-6.5KD	3-10
TCA/ Acetone	1,567 +/- 57	>200KD-6.5KD	3-10
Multi-Detergent	1333 +/- 306	>200KD-6.5KD	3-10

Comparing the Numbers:

- In total 1589 protein spots found
  - 1259 similar concentration in each extract
- TCA: 122 differentially extracted spots
  - 36 in Phenol, 36 in MD, 50 unique
- Phenol: 128 differentially extracted spot
  - 36 in TCA, 16 in MD, 76 unique
- Multi-detergent: 80 differentially extracted
  - 36 in TCA, 16 in phenol, 28 unique

Conclusion:

- If multiple methods are tested one will be best, but…
- No single extraction protocol is sufficient.

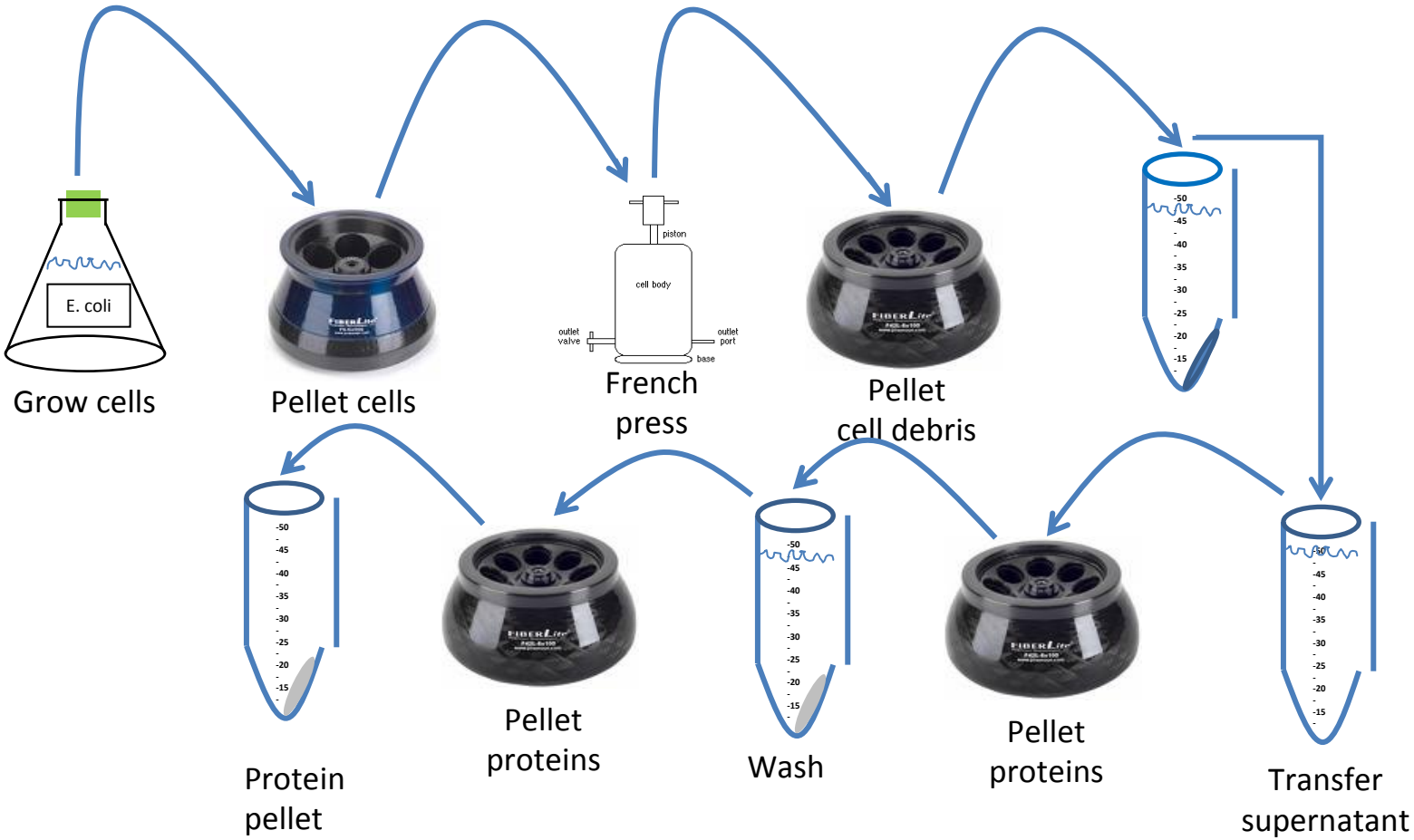
Proteomic samples are complex:

- How complex?
  - Arabidopsis has 2.5 $x10^4$ genes
  - Splice variants, PTMs, etc. → 10^5 -10^6 Proteins
  - Area of a Spot ≈ 7 mm^2
  - Area of a large format 2-D gel = 50,000 mm^2
  - Spot capacity of gel ≈ 7,000
  - Thus, insufficient to resolve the components of an entire proteome

Strategies to reduce complexity

- Isolate the tissue/cell type/organelle of interest
  - Arabidopsis has 2.5 x10^4 genes
    - Chloroplast has only 3.5 x10^3 genes: much smaller problem
- Separate based on physicochemical properties:
  - Protein-centric methods:
    - Sub-fractionate proteins → digest → MS
  - Peptide-centric methods:
    - Digest proteins → fractionate peptides → MS
  - Mixed methods
    - Fractionate proteins → digest → fractionate peptides → MS

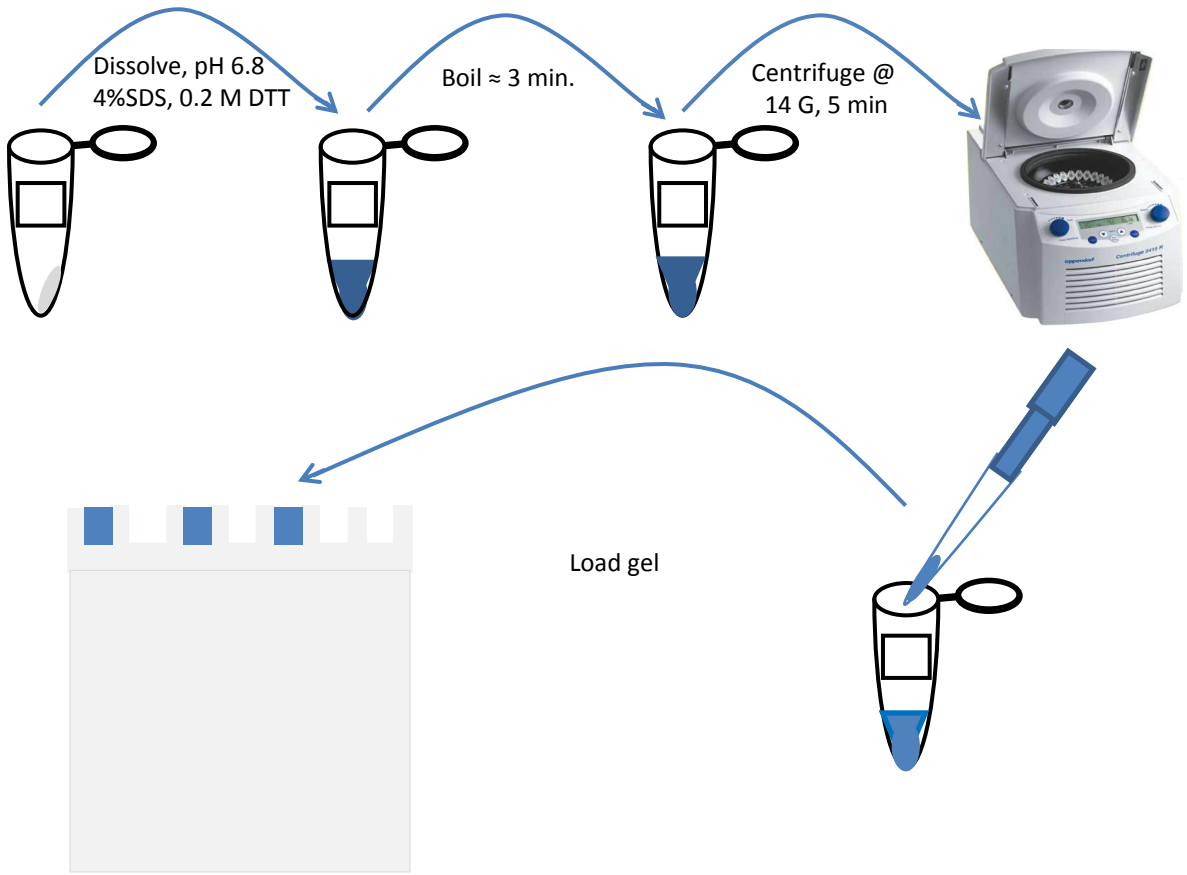
Schematic of protein isolation:



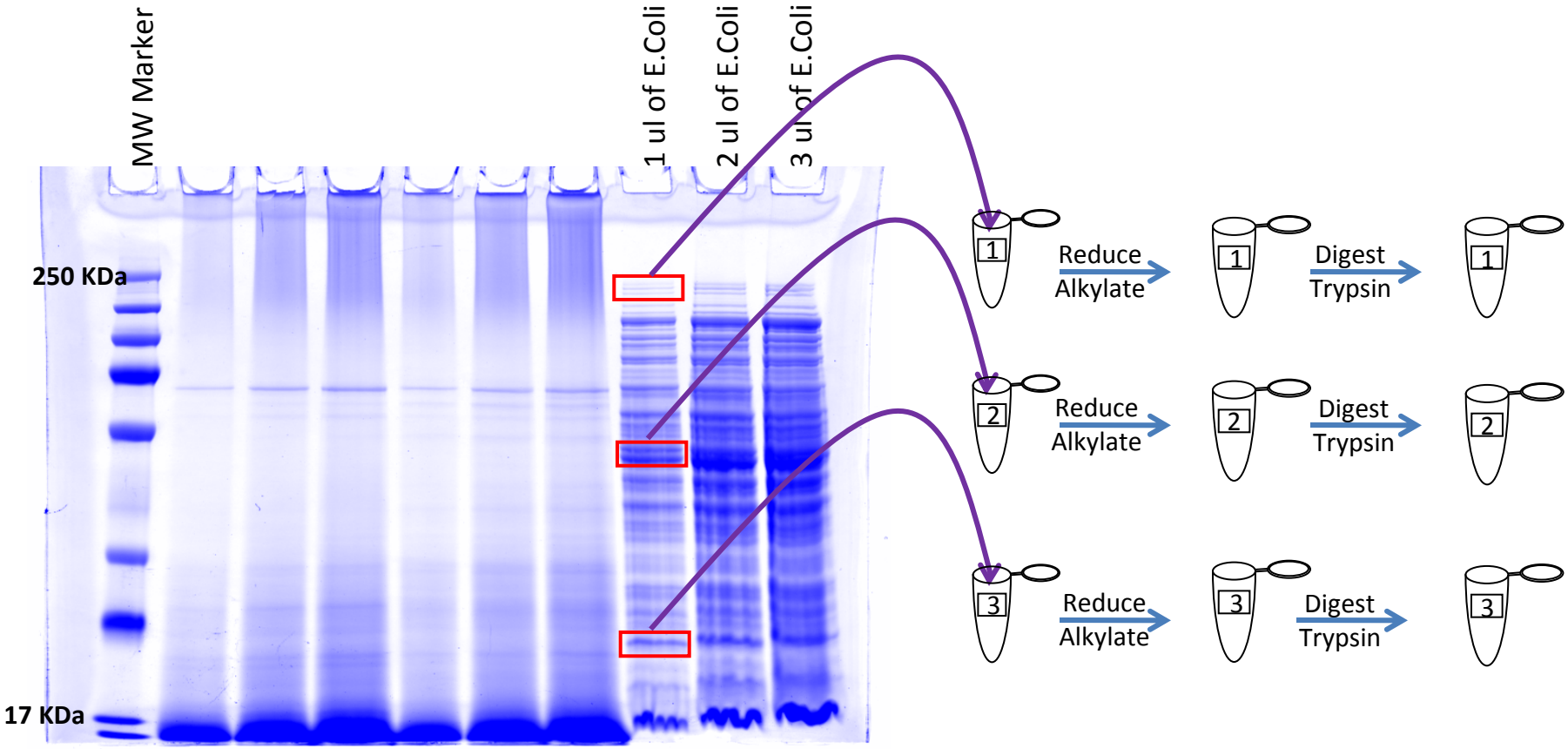
1st Dimension Separations:

- SDS-PAGE
- SCX
- OffGel IEF

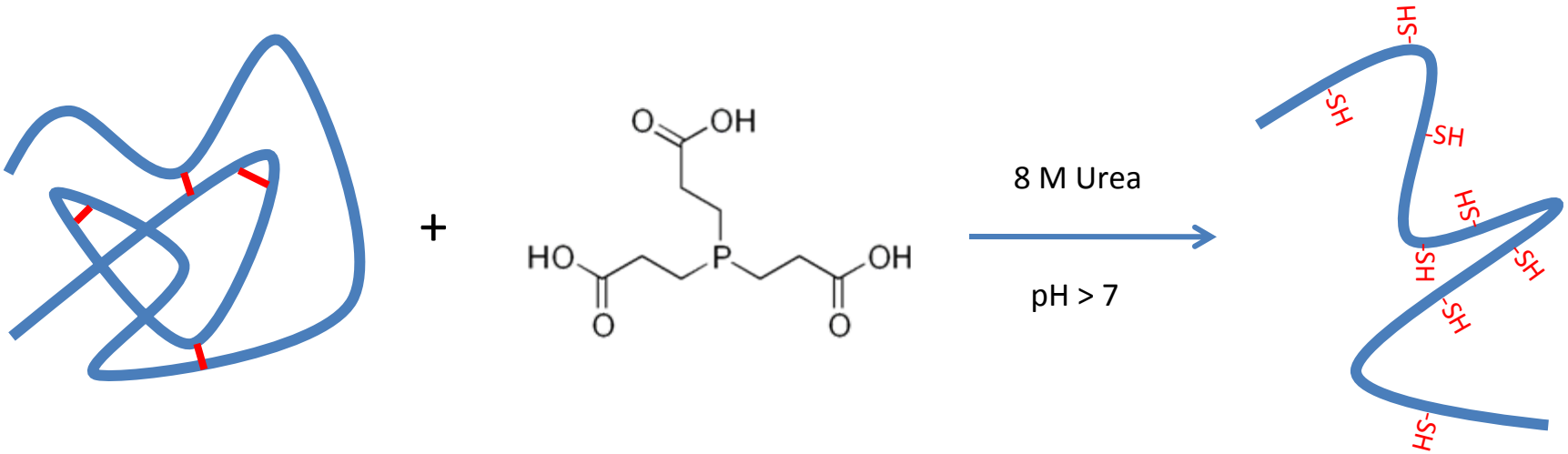
Protein Centric Method:
SDS-PAGE



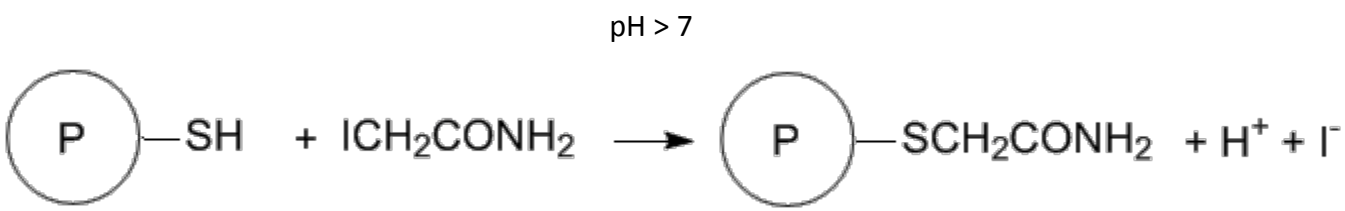
Schematic of workflow



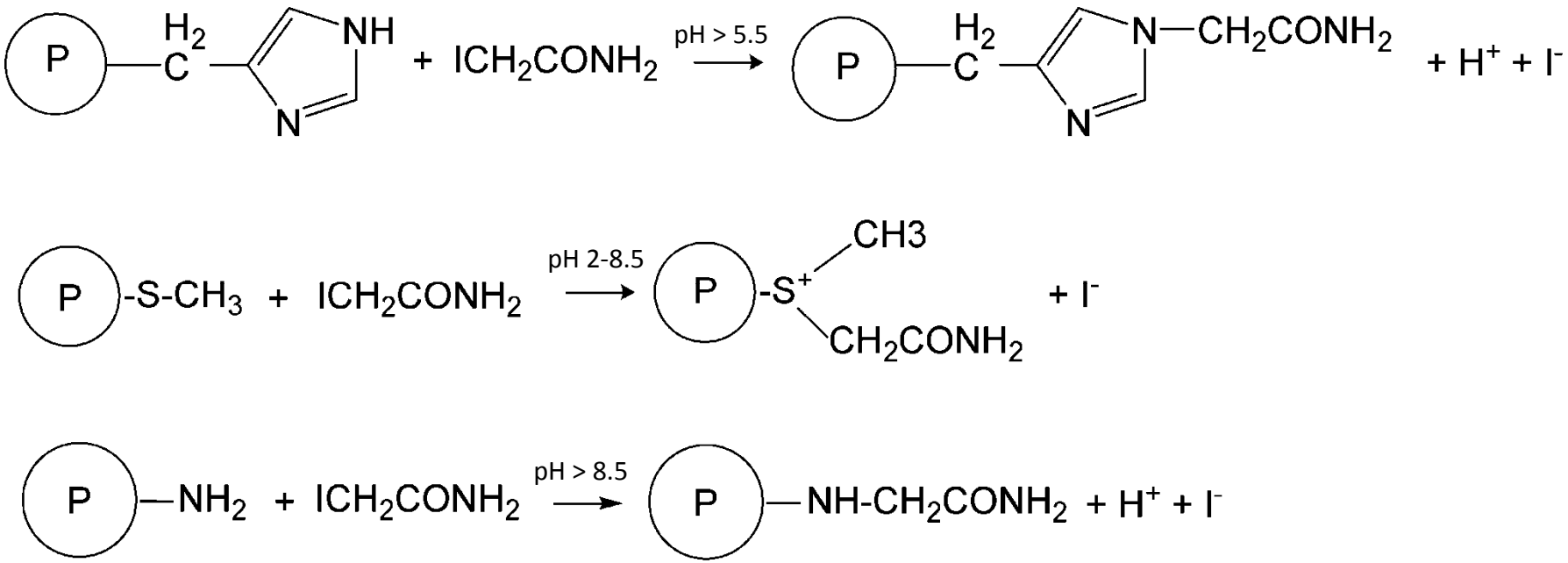
Protein reduction with excess TCEP



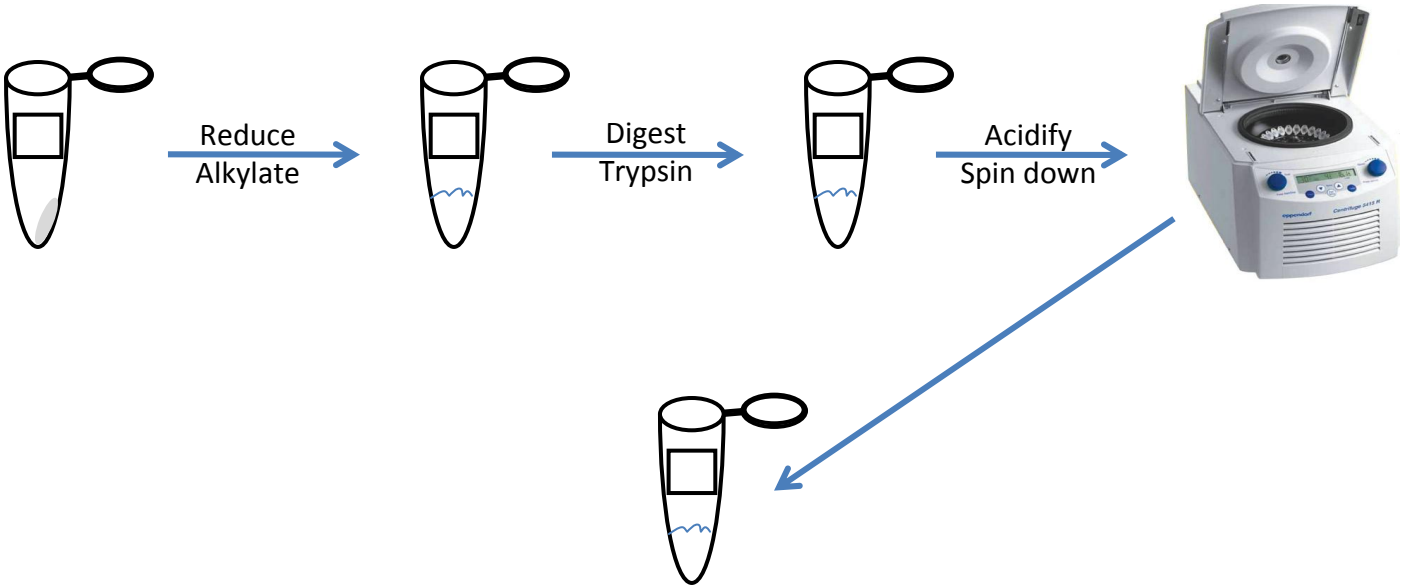
Alkylation with Iodoacetamide



Potential side reactions w/activated halogen reagents:

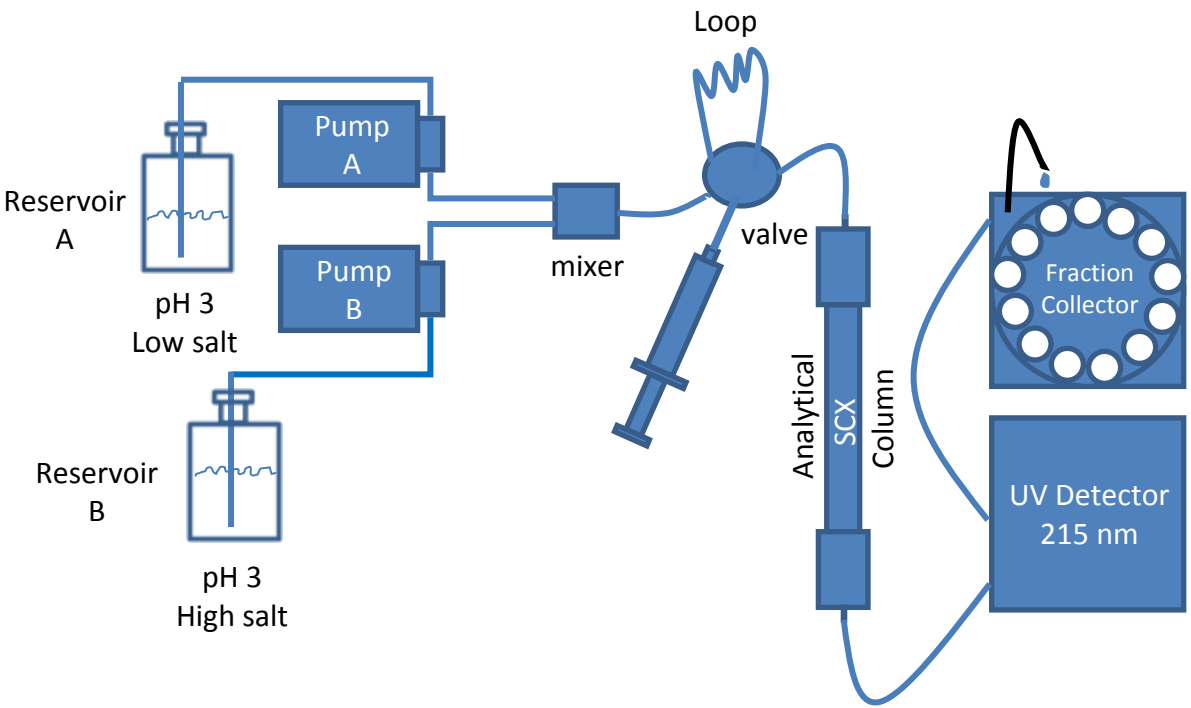


MuDPIT:
Shotgun approaches

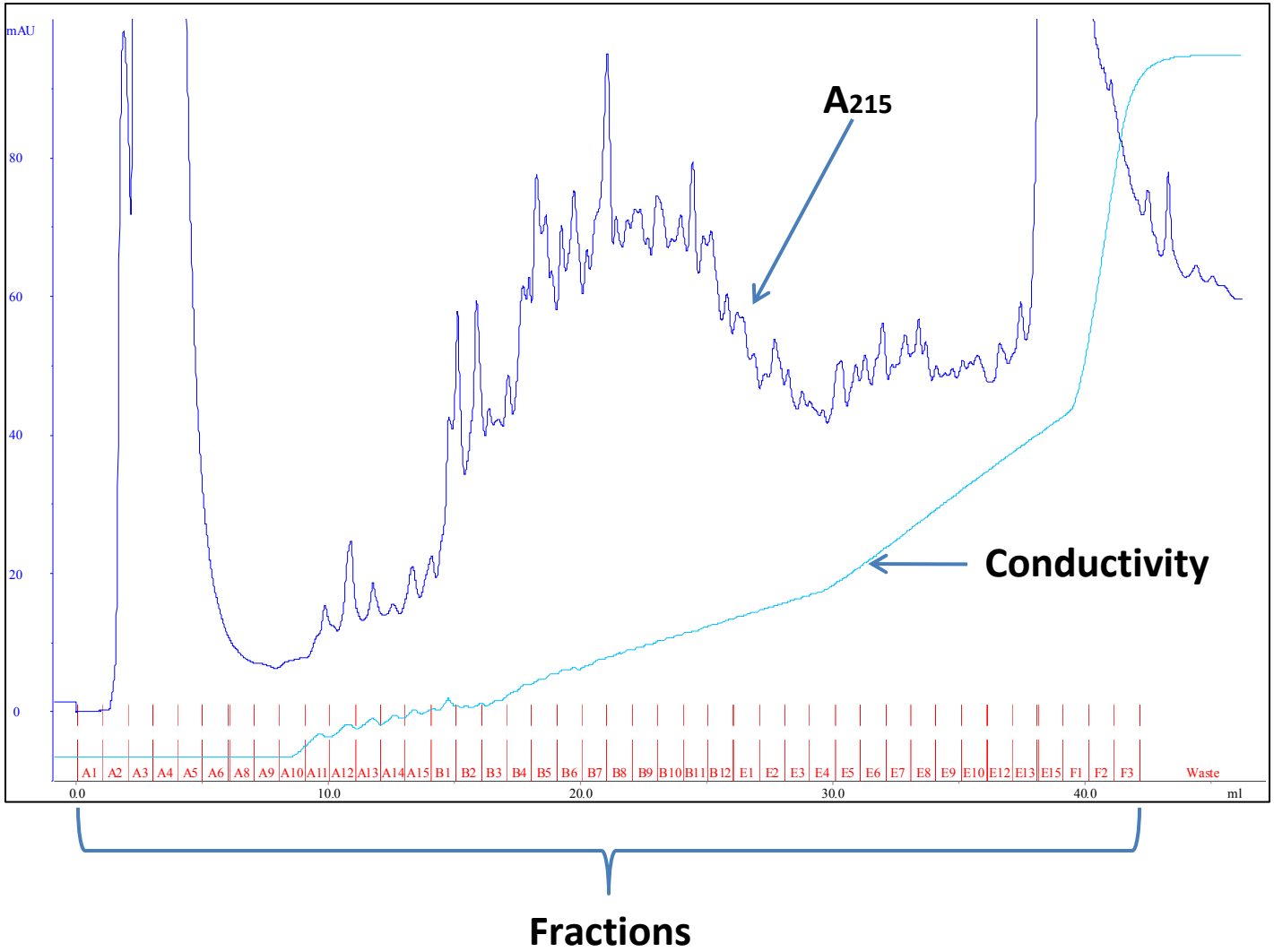


Ready for 1st dimension separation

Schematic of SCX pre-fractionation of peptides



Strong Cation eXchange (SCX) separation of tryptic peptides:



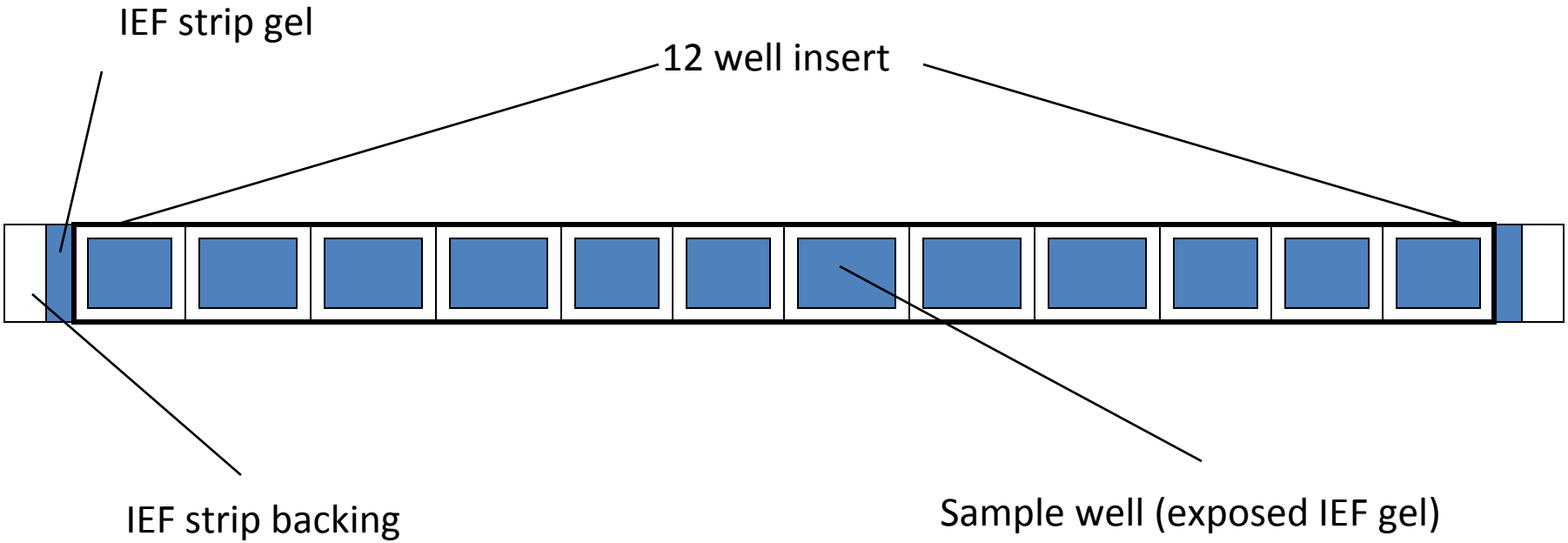
OFFGEL Fractionation

- Employs isoelectric focusing (IEF) as a first dimensional peptide separation
- Uses standard 13 or 24cm IEF strips
- Wells sit on top of strip, allowing peptides to diffuse into the strip, focus to their isoelectric point (pI), and diffuse back out

Agilent 3100 OFFGEL Fractionation

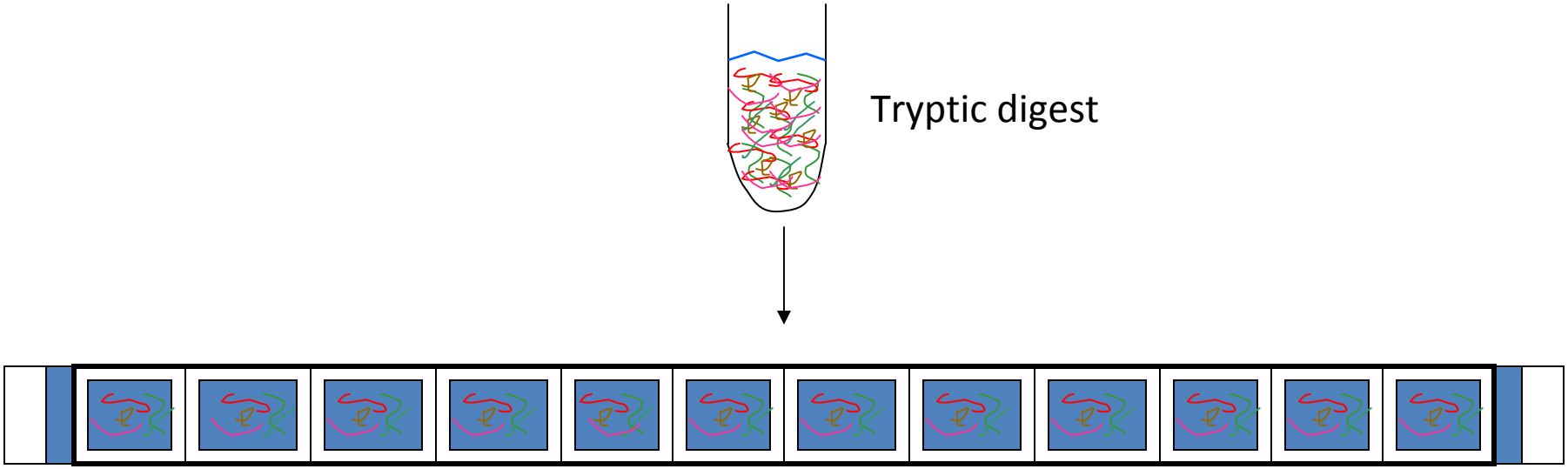
OFFGEL Fractionation

Sample OFFGEL setup using a 13cm (12 well) strip



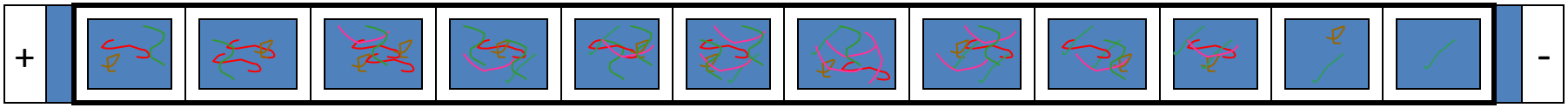
OFFGEL Fractionation

Sample is digested with trypsin (or other suitable enzyme), cleaned up with a reverse-phase cartridge and re-suspended in OFFGEL buffer. An equal aliquot is pipetted into each of the 12 wells.

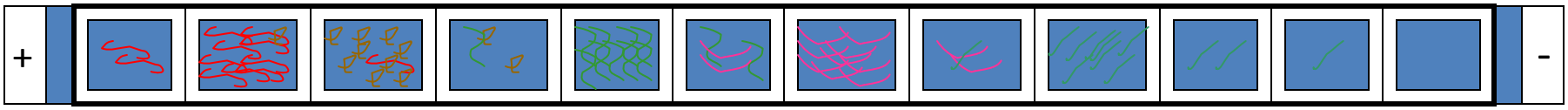


OFFGEL Fractionation

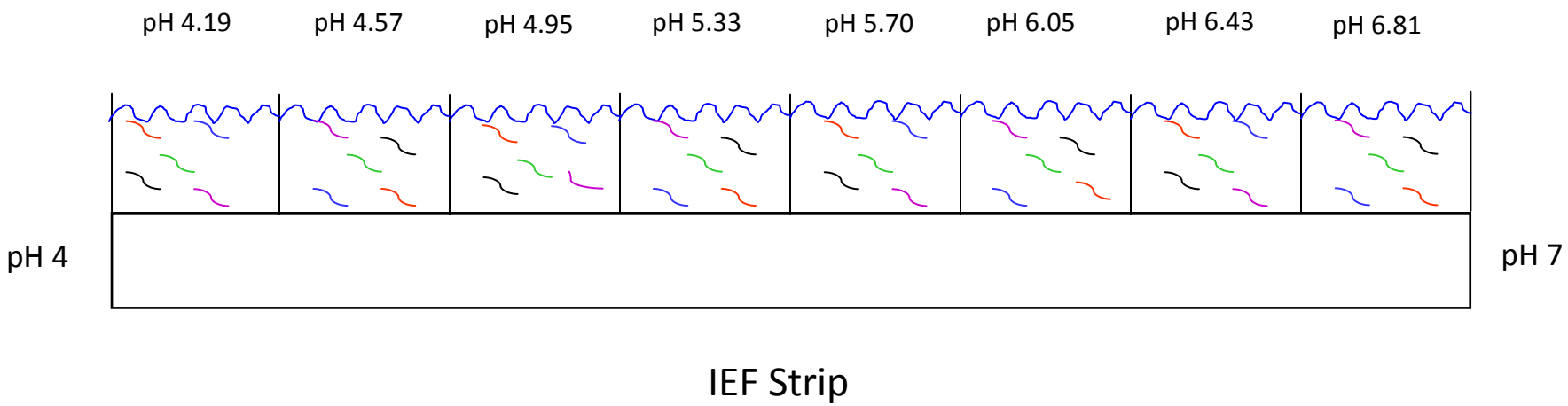
Voltage is turned on and peptides begin to migrate through the strip towards their pI



After several hours the peptides have focused at their pI on the strip and diffused into solution. Approximately 70% of the peptides find their way into the “correct” well. 30% end up in the wells to either side

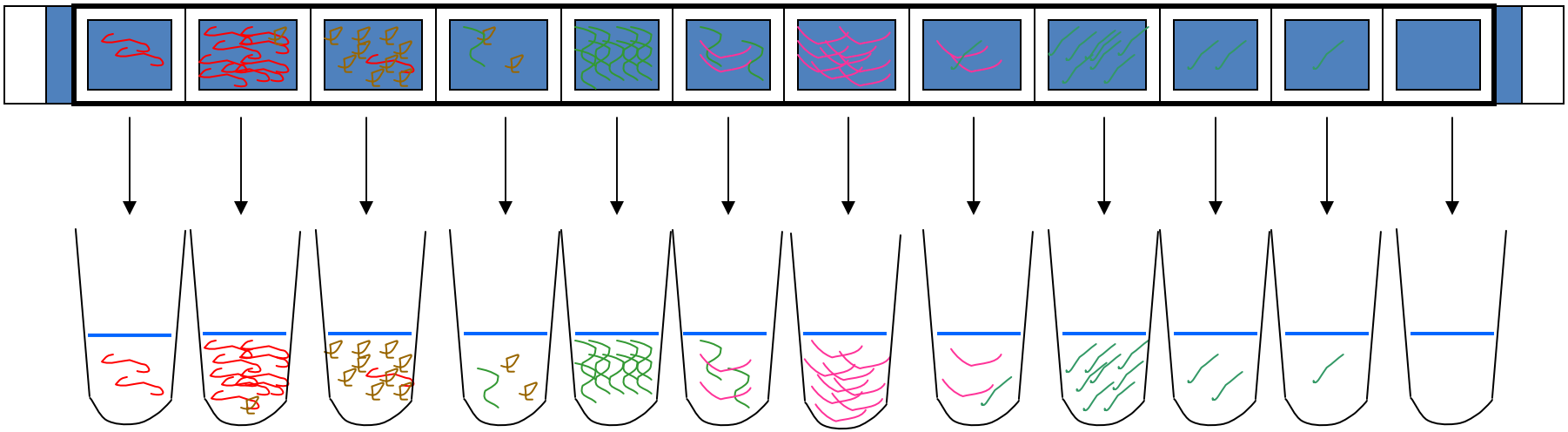


OffGel Electrophoresis: (The Movie)

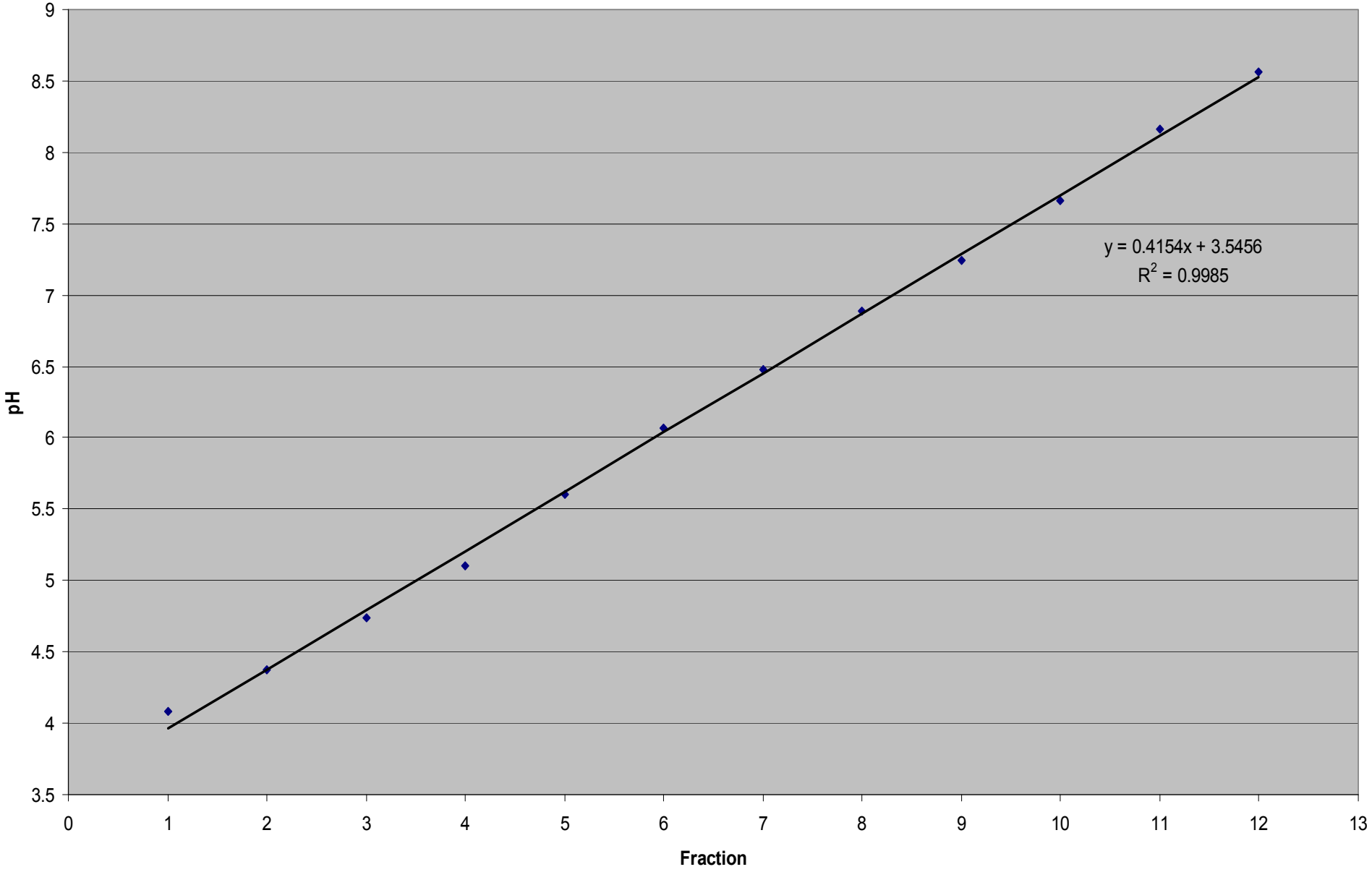


OFFGEL Fractionation

After focusing is complete, the buffer (containing the focused peptides) is removed from each well to its own separate tube. The pH of each tube is measured, and samples are again cleaned by reverse-phase extraction



Sample pH graph for 12 well OFFGEL fractionation on a 3-10 linear IEF strip



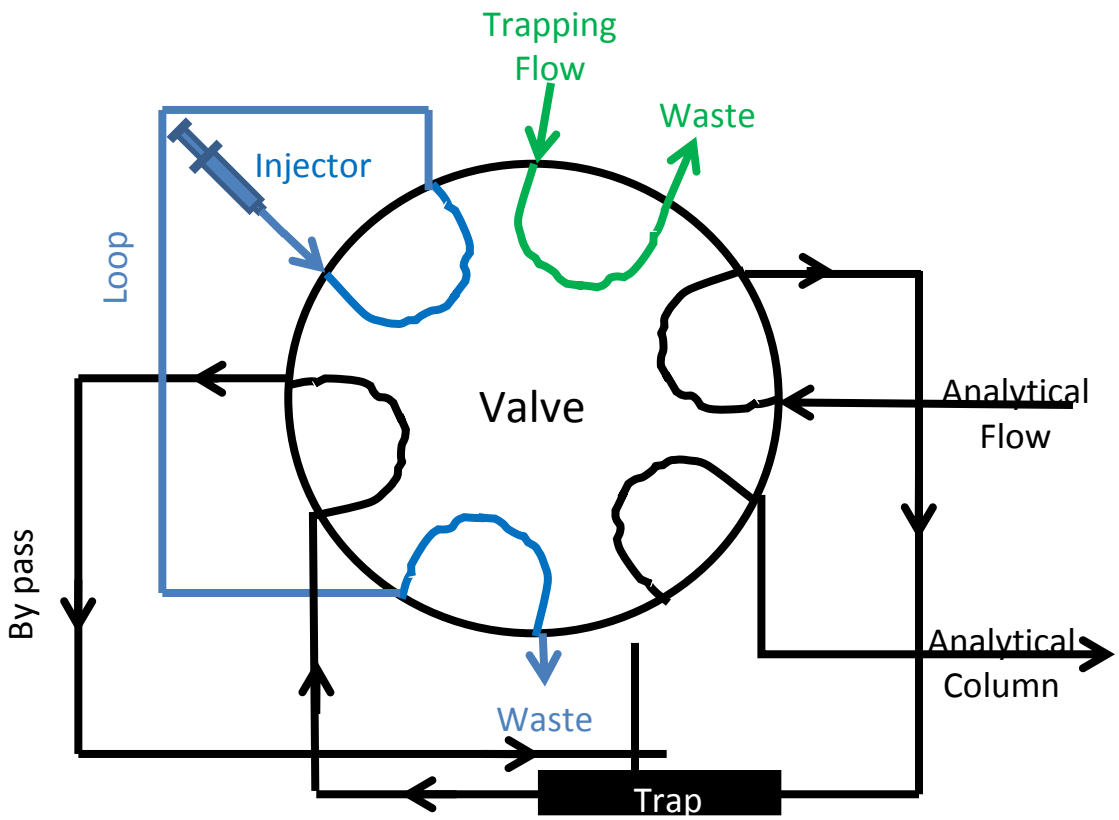
OFFGEL Fractionation

- Compare measured pI to predicted pI of identified peptides.
- Reduces predicted FDR by a factor of 2-3.
- More importantly:
  - Highlights specific peptide identifications which are likely to be false.
  - Increases number of identifications by 30-40%
  - Peptides identified are complementary to those identified using other 1st dimension techniques (i. e. SCX).

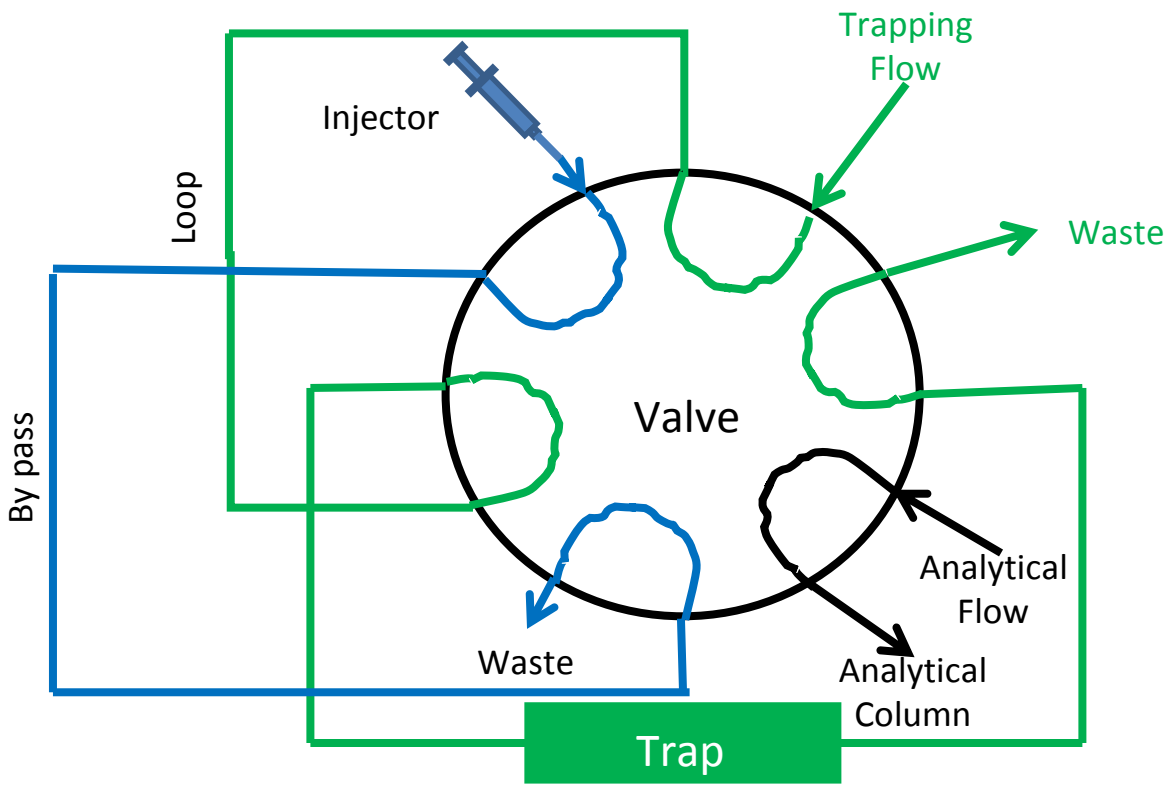
2nd Dimension Separation

- Almost always reverse phase chromatography at low pH:
  - 0.1% formic acid, pH ≈ 2
  - Nano scale:
    - Analytical column dimensions: 150 mm x 75 µM
    - Typical flow rate 300 nl/min
  - Usually involves trapping
    - Seldom uses direct injection
  - Valve configuration critical

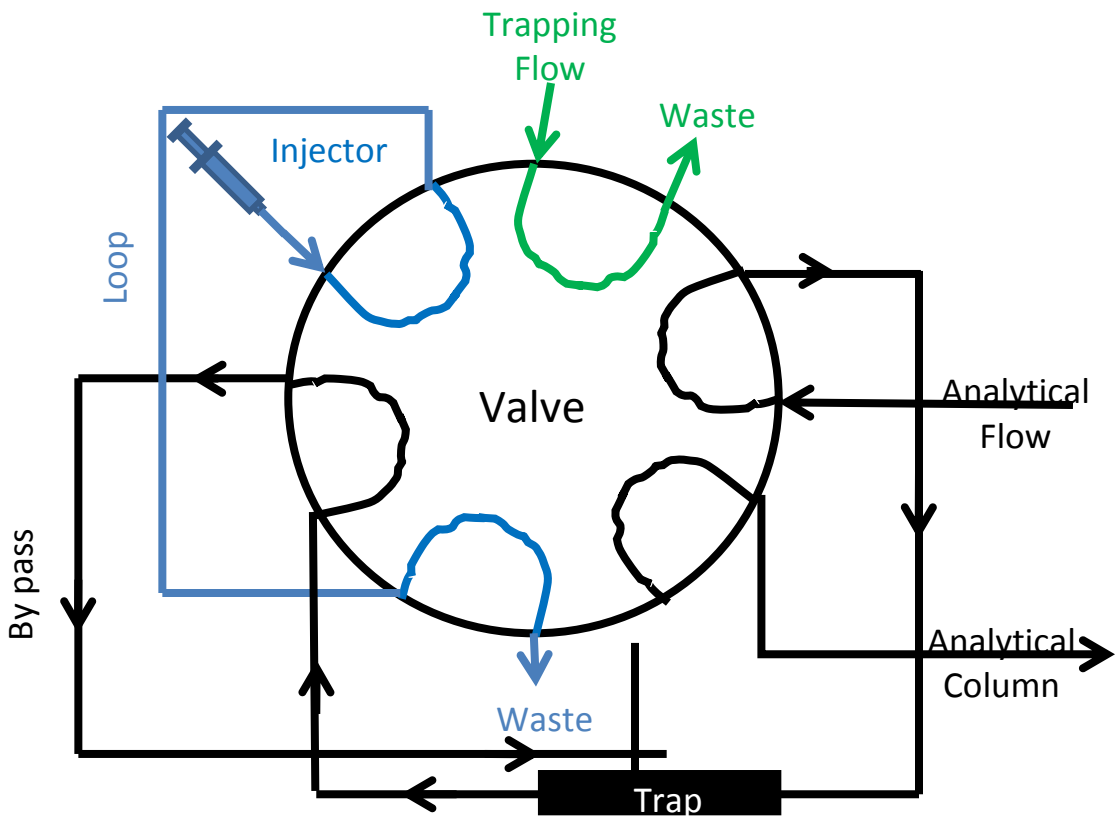
Injection Configuration:



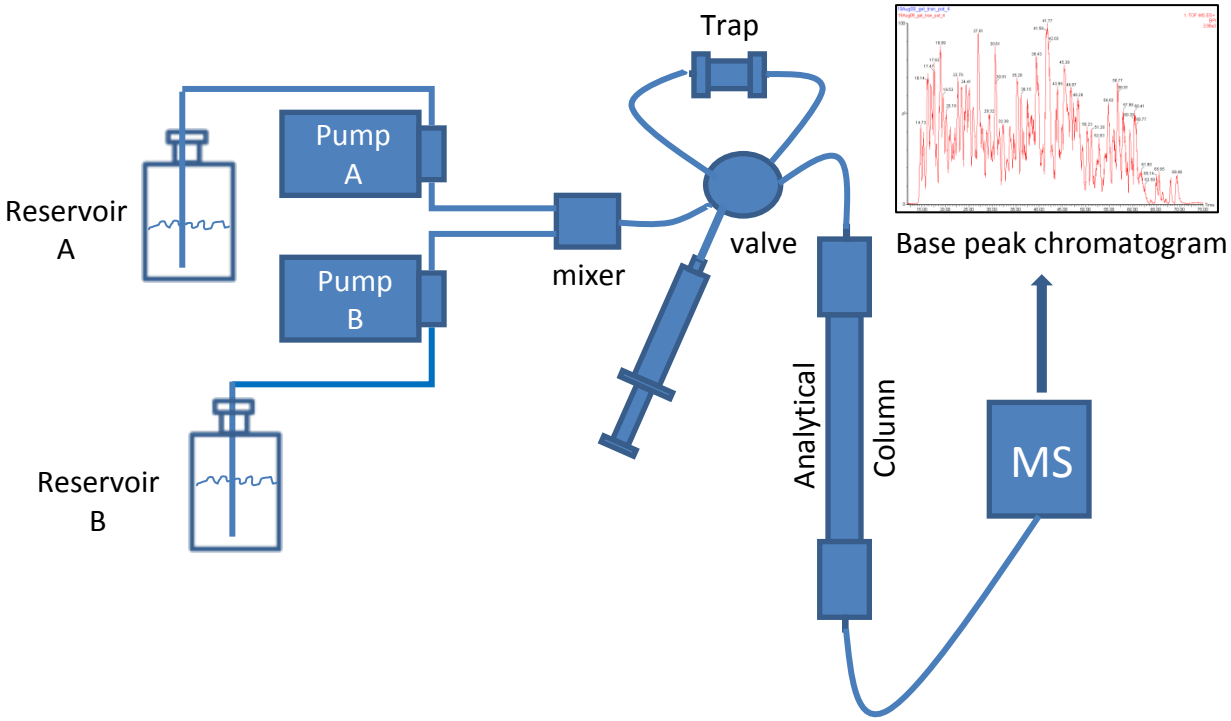
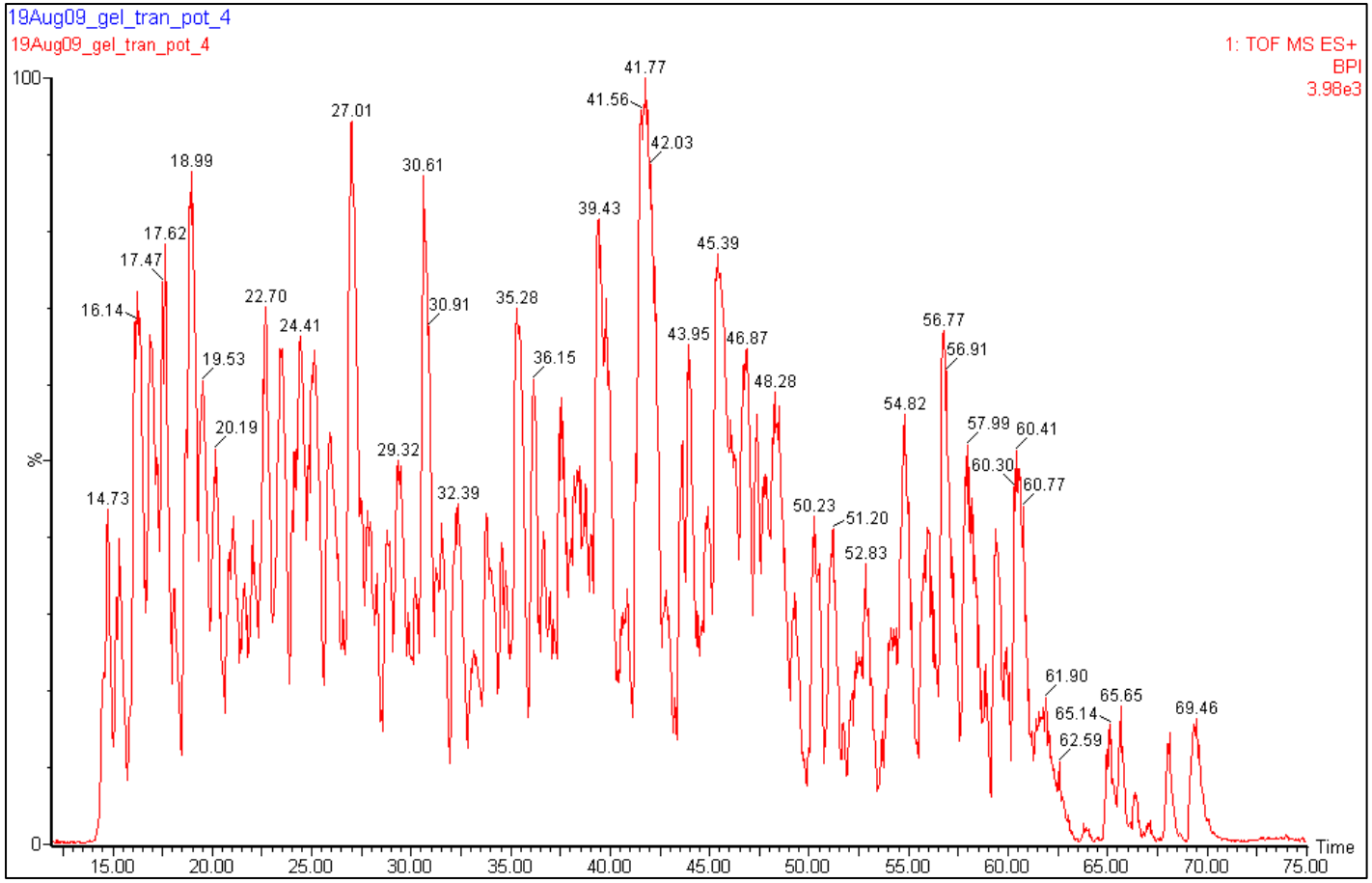
Trapping Configuration



Running Configuration:



Schematic of direct flow nano-LC



Base Peak Chromatogram