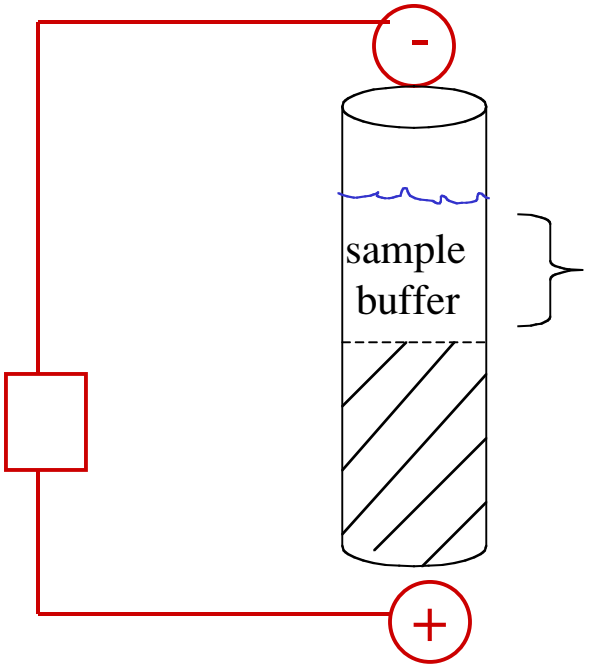
Stacking and Discontinuous Gels

Zonal analysis depends on having sharp, well defined bands

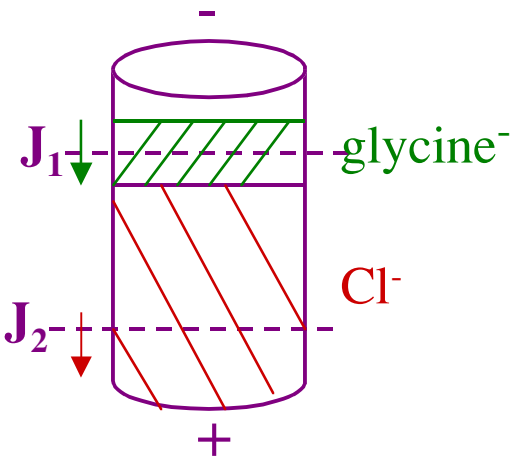
1 create a large electric field (voltage drop) in the sample buffer to concentrate the protein prior to separation by the gel



want large Electric Field to rapidly pile up protein at the surface of the gel.

Stacking

current (ion flux) must be constant throughout the gel



$J_1 = J_2$

$C_1V_1 = C_2V_2$

$NH_2CH_2CO_2^-$ (charged)

pH-dependent mobility

$NH_3^+CH_2CO_2^-$ (neutral)

C = concentration

V = net velocity

$$U = \text{Mobility} = \left(\frac{V}{E}\right)$$

$$J_1 = C_1U_1E_1 = C_2U_2E_2 = J_2$$

glycine → $C_1U_1E_1$; $C_2U_2E_2$ ← Cl$^-$

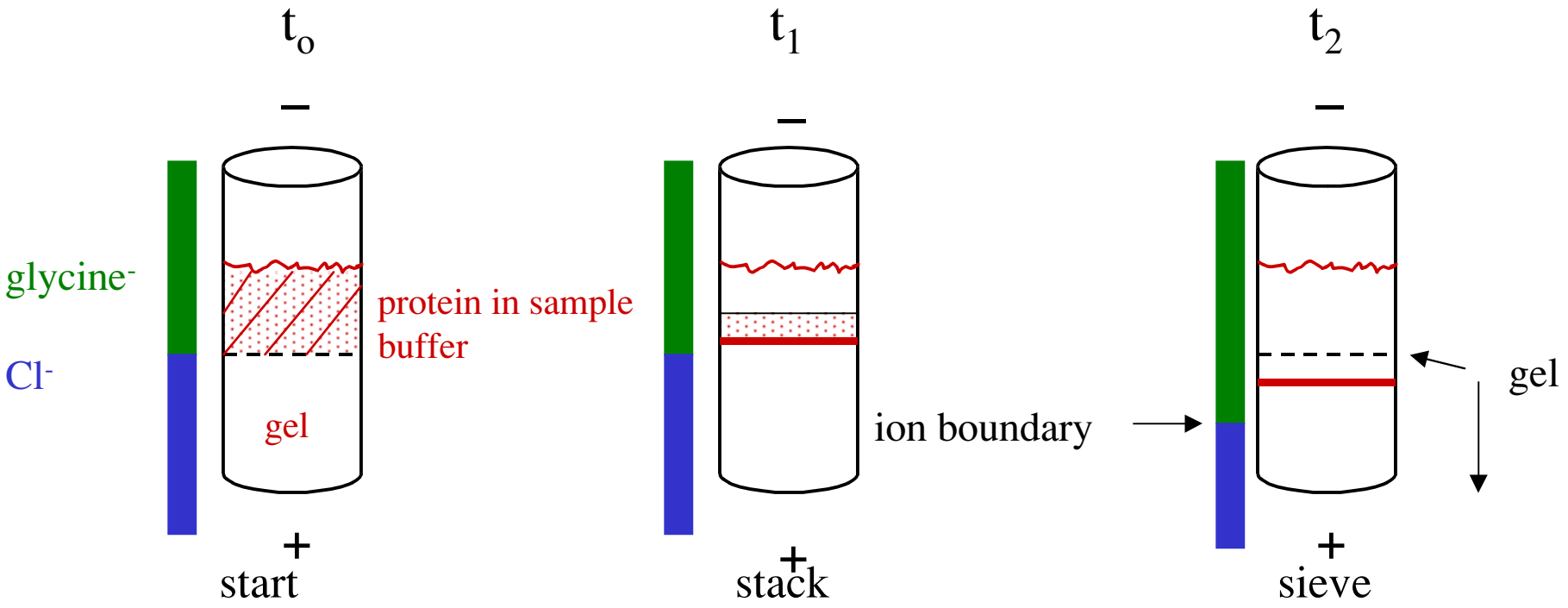
if $(C_1\mathbf{U_1}) << (C_2\mathbf{U_2}) \Rightarrow E_1 >> E_2$

Protein in the glycine buffer experiences a high electric field because of the low electrophoretic mobility of glycine at the pH used

-the protein moves to the ion boundary if

$U_{gly} < U_{prot} < U_{Cl-}$

Stacking



$U_{glycine^-} < U_{Cl^-}$

(depends on pH)

Alternative: Dilute running buffer (1/10) to make sample buffer ($U_1 = U_2$)

$$J_1 = \mathbf{C_1} U_1 \mathbf{E_1} = \mathbf{C_2} U_1 \mathbf{E_2} = J_2$$

(low) (high) in sample region

Two variants of gel electrophoresis

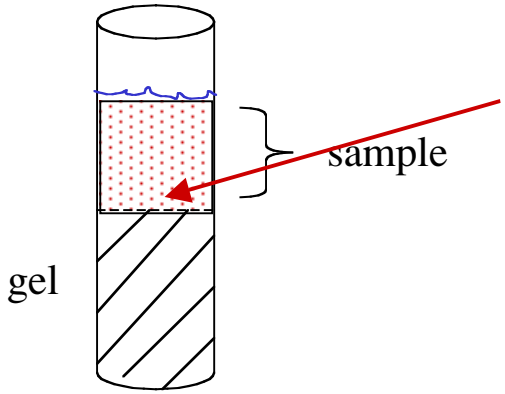
1. **Gel mobility-shift assay for protein-DNA interactions**

2. **Pulsed field gel electrophoresis for separating very large DNA (chromosomes)**

Gel Mobility - Shift Assay

for quantitative and qualitative characterization of DNA-protein interactions

DNA + protein (eg lac repressor) ⇌ DNA-protein complex

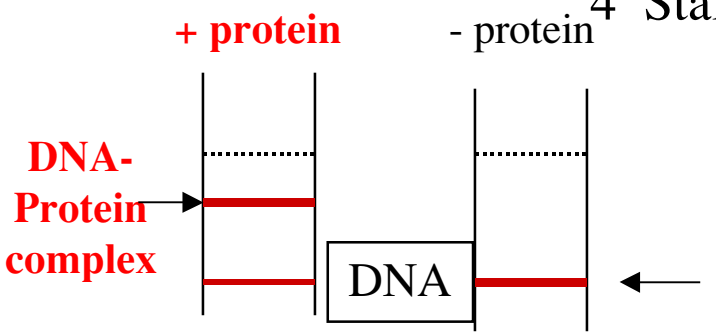


1 Equilibrate Reaction Mixture

2 Load onto gel (e.g., 7.5% Polyacrylamide Tris-Borate-EDTA buffer)

3 Run gel

4 Stain with ethidium bromide for DNA (or detect using radioactive labels)

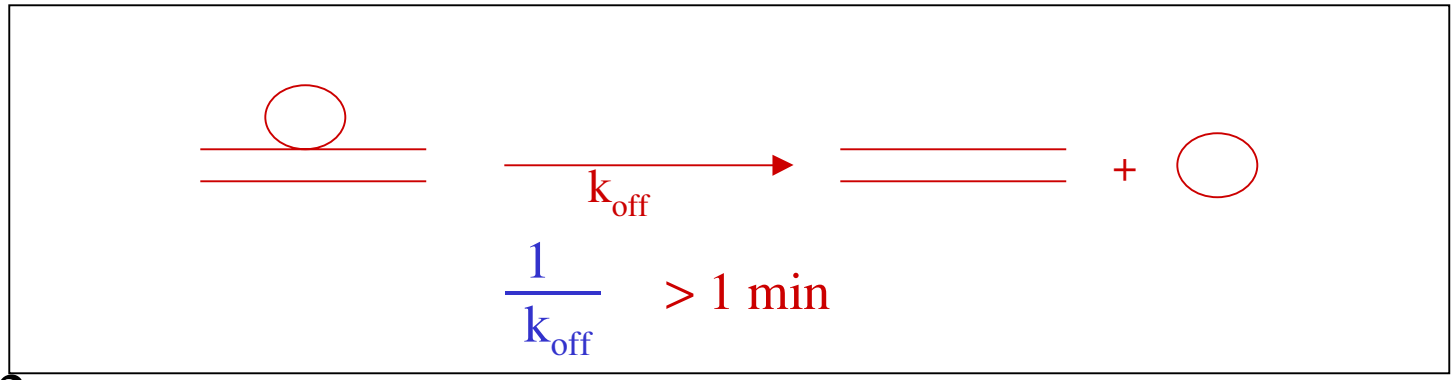


Gel Mobility shift assay

Why does this work?

1. Free DNA and **Bound DNA** species (along with free protein) are stacked and moved into the gel **before there is any time for protein dissociation**

Takes ~ 1 min to complete this process



2 Once in the gel, the DNA-protein complexes are virtually **locked together**

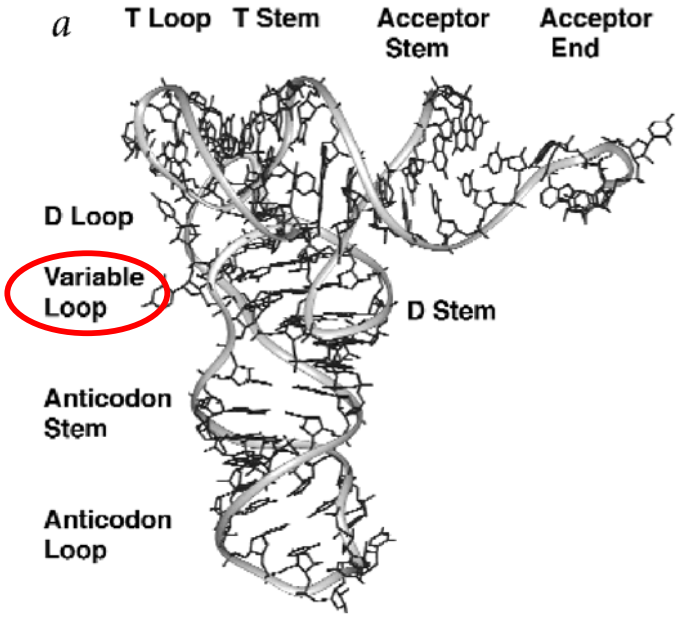
WHY?

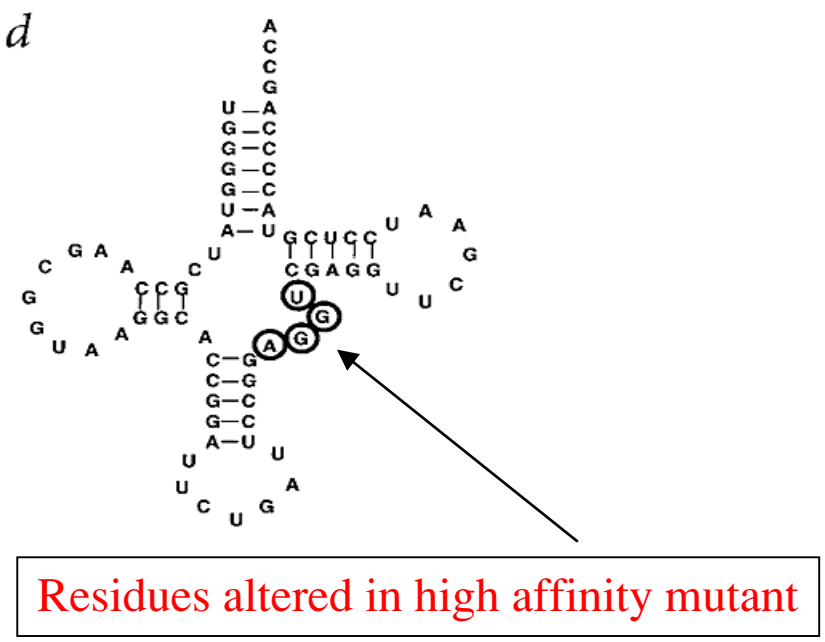
- low salt
- excluded volume effect of gel
- "cage" effect of the gel

(not fully understood)

Example: Gel Mobility Shift Assay of a Protein-RNA Complex

Goal is to measure the K_d of the complex formed between a t-RNA^{gln} mutant and the glutaminyl-tRNA synthetase

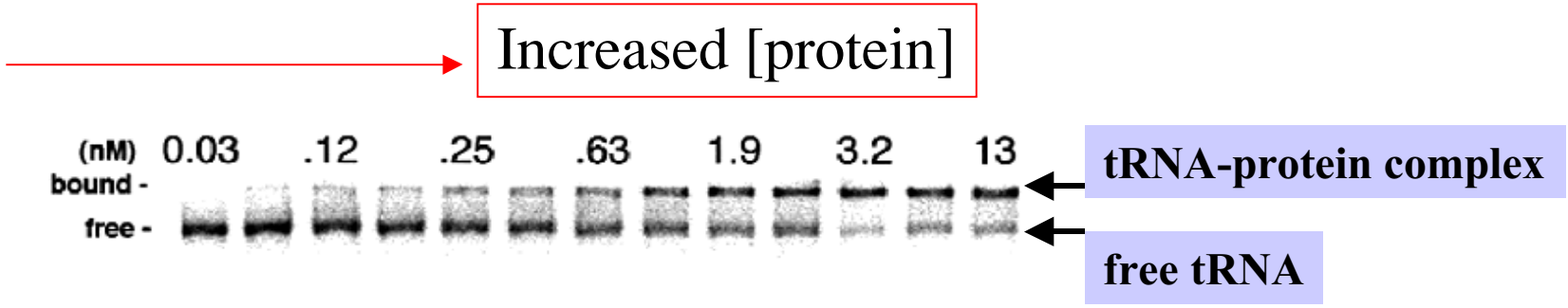




Residues altered in high affinity mutant

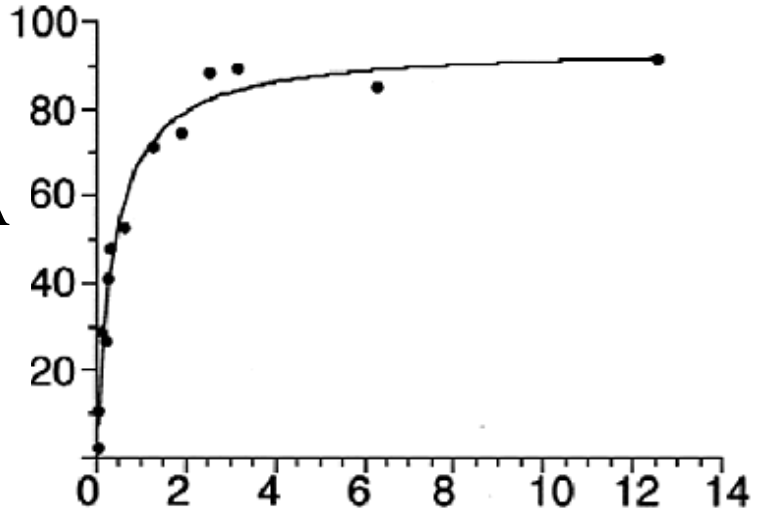
Gel shift assay

1. Use radioactive label on tRNA to detect on the gel
2. Incubate tRNA/protein mixture for 15 min
   - 200 pM tRNA
   - 6.6 nM to 66 pM Gln-tRNA Synthase (GlnRS)
3. Load onto 20% polyacrylamide gel and run for 5 h at 4° C
4. Autoradiography to determine bound and free tRNA
5. Fit to binding isotherm for 1:1 complex formation



Binding isotherm for tRNA/protein Complex

K_d = 0.27 nM



% of tRNA bound to protein

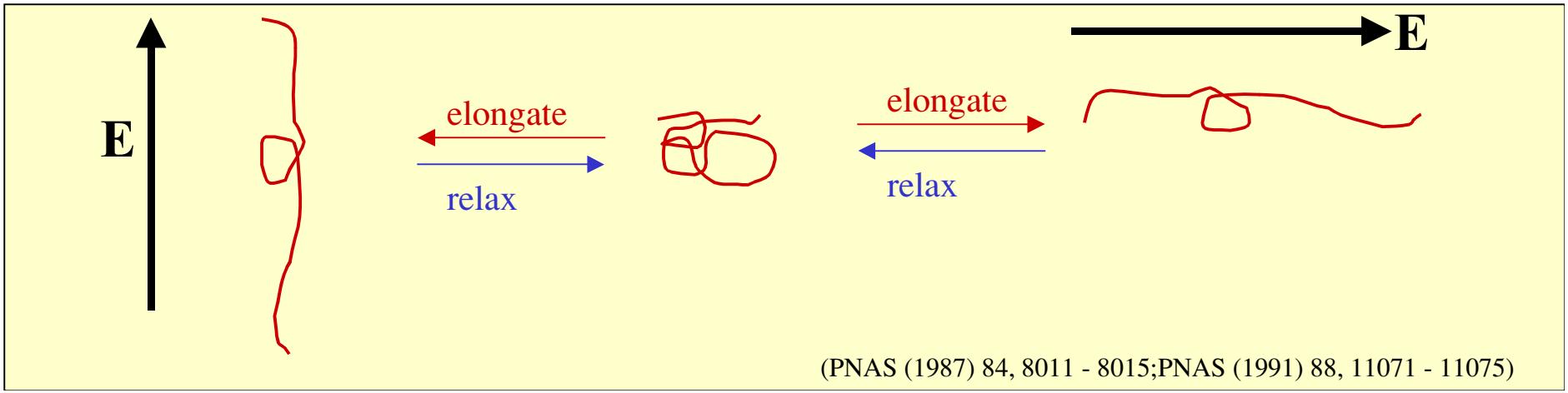
(free protein concentration)

Pulsed Field Gel Electrophoresis

In normal electrophoresis - electrophoretic mobility is **independent** of molecular weight for large DNA (> 50 kbp)

Pulsed field gel electrophoresis is a collection of techniques to increase the resolution of DNA fragments to extend to very large DNA fragments (>>100 kbp) based on the size-dependent relaxation properties of DNA

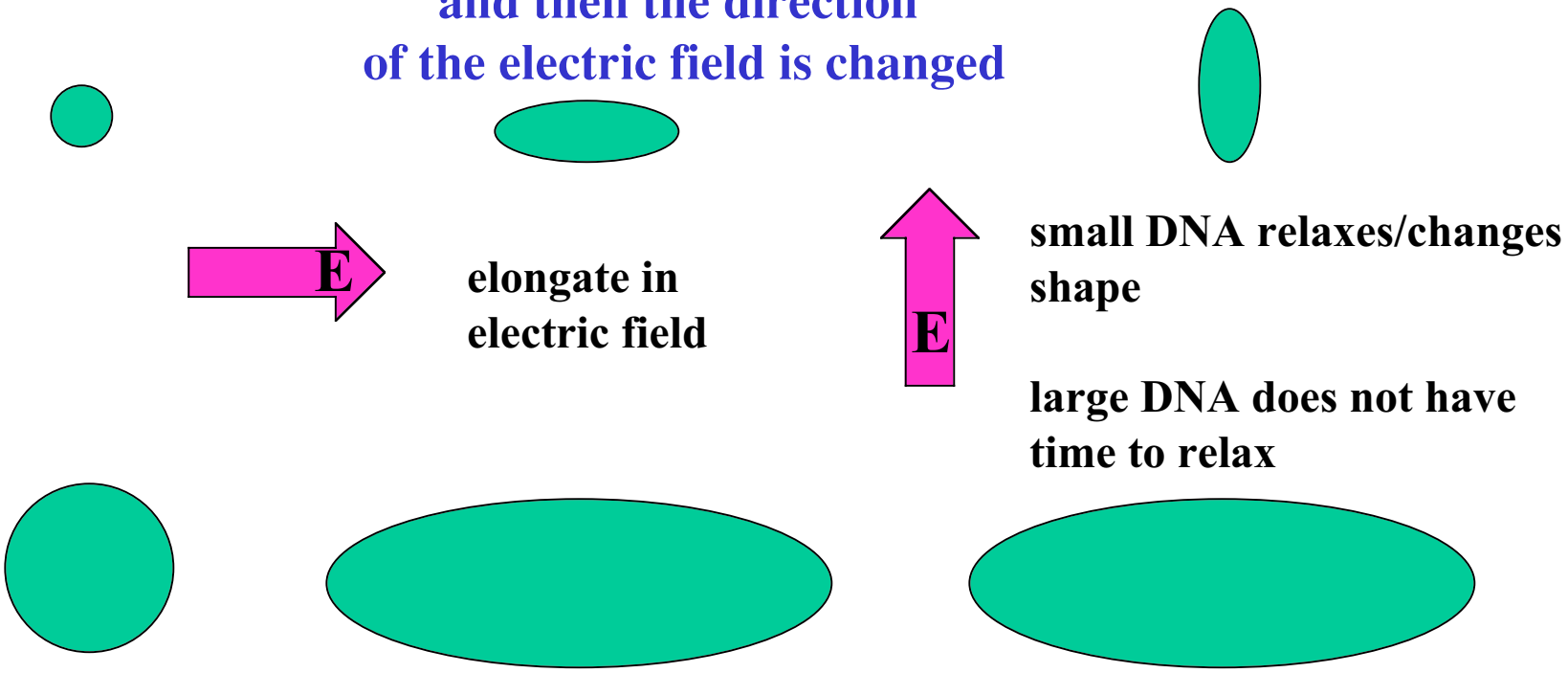
DNA elongates along the direction of the electric field (E)
DNA relaxes when the field is turned off
DNA shape changes when field direction is changed
DNA mobility depends on the shape/determined by previous pulse sequence



(PNAS (1987) 84, 8011 - 8015;PNAS (1991) 88, 11071 - 11075)

Pulsed Field Gel Electrophoresis

After a brief pulse of electric field the DNA is allowed to "relax" and then the direction of the electric field is changed



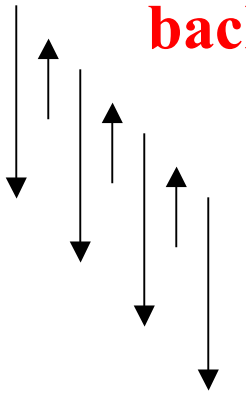
critical parameters

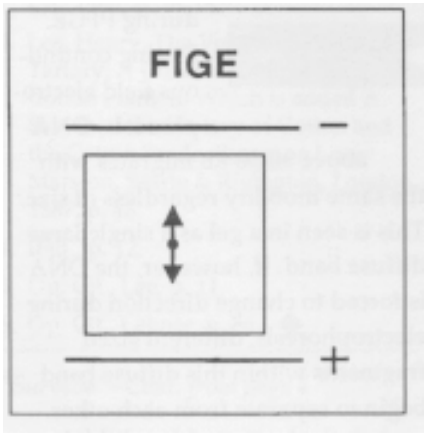
- DNA relaxation time (T_R) vs electrophoresis pulse time (T_P)

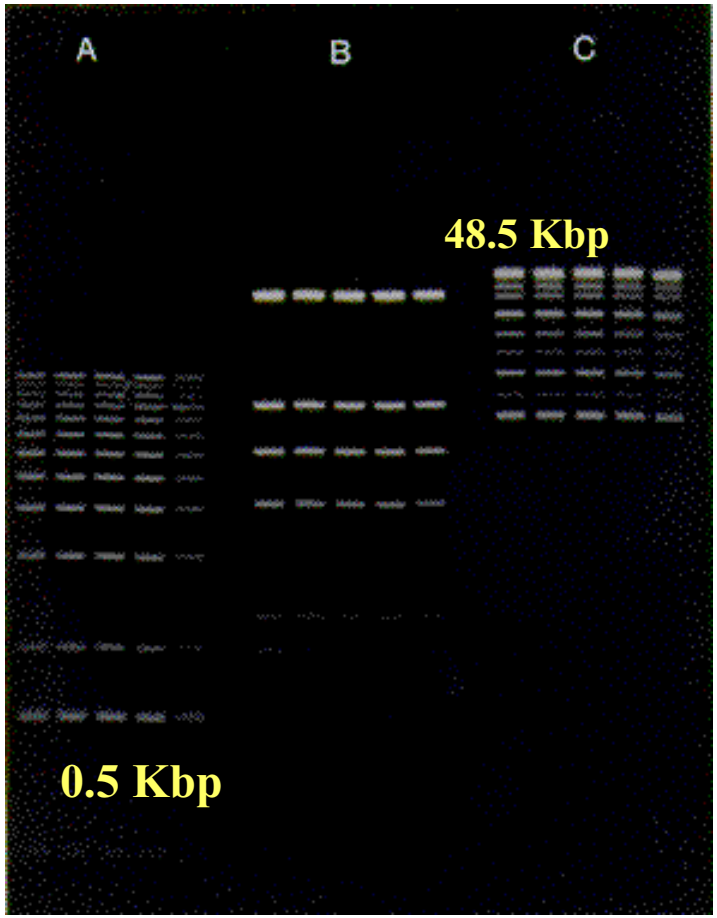
Field Inversion Gel Electrophoresis: FIGE

reverse direction of the field: forward/reverse pulse ratio = 2.5/1

small DNA goes backwards part of the time





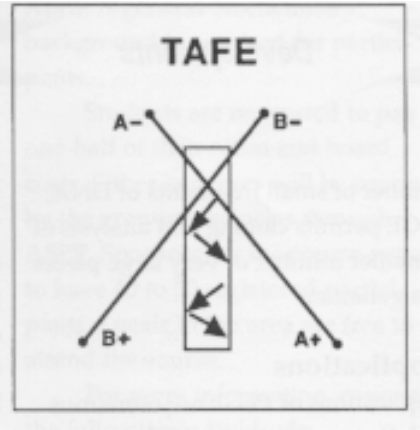


Increased separation of the 20-50 kb range with field inversion gel electrophoresis (FIGE).
Run conditions: 230 V, 7.9 V/cm, 16 hrs., 50 msec. pulse, forward:reverse pulse ratio = 2.5:1,
1% GTG agarose, 0.5X TBE, 10 C.
a) 1 kb ladder, 0.5-12 kb;
b) Lambda/Hind III, 0.5-23 kb; and
c) High molecular weight markers, 8.3-48.5 kb.

http://www.nal.usda.gov/pgdic/Probe/v2n3/puls.html

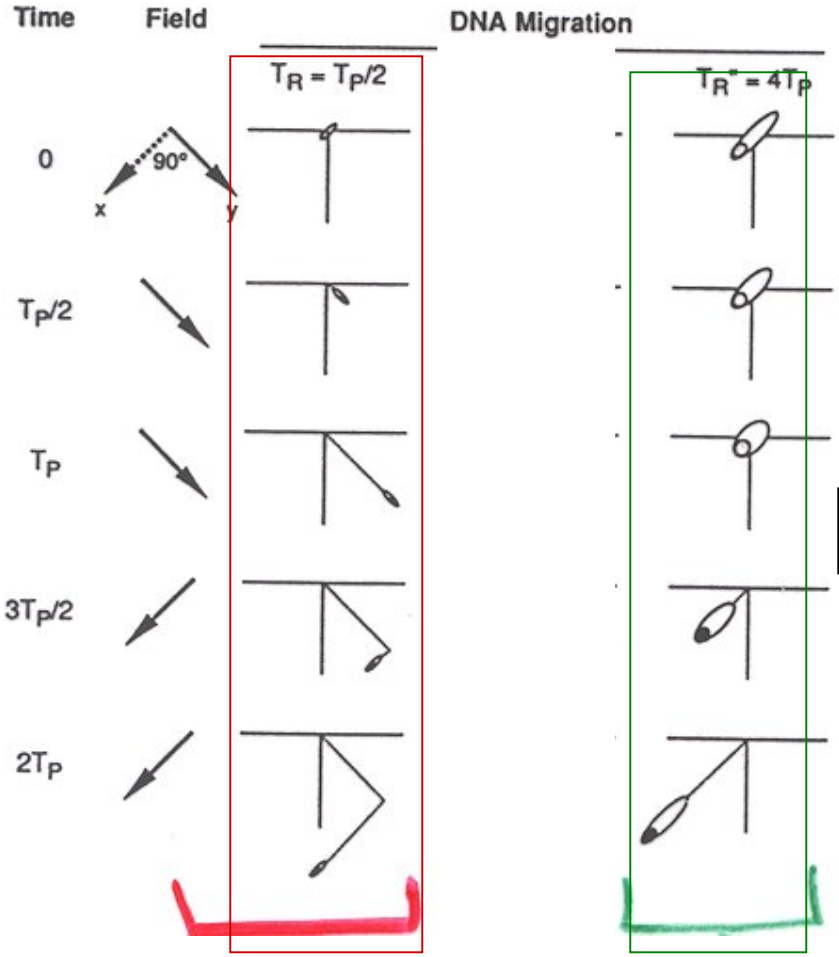
Transverse Alternating Field Electrophoresis

switch field at an angle to get DNA migration in a zig-zag forward direction



important considertion is the pulse time compared to the relaxation time of the DNA

Example: Alternate field direction by 90°
Direction of DNA migration will also depend on DNA size



small DNA responds rapidly to changing field

Very large DNA

PNAS 88 11071

Mass Spectrometry

Separates charged atoms or molecules according to their mass-to-charge ratio

Frequently used for determination of molecular mass of proteins and nucleic acids and other macromolecules

very accurate fast requires very little material

http://www.bmss.org.uk/what_is/whatisframeset.html

Basic steps in mass spectrometry:

1. **Ionize the sample in an ion source**

   Many methods are available to ionize molecules

   Molecules are brought into the gas phase either prior to or at the time of ionization

   The molecular ion can be fragmented, depending on the procedure use. This yields structural information about the molecule

2. Ions are repelled out of the ion source and accelerated towards an analyzer.

   Must chose whether to observe positive or negative ions. Neutral species are not observed.

http://www.bmss.org.uk/what_is/whatisframeset.html

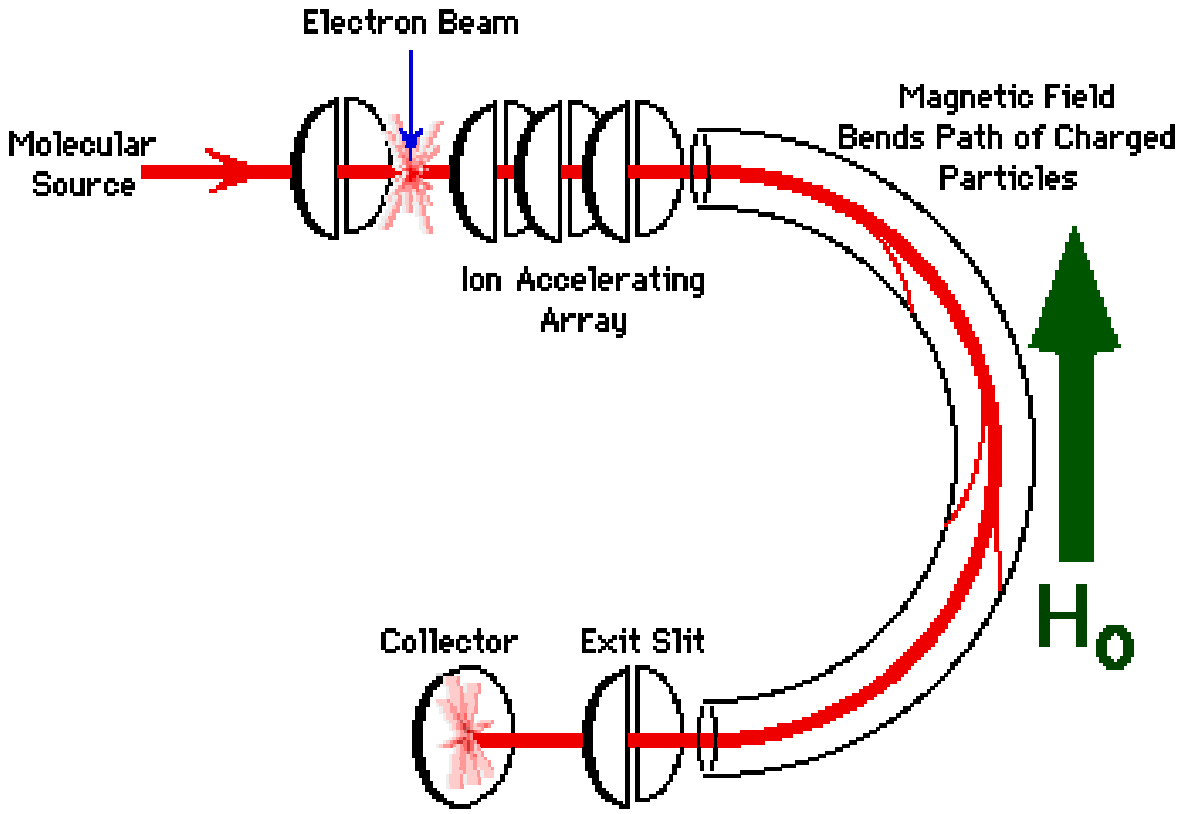
Types of mass spectrometry analyzers

1. **Field analyzer**: deflects ion depending on mass/charge ratio
   Field strength is scanned so ions hit the deflector at different times.

2. **Time of flight analyzer**: Measures the time it takes for the ionized molecule to reach the detector. This depends on the charge/mass ration

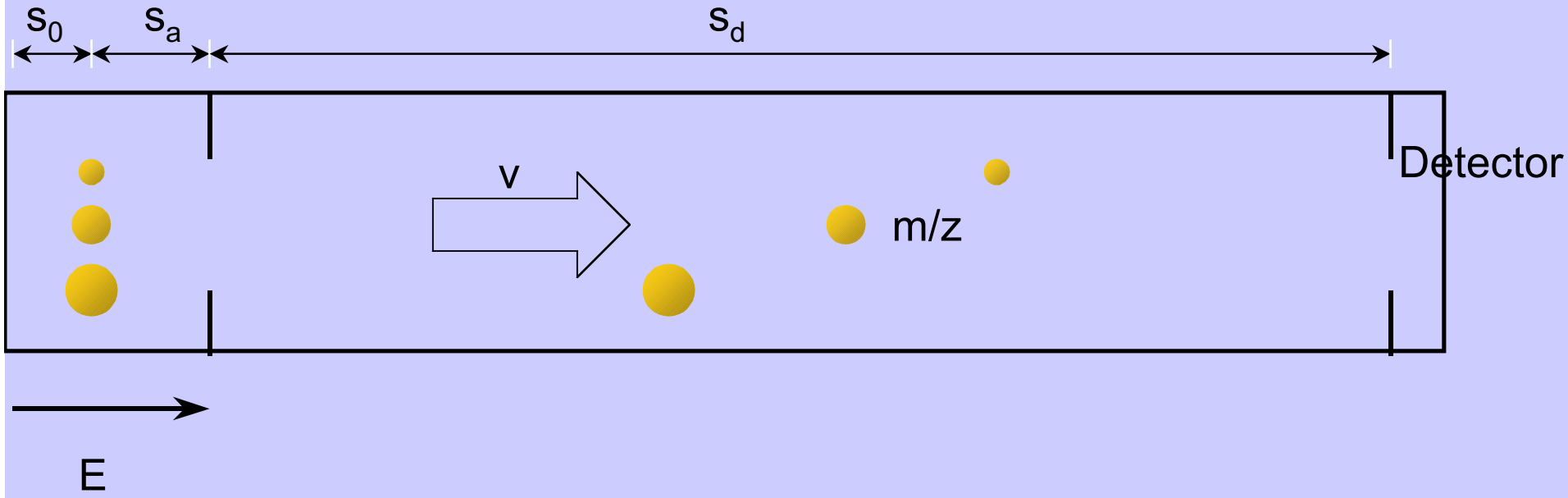
http://www.bmss.org.uk/what_is/whatisframeset.html

Field Analyzer



http://chipo.chem.uic.edu/web1/ocol/spec/MS1.htm

Time-of-Flight Analyzer

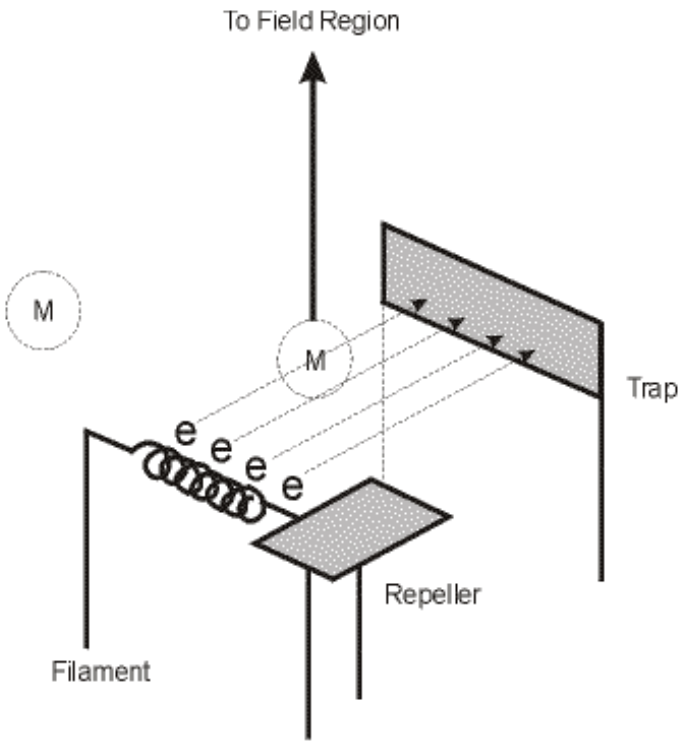


$$t = t_0 + t_a + t_d + t_m$$

http://ms.mc.vanderbilt.edu/tutorials/maldi/maldi-ie_files/frame.htm

There are many types of ionization methods

1. Electron ionization (EI) is the original method (still widely used)



E I Ion Source

A high energy beam of electrons is generated at a heated filament

Sample molecules in the gas phase pass through this beam. Electrons are stripped off and go to the positive "trap"

Result is sample molecules that carry positive charge. Molecules often fragment

http://www.bmss.org.uk/what_is/whatisframeset.html

Most frequently used ionization methods used for the analysis of macromolecules

1. MALDI: matrix assisted laser desorption ionization

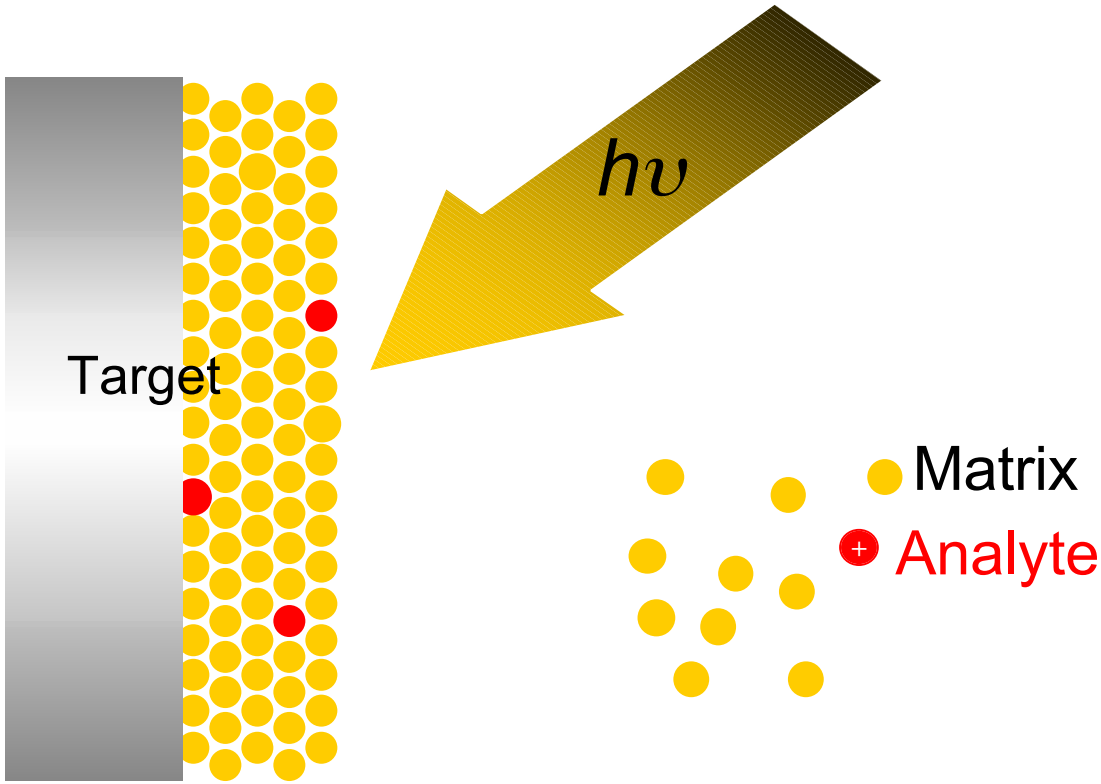
Dissolve the sample in a "matrix" that is allowed to crystallize on a stainless steel target.

matrix material: 2,5 dihydroxybenzoic acid or sinapinic acid

Use a pulsed laser (337 nm) to desorb the sample into the gas phase and ionize it. Ionization is usually by protonation or deprotonation

Usually little fragmentation. Mass range is virtually without limit

MALDI



can get resolution of 1 part in 50,000 by MALDI MS

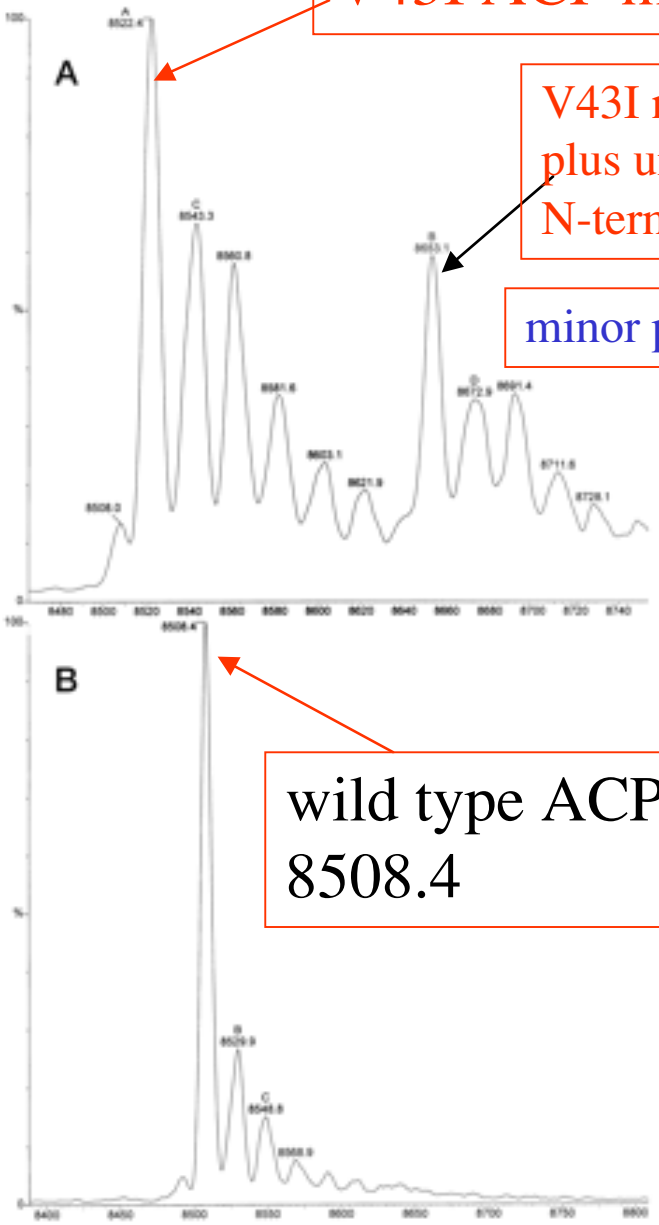
V43I ACP mutant: 8522.4

example of MALDI MS

Acyl Carrier Protein from *E. coli*

V43I mutant
plus unprocessed
N-terminal Met: 8653.1

minor peaks are protein plus monovalent and divalent cations



wild type ACP
8508.4

Unusual properties of the acyl carrier protein (ACP) was discovered to be due to a single mutation: V43I

The change in molecular weight is evident in **MALDI** mass spec of the purified proteins

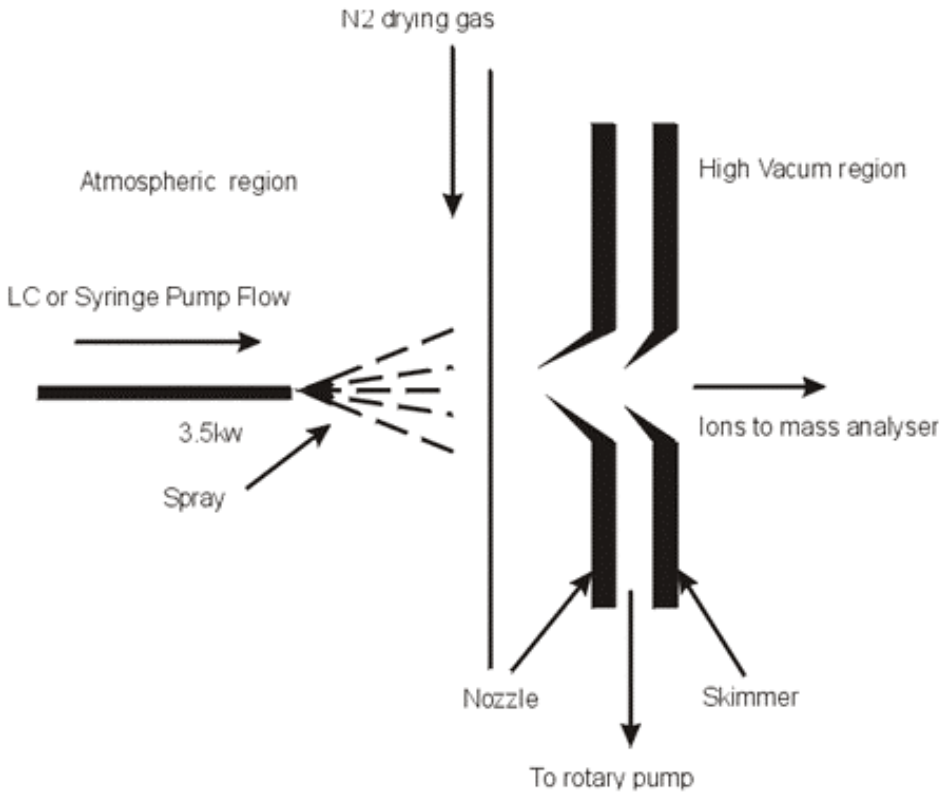
part of sequence of *E. coli* Acyl Carrier Protein

```
E.coli Fast          DLGADSLDTVELIMALEEEF
E.coli Wild type     DLGADSLDTVELVMALEEEF
R.mel                DLGADSLDTVELVMA+EEEF
A.thal.              DLGADSLDTVE+VM+LEEEF
S.ole                  GADSLDTVE+VM+LEEEF
G.gal                DLG DSL  VE+ + LE
```

J. Biol Chem (1996) 271, 15905-

Most frequently used ionization methods used for the analysis of macromolecules

2. Electrospray ionization (ESI)



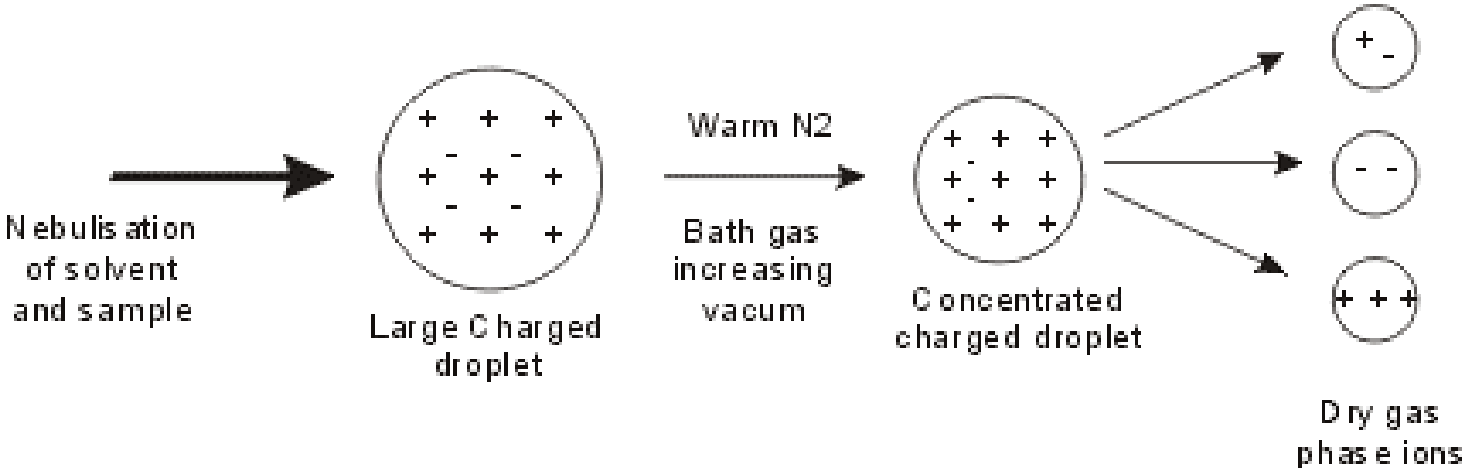
Sample is dissolved in liquid,
e.g., 1:1 water/methanol

Squirted through a capillary held at very high potential at atmospheric pressure, generating a spray of charged droplets

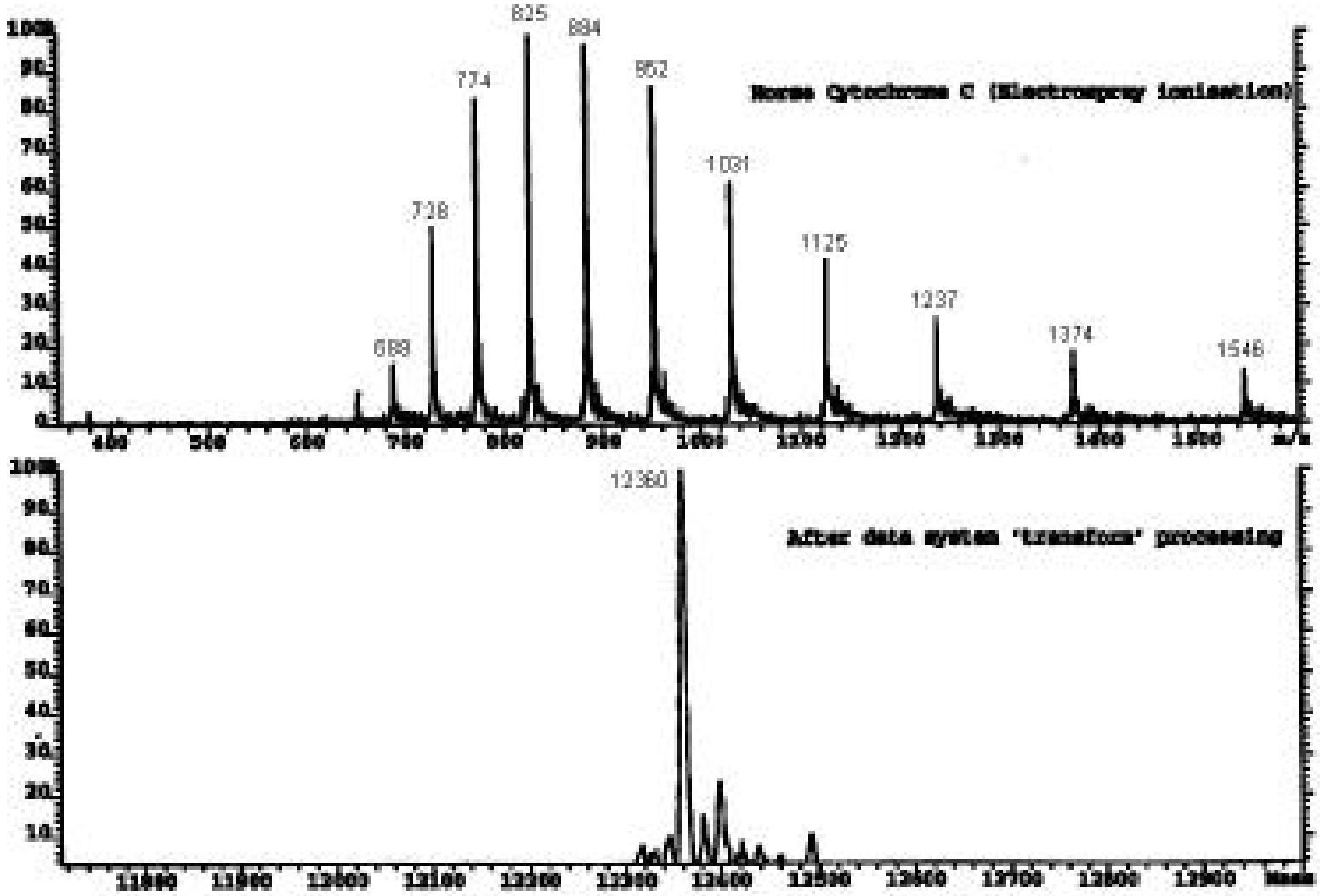
Nitrogen gas directs the droplets into chambers of increasing vacuum, causing loss of solvent and concentration of charge

End up with sample molecules in multiple ionization states, but not fragmented

Electrospray ionization



ESI of horse cytochrome c: multiple ionization states of a single molecule results in a complex pattern, but this can be mathematically analyzed and combined to reveal the mass of the sample molecule



Tandem Mass Spectrometry (MSMS)

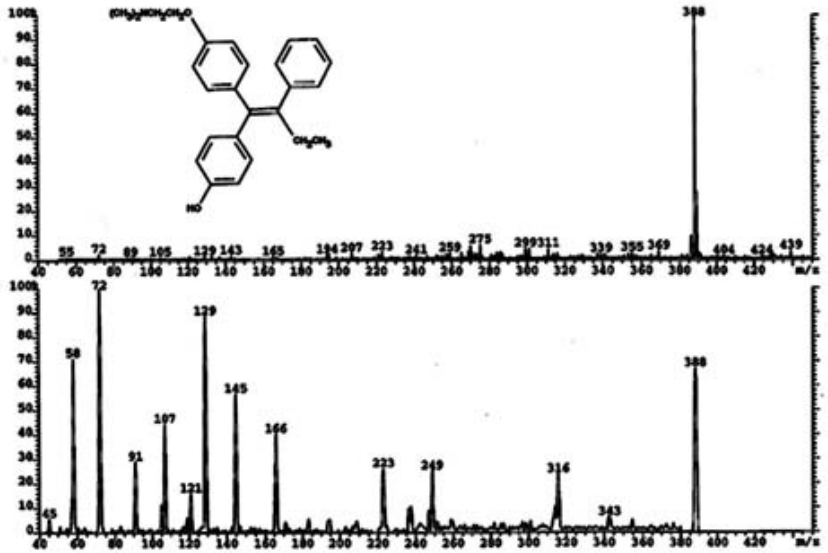
Essentially two mass spectrometers in series.

First spectrometer is used to select a designated ion, which is then directed to a collision chamber.

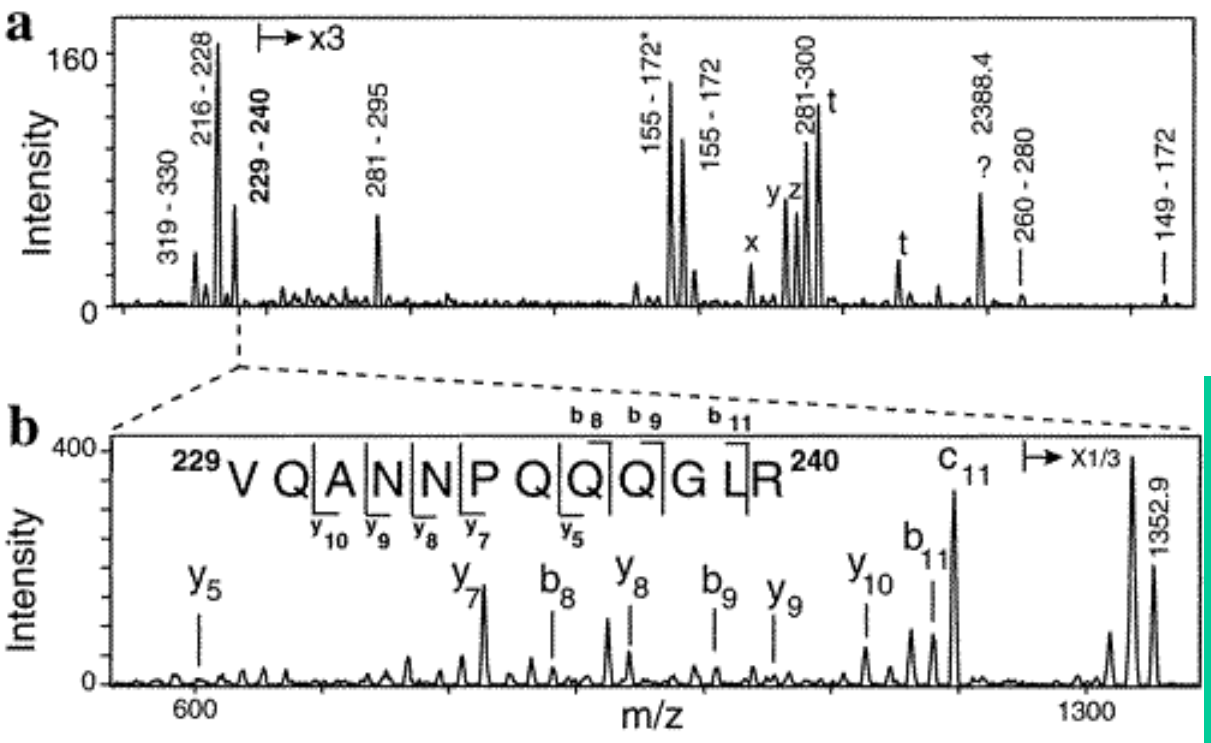
Pressurized inert gas (Argon) is used to cause collision induced dissociation resulting in product ions (fragments)

The product ions are then sent to the second analyzer

MS-MS of 4-hydroxy Tamoxifen



Assignment of peptides of the gag HTLV-1 protein (22 KDa) to the trypsin fragment mass spectrum and confirmation by Tandem MALDI mass spectrum of the 229-240 fragment



MALDI mass spectrum from an in-gel trypsin digest after SDS-PAGE

Tandem MALDI of the m/z 1352.8 fragment, confirming its assignment as 229-240 of the gag protein.

fragments yield sequence information