

US EPA ARCHIVE DOCUMENT

# T-RFLP for the Bacterial Source Tracking of *Escherichia coli* Sourced from Feral Pigeons and Cattle in Agricultural Roof Runoff

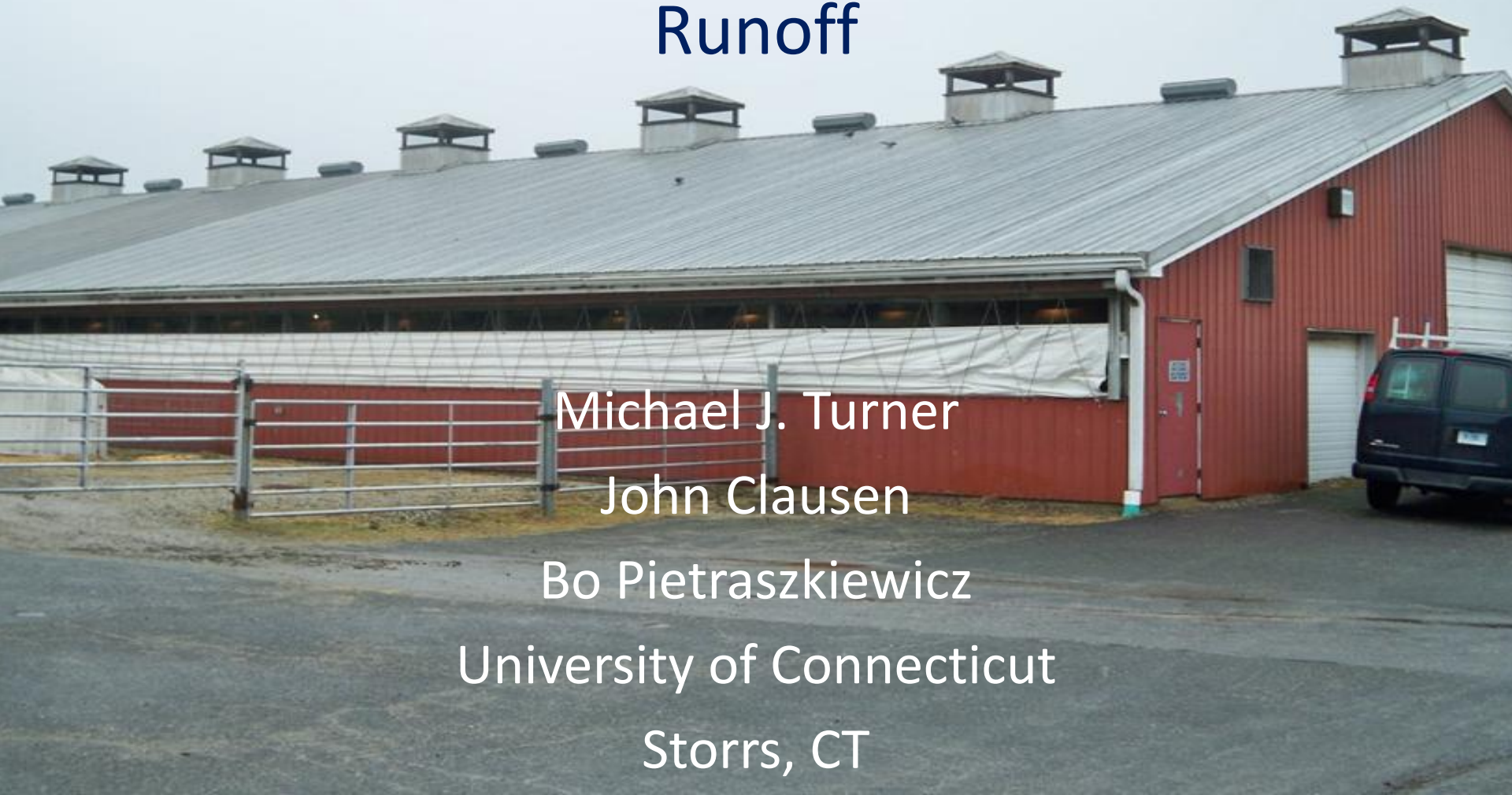
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University of Connecticut

Storrs, CT



# Overview

- Problem
- Objective
- Study area
- Methods
- Results
- Future directions

# Problem

- pathogens : leading cause of impairment in streams, bays, and estuaries (USEPA, 2004)
- Fecal indicator bacteria designated use criteria exceeded in 13% of monitored surface waters (USEPA, 2005)
- Beach closures, drinking water cutoff, and shellfish bed closures (Schueler, 1999)
- 12-20 million cases of illness in US (Colford et al., 2006; Reynolds et al., 2008)
- Pathogens may impact aquatic biota (Trombulak and Frissel, 2000)

# Problem

- Bacterial counts don't reveal contamination source
- Focus on 0157:H7 and Shiga-toxin
- Vague literature on method development
- Efficient *E. coli* attenuation shown at study site
  - Source of *E. coli* remains unknown

# Objective

- Track the source of *E. coli* in a constructed wetland to pigeons or cattle
  - Wetland once treated dairy wastewater
  - Now treats roof runoff
  - Legacy effect from cattle?
  - Contamination from pigeons on roof?



# Study Area

- Constructed treatment wetland
- Kellogg Dairy Center, University of Connecticut, Storrs, CT
- Roof runoff from the animal facility
- *E. coli* attenuation previously studied at site (Hier, 2007)



# Methods

- Terminal Restriction Fragment Length Polymorphism Analysis (T-RFLP)
- A molecular biological assay
- Used for classifying microbial communities
- Used for Bacterial Source Tracking (BST)

# Method development

- DNA extraction
- PCR inhibitors
- Rigorous experimentation to arrive at:
  - PCR conditions
  - PCR Cycling temperatures
  - Restriction enzyme choice
- 1 year process

# DNA

- Deoxyribonucleic acid
- Double helix
- Made up of deoxynucleotides and a phosphate and sugar “backbone”
  - “rungs in the ladder” of the double helix
  - Adenine and Thymine (As and Ts)
  - Cytosine and Guanine (Cs and Gs)
- Organized into chromosomes
- *E. coli* chromosome is circular



# T-RFLP analysis

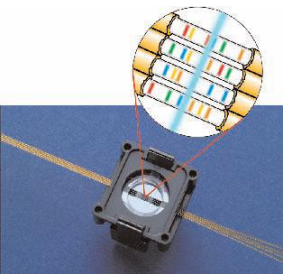
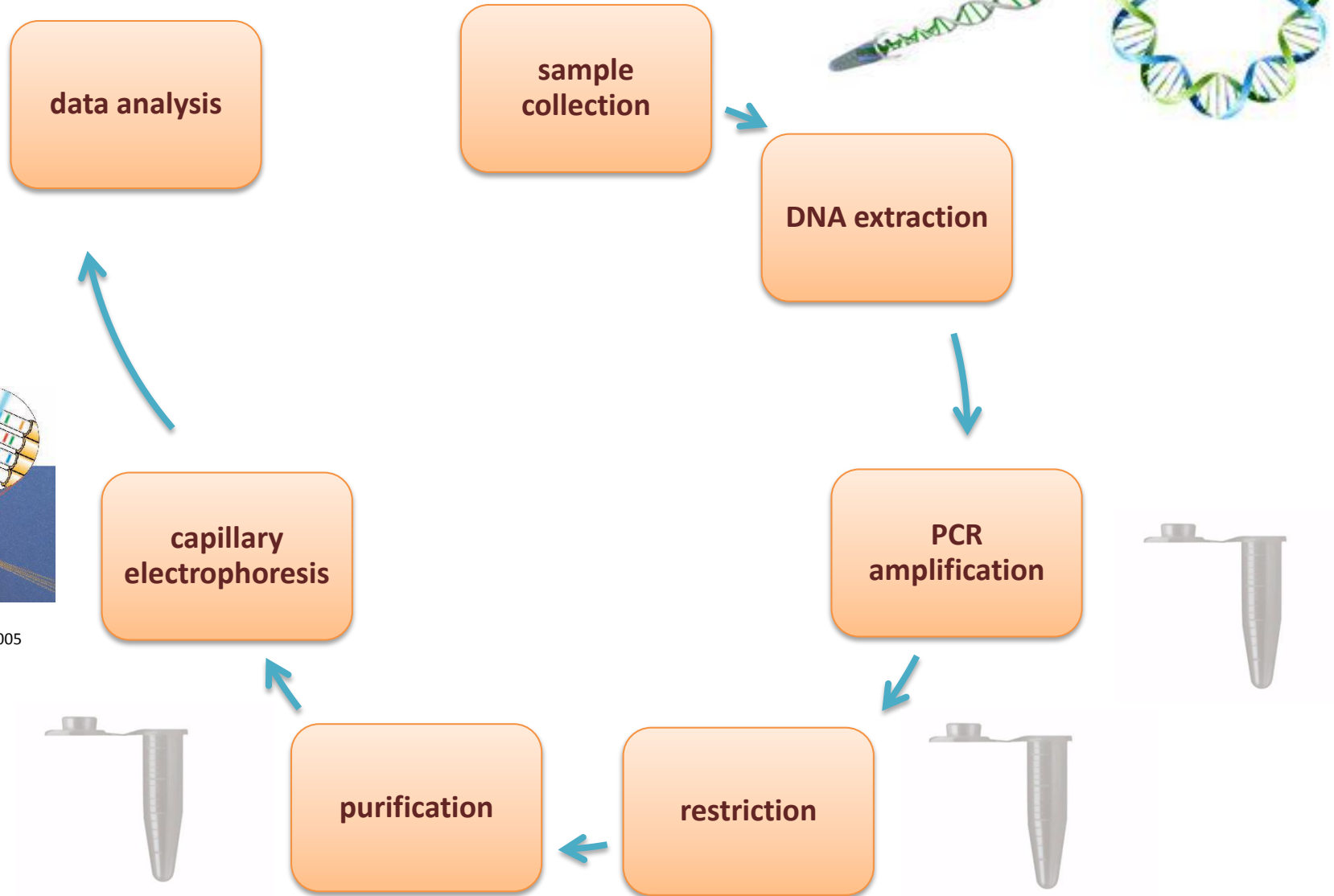


Image borrowed from Applied Biosystems, 2005

# T-RFLP analysis

data analysis

sample collection

DNA extraction



PCR amplification



restriction



purification



capillary electrophoresis

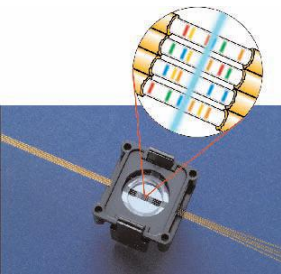
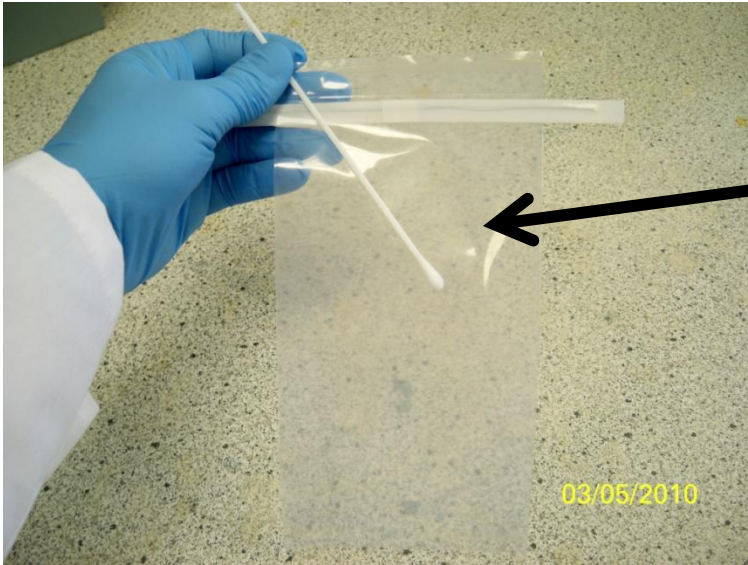


Image borrowed from Applied Biosystems, 2005

# Sample Collection



Fecal samples collected with sterile swabs  
Placed into sterile sample bags

Water grab samples collected into sterile sample bags



# T-RFLP analysis

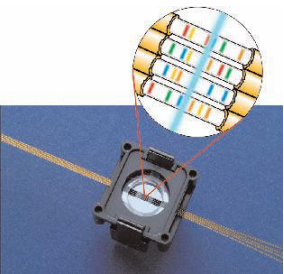
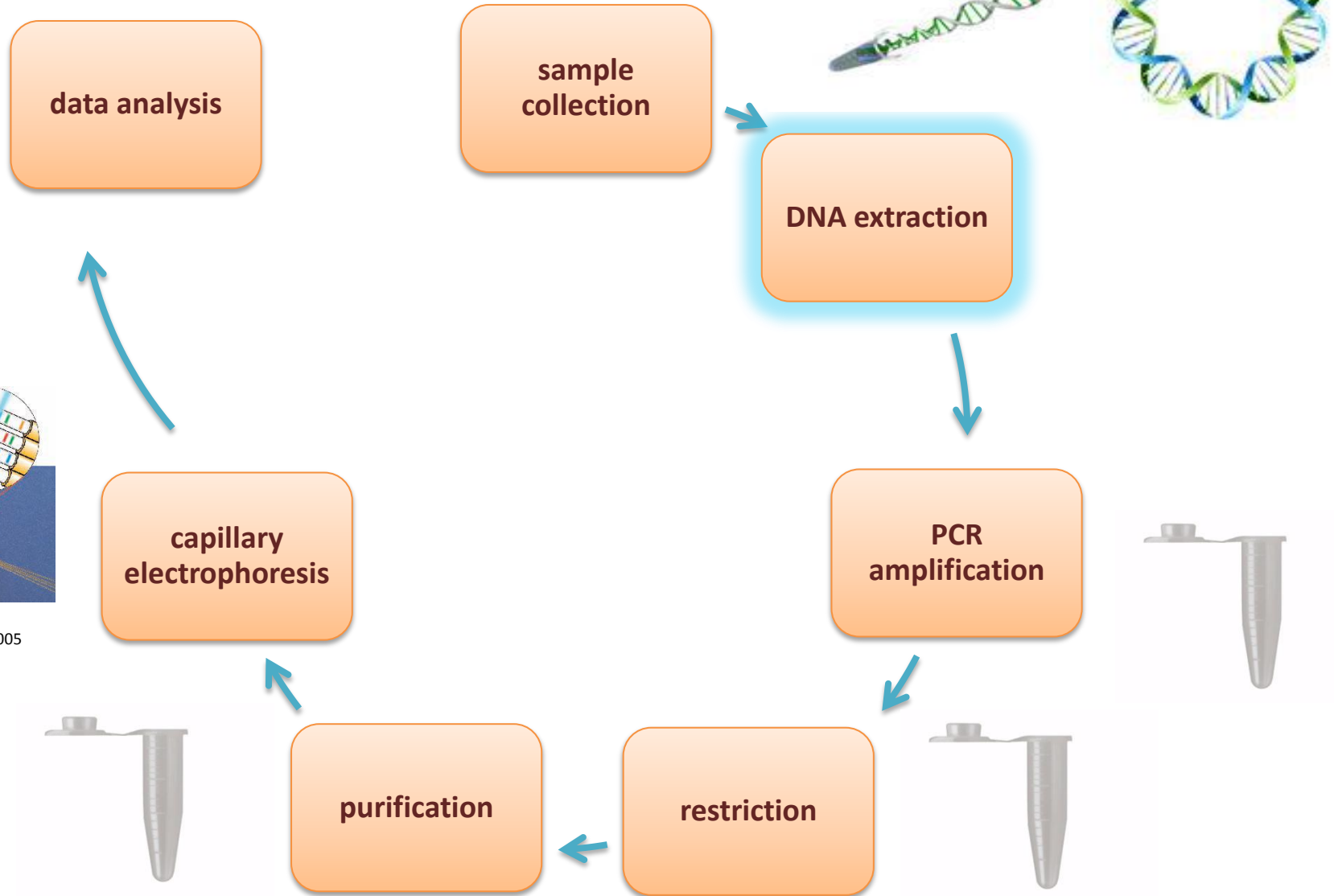


Image borrowed from Applied Biosystems, 2005

# DNA extraction



*E. Coli* cells existing in water or fecal samples



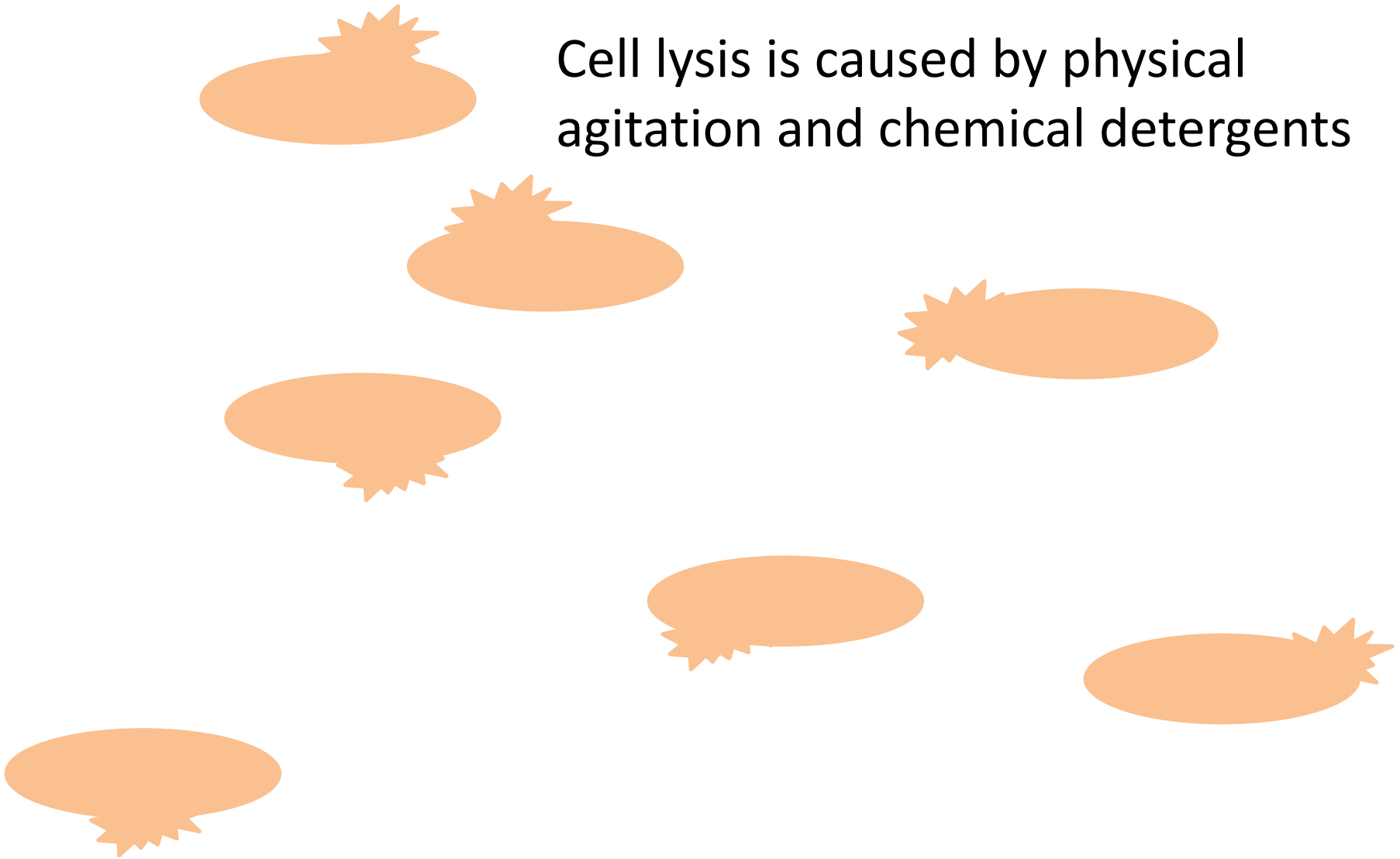
Fecal samples weighed into microcentrifuge tubes



Water samples filtered and filters placed in microcentrifuge tubes

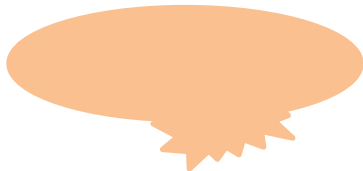
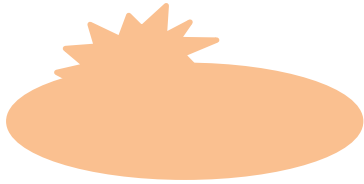
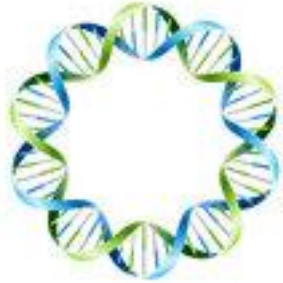
# DNA extraction

Cell lysis is caused by physical agitation and chemical detergents



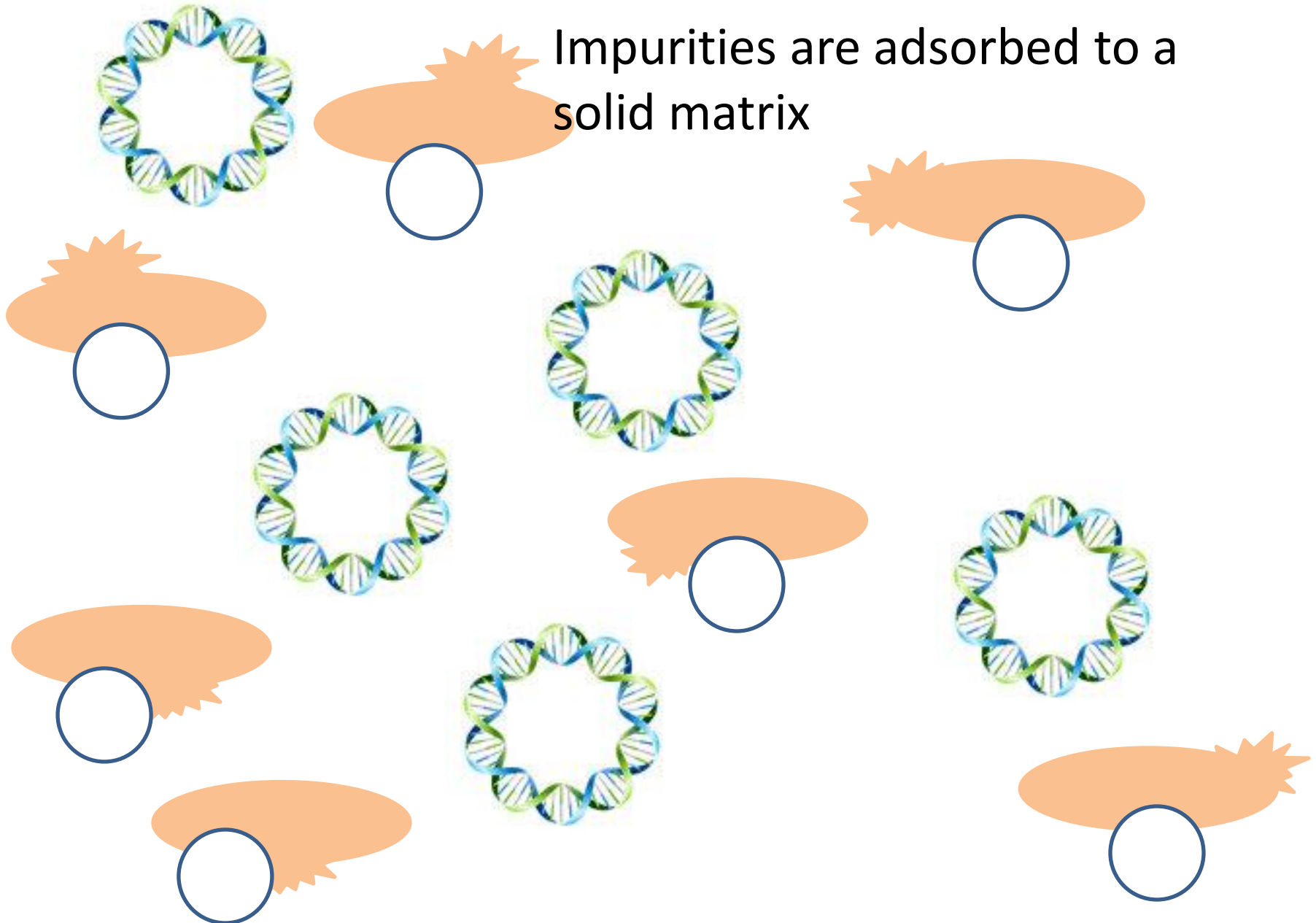
# DNA extraction

DNA, proteins, polysaccharides,  
and many impurities are  
suspended in the solution

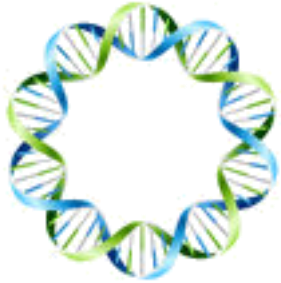


# DNA extraction

Impurities are adsorbed to a solid matrix



# DNA extraction

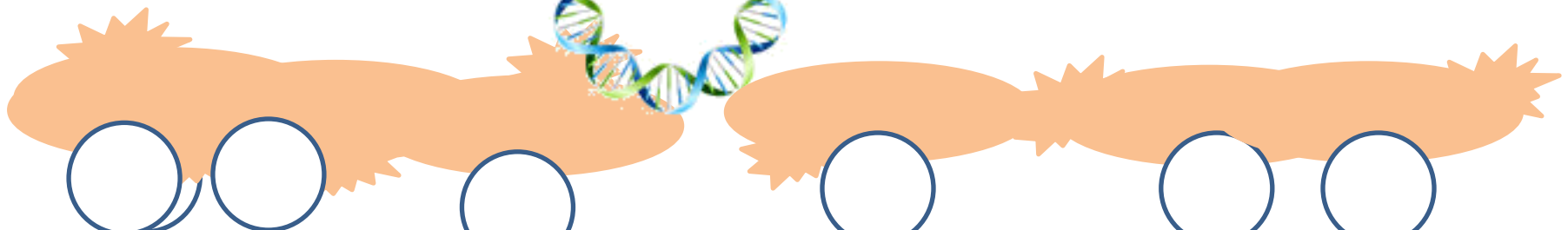


Proteins, polysaccharides, and other impurities are pelleted by centrifugation

Impurities are adsorbed to a solid matrix



DNA is bound to a silica membrane



# DNA extraction



Proteins, polysaccharides,  
and other impurities are  
pelleted by centrifugation

Impurities are adsorbed to a  
solid matrix

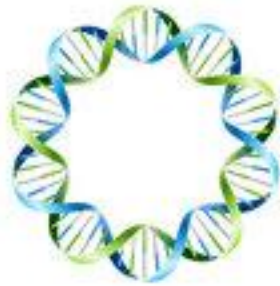


DNA is bound to a  
silica membrane and  
washed to remove  
remaining impurities

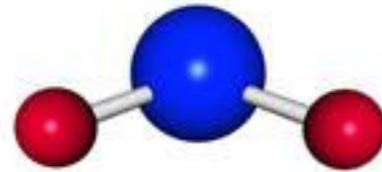


# DNA extraction

- DNA is then eluted in sterile deionized water



+



# T-RFLP analysis

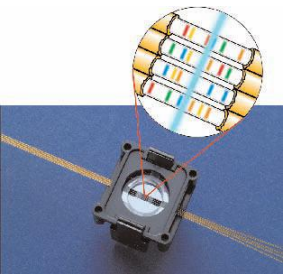
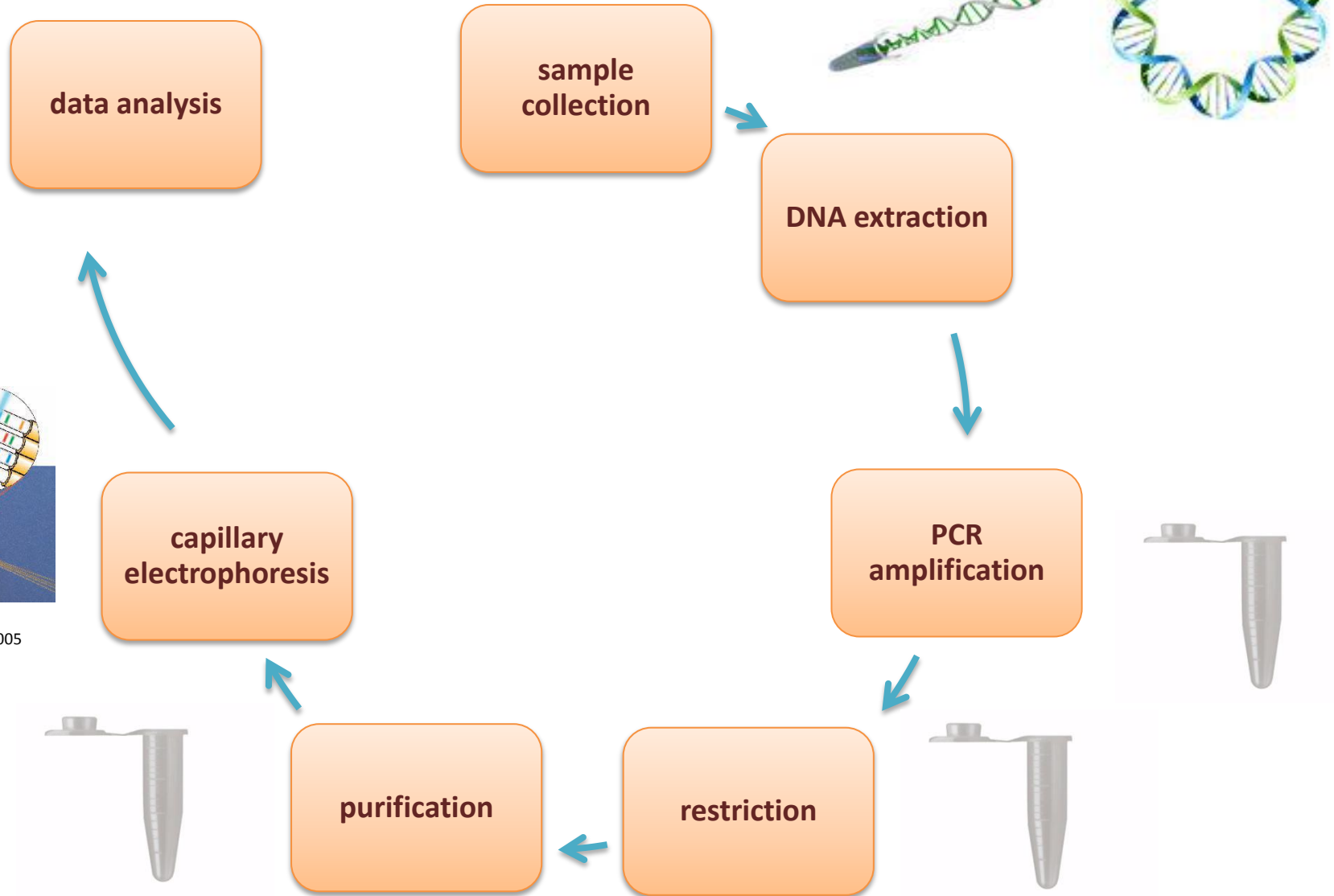


Image borrowed from Applied Biosystems, 2005

# Quantification and Dilution

- DNA is quantified
- Gel electrophoresis
- Spectrophotometry
  
- DNA diluted to standard concentration



# T-RFLP analysis

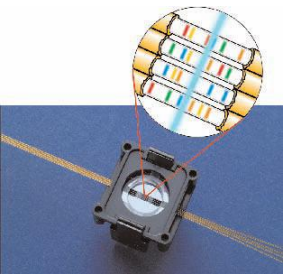
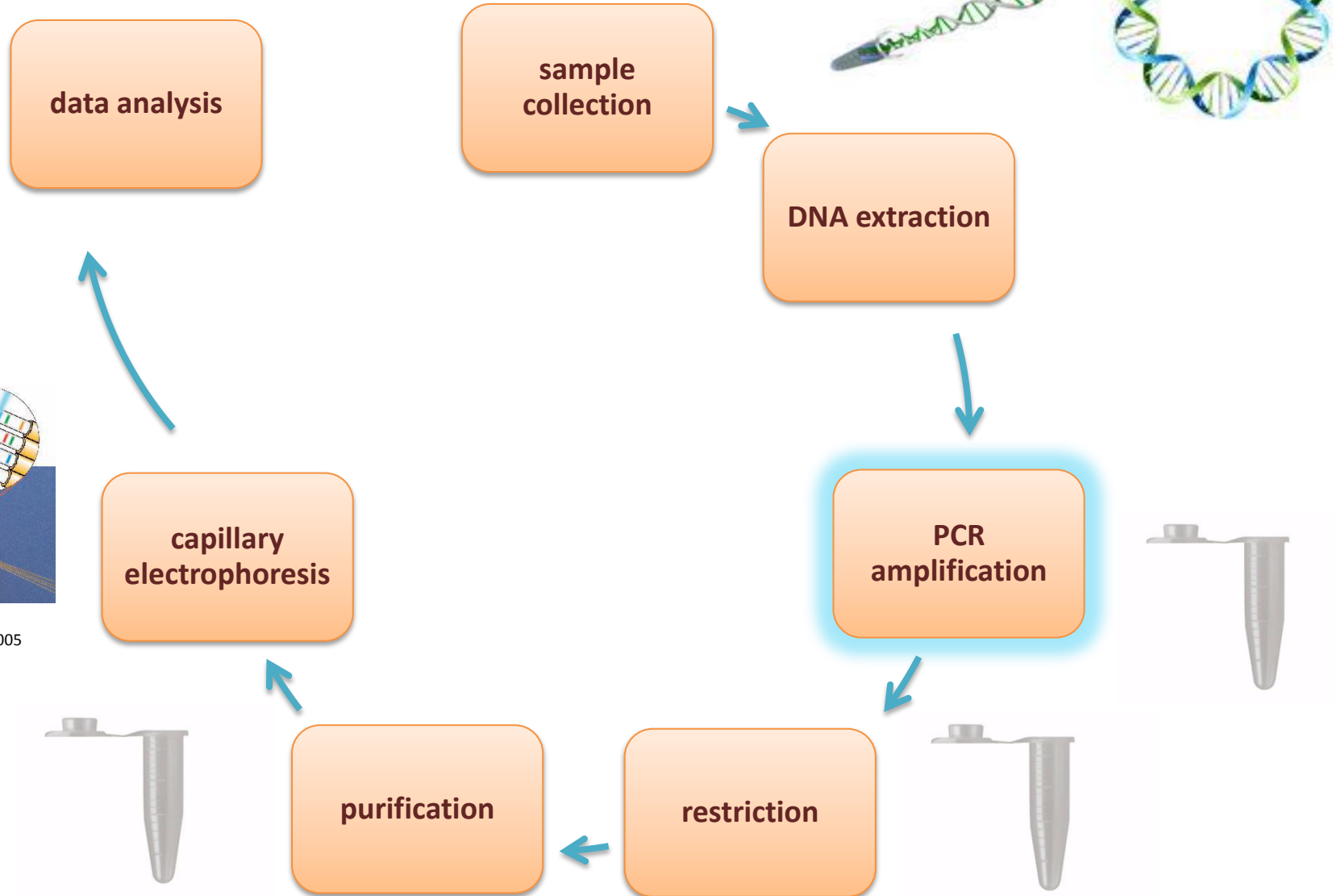


Image borrowed from Applied Biosystems, 2005

# Polymerase Chain Reaction (PCR)



# Polymerase Chain Reaction (PCR)



Double-stranded DNA “template”

Mixture contains:

- “template” DNA
- Taq polymerase
- $\text{MgCl}_2$
- Deoxynucleoside triphosphates (dNTPs)
- Primers (20 bp synthetic DNA)
- PCR buffer
- Cresol red
- Water

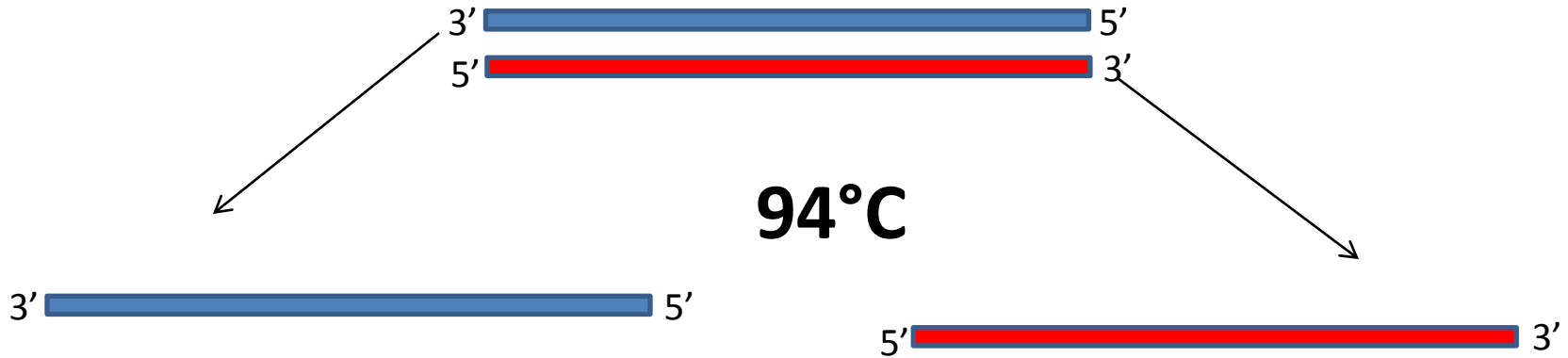
# Polymerase Chain Reaction (PCR)



Double-stranded DNA “template”

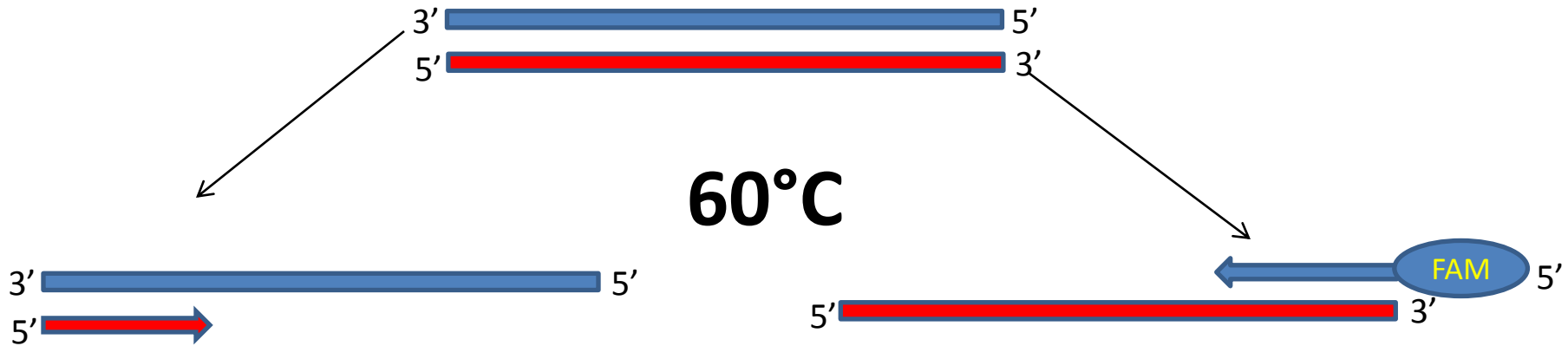
Reaction occurs in a thermal cycler  
(thermocycler)

# Polymerase Chain Reaction (PCR)



Denaturation—Double-stranded DNA  
“unzips” into single-stranded DNA

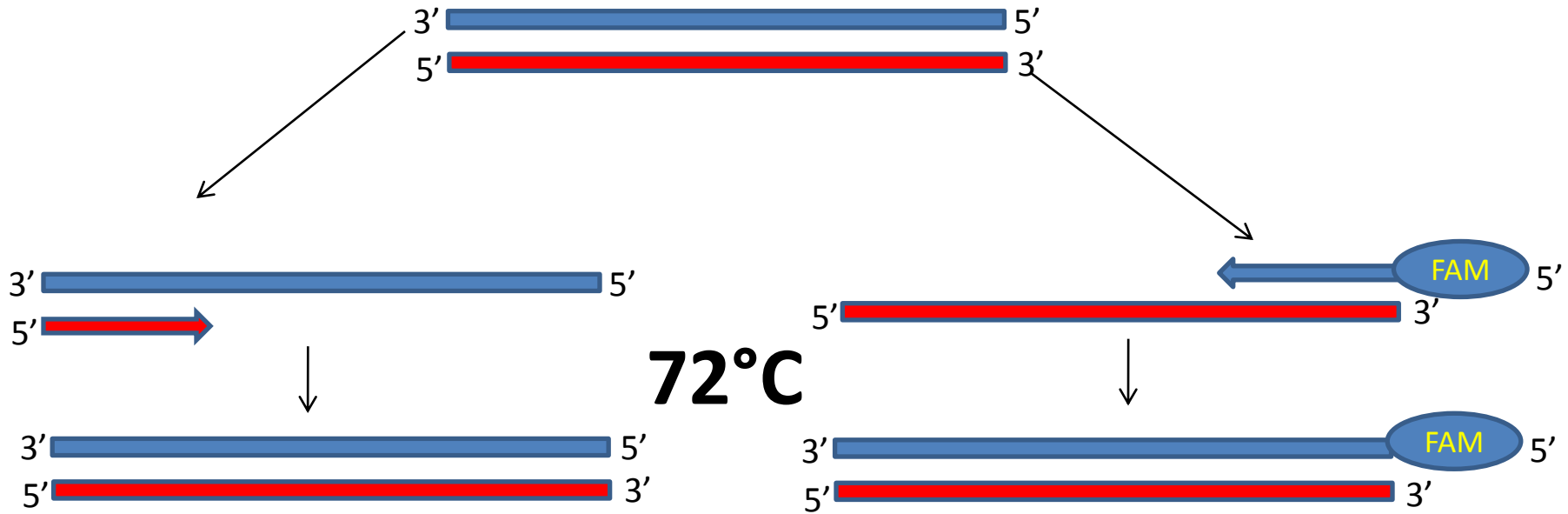
# Polymerase Chain Reaction (PCR)



Annealing—Primers attach to complementary sections of single-stranded DNA

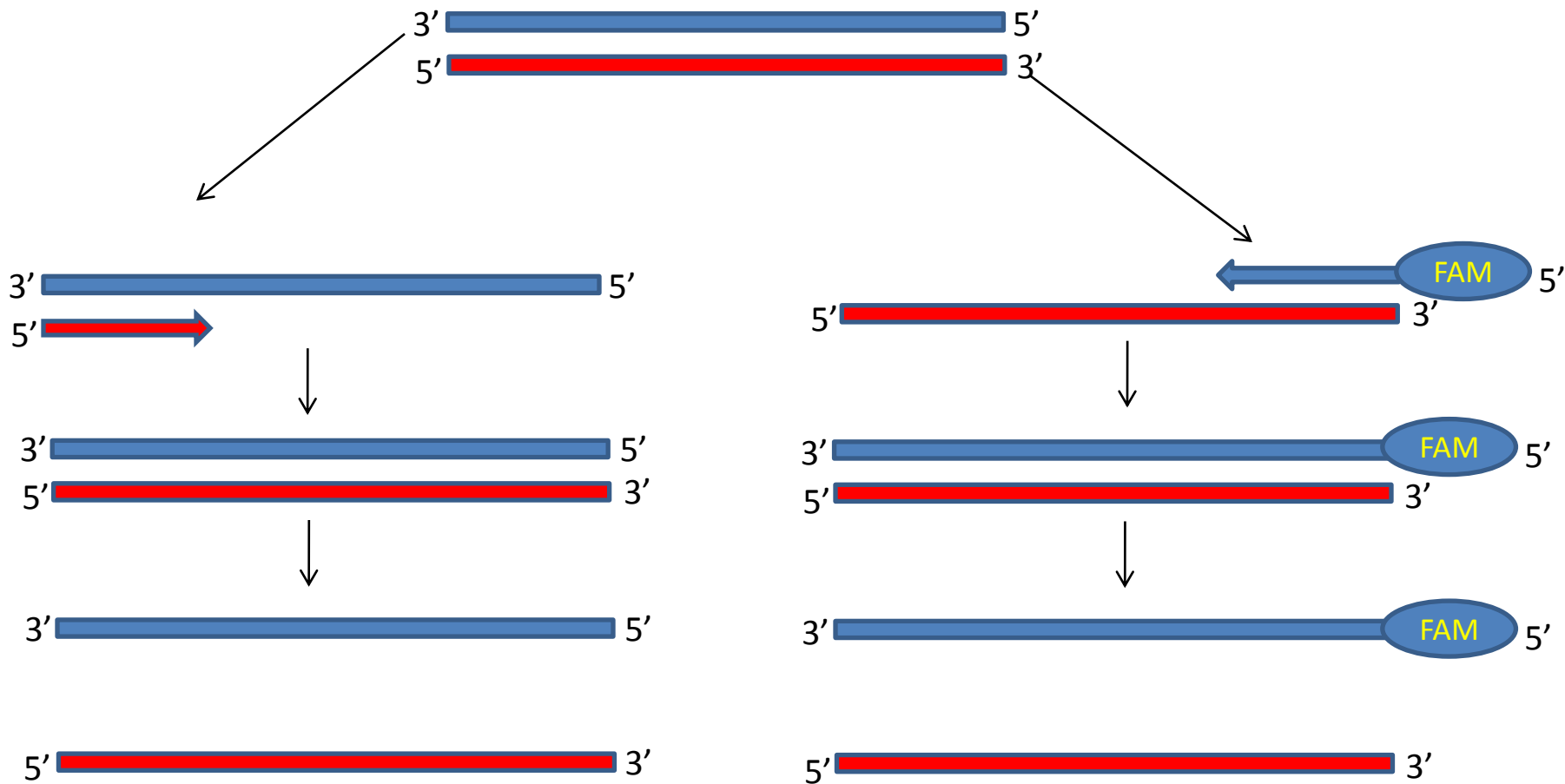
One primer labeled with a flourophore:  
FAM

# Polymerase Chain Reaction (PCR)

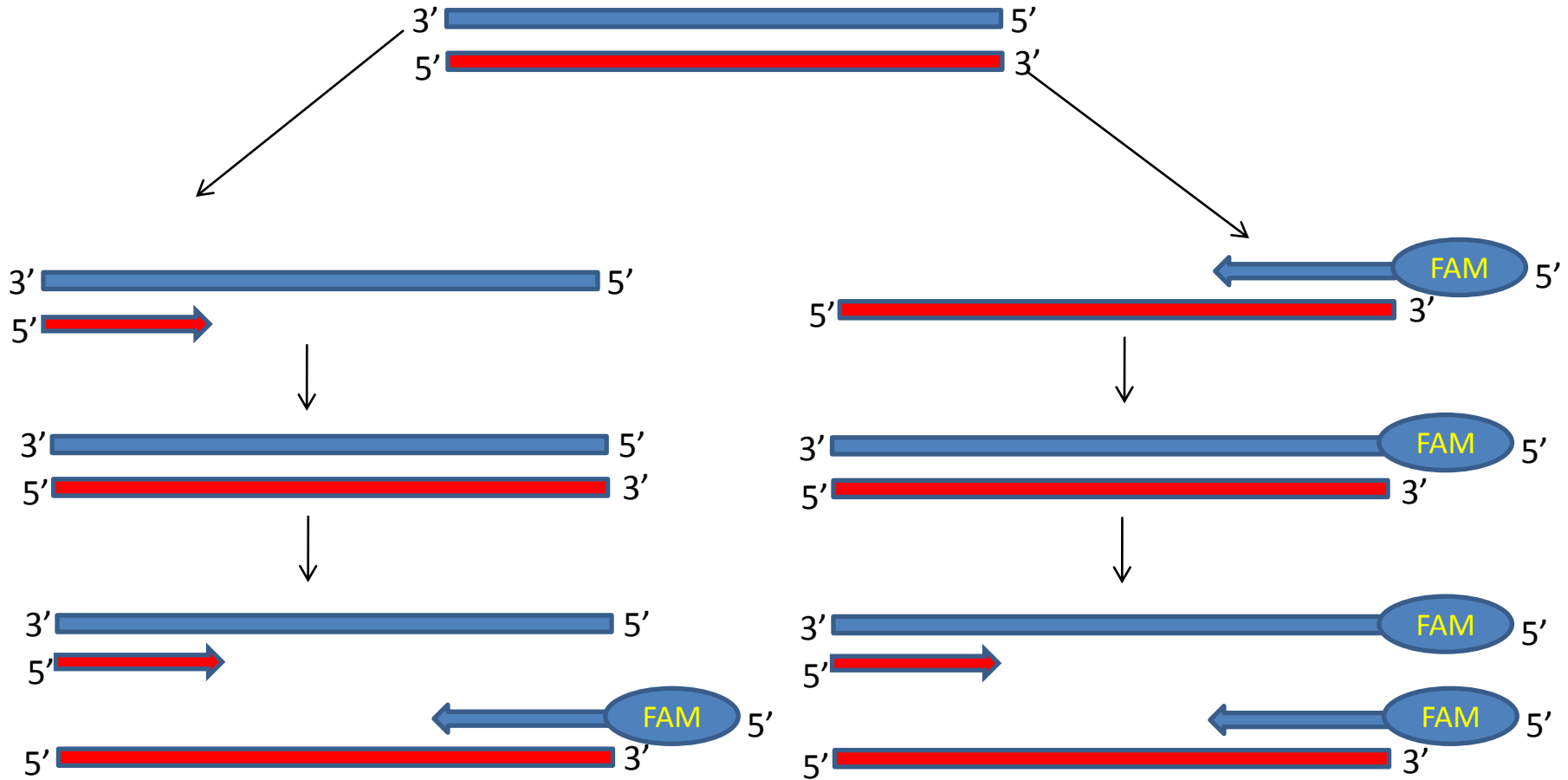


Elongation (Extension)—Taq polymerase enables dNTPs (Cs, Gs, As, and Ts) to build complimentary sequences from 5' to 3', beginning with the primers. This forms more double stranded DNA.

# Polymerase Chain Reaction (PCR)



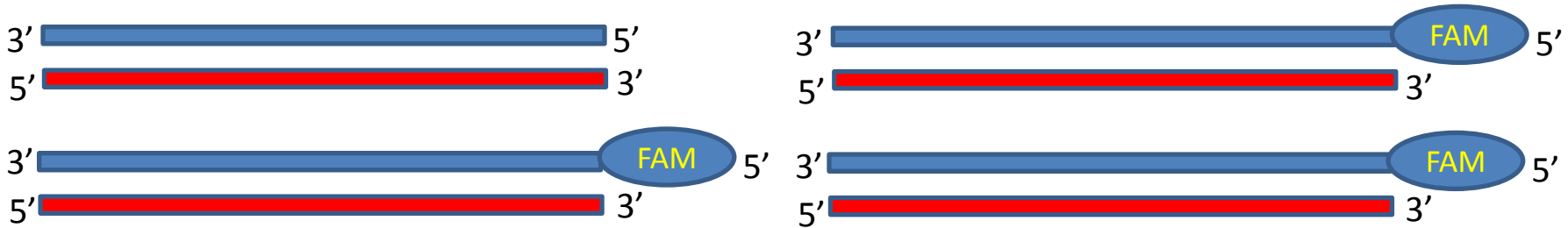
# Polymerase Chain Reaction (PCR)



# Polymerase Chain Reaction (PCR)

884 bp product

Billions of copies



# T-RFLP analysis

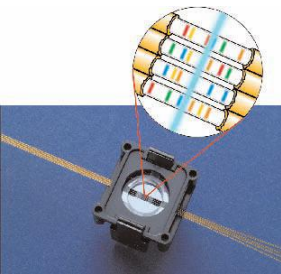
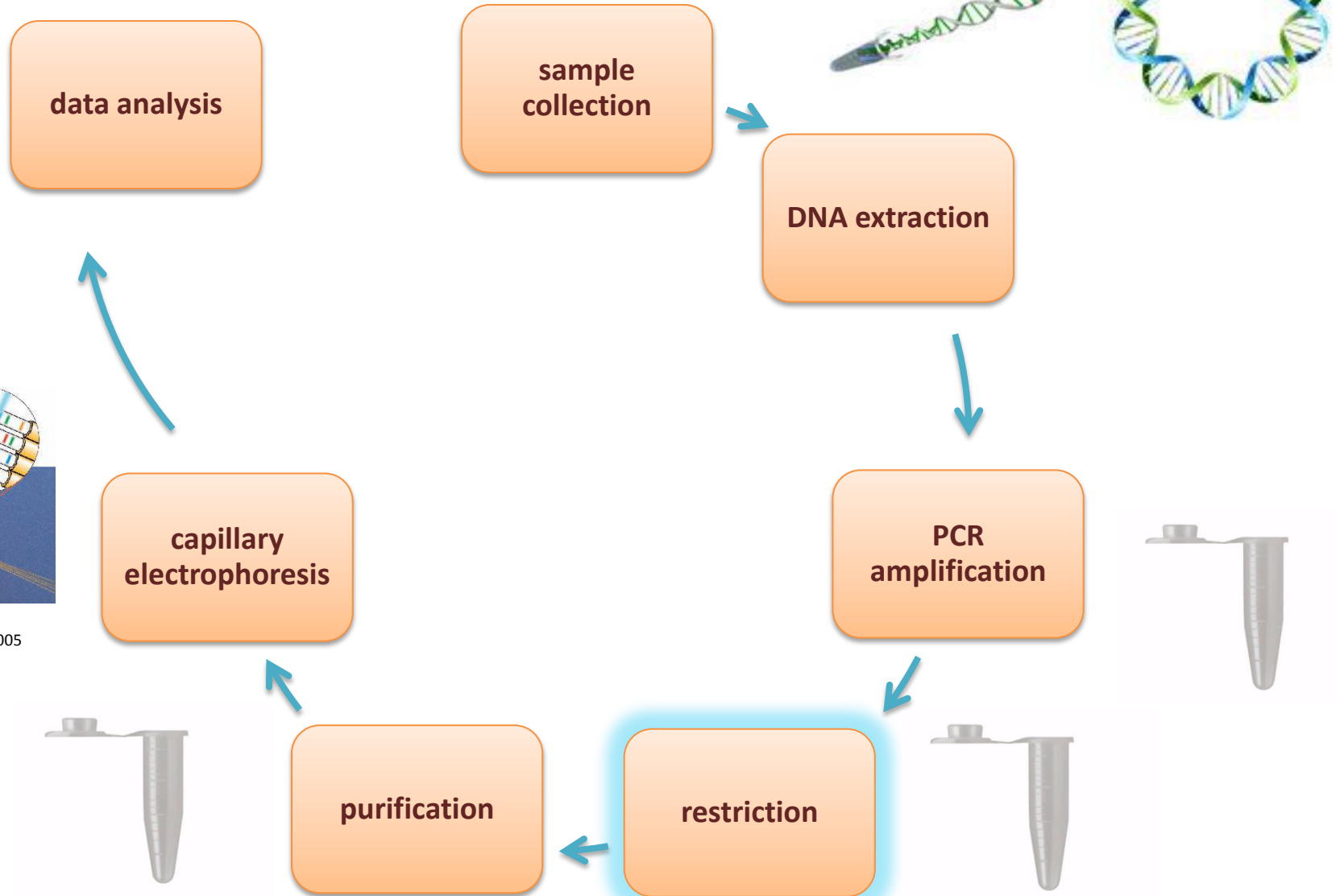
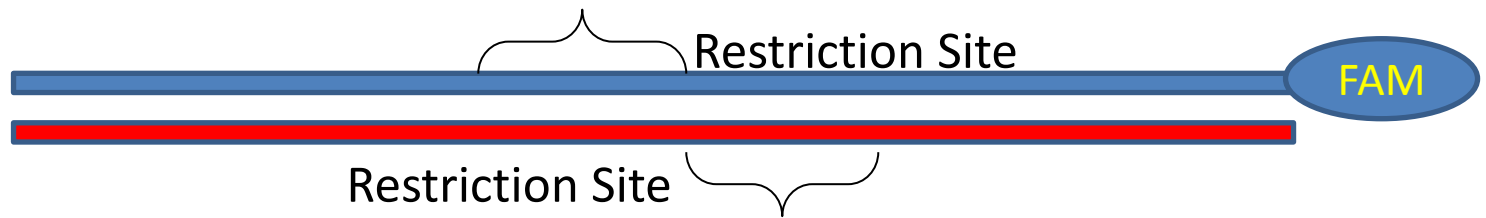


Image borrowed from Applied Biosystems, 2005

# Restriction



Amplicons produced in PCR are digested with restriction enzymes

Restriction enzymes “cut” DNA wherever their specific 4 base pair restriction site occurs

# Restriction



**37°C**

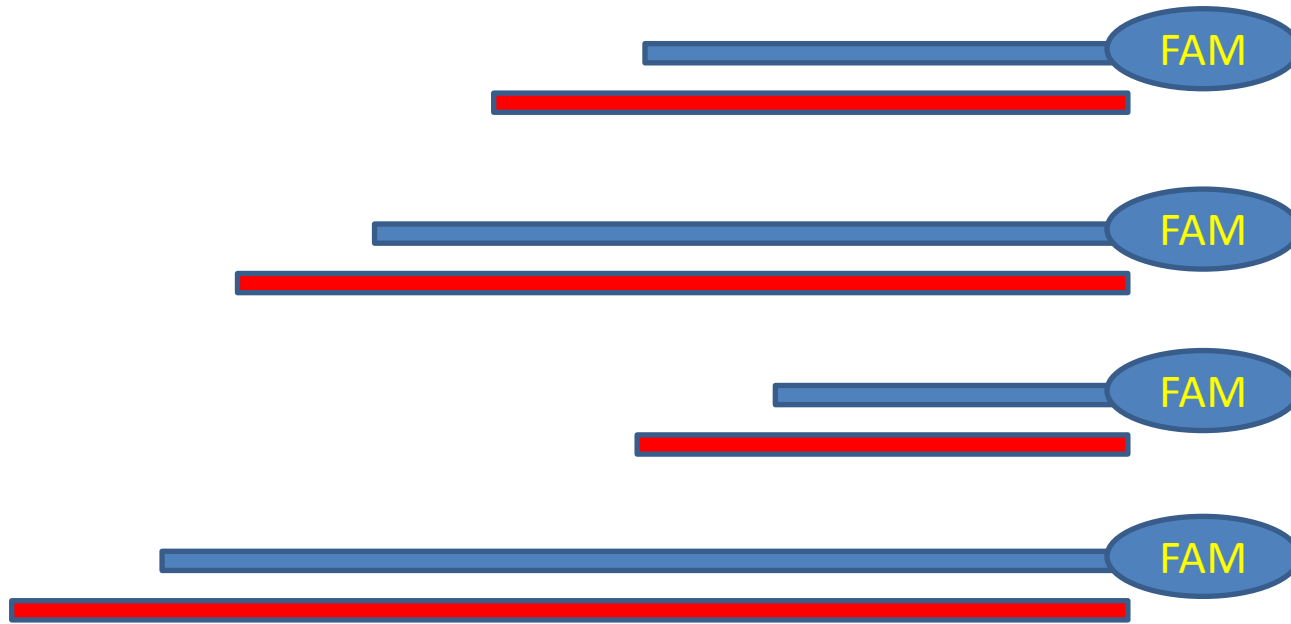
**Incubation**

**16 hours**

# Restriction



# Restriction



Terminal restriction fragments (T-RFs) of varying lengths are produced from novel amplicons

# T-RFLP analysis

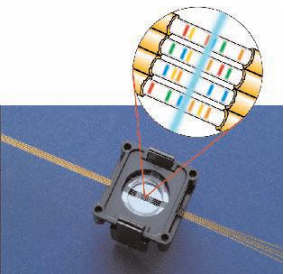
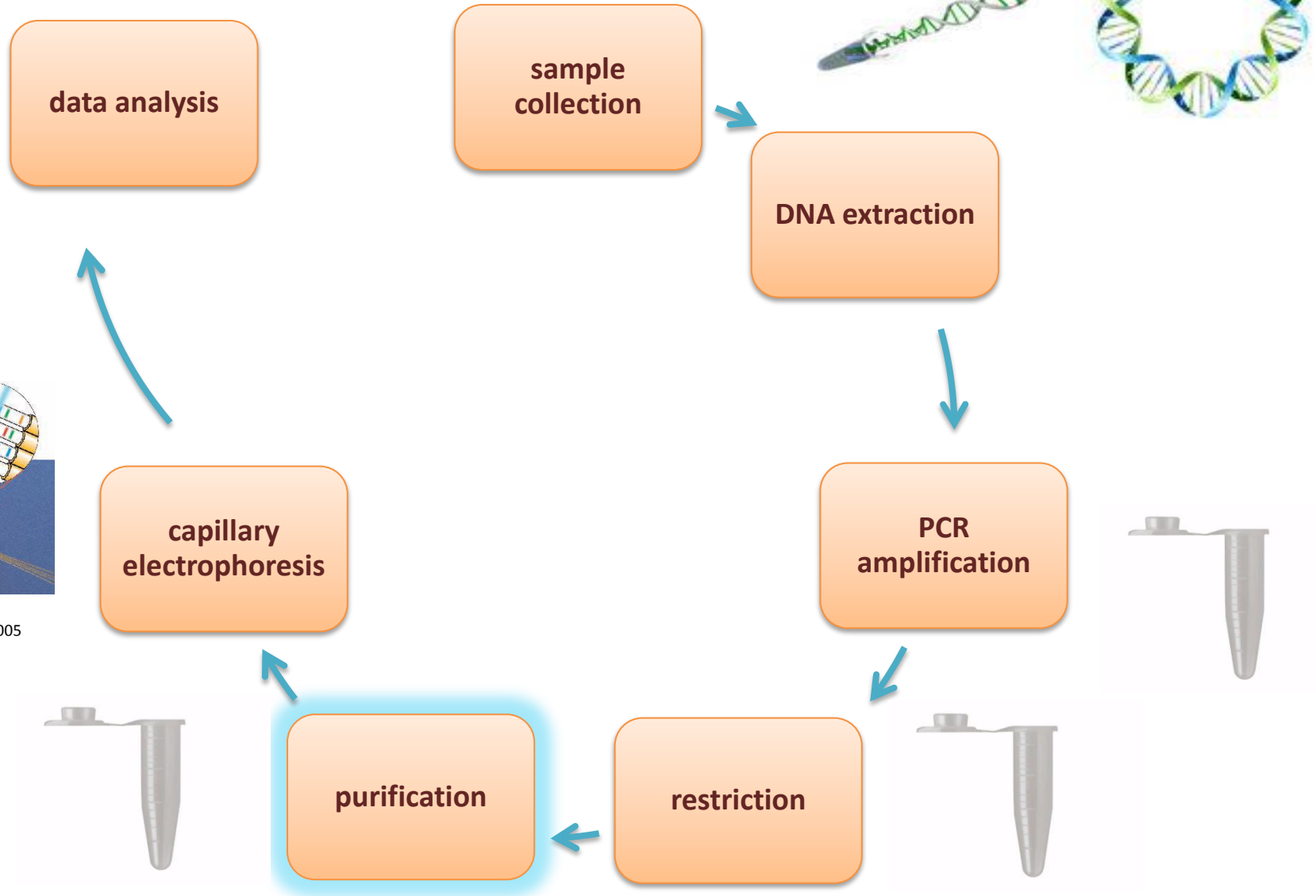


Image borrowed from Applied Biosystems, 2005

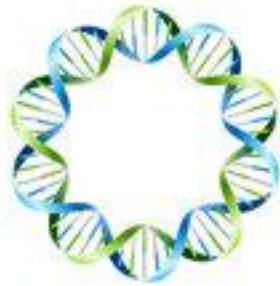
# DNA Purification



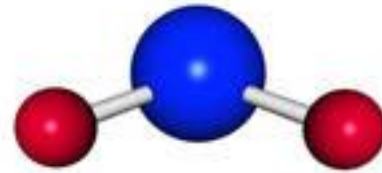
DNA is bound to a silica membrane and washed to remove reagents from PCR and restriction

# DNA Purification

- DNA is then eluted in sterile deionized water



+



# T-RFLP analysis

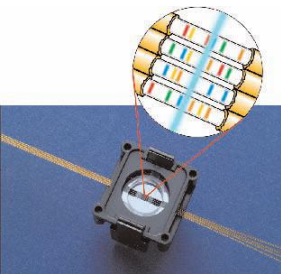
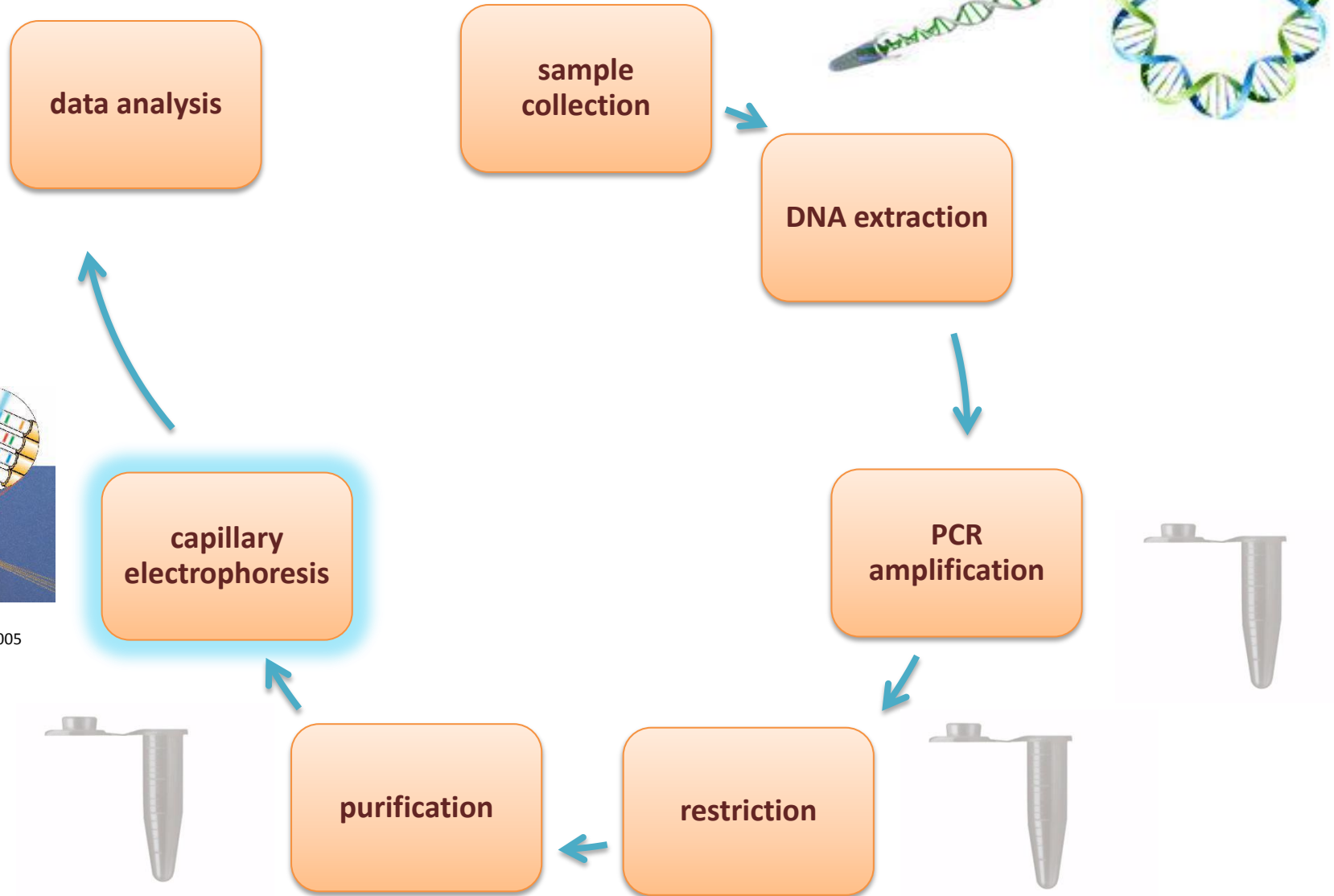
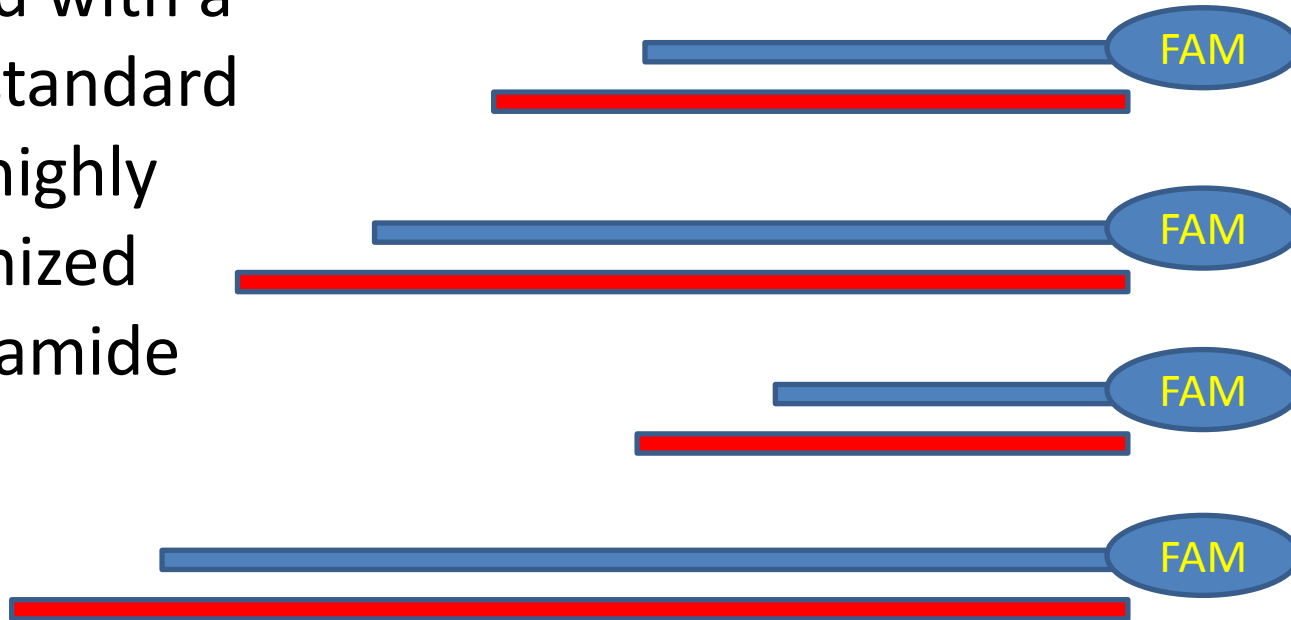


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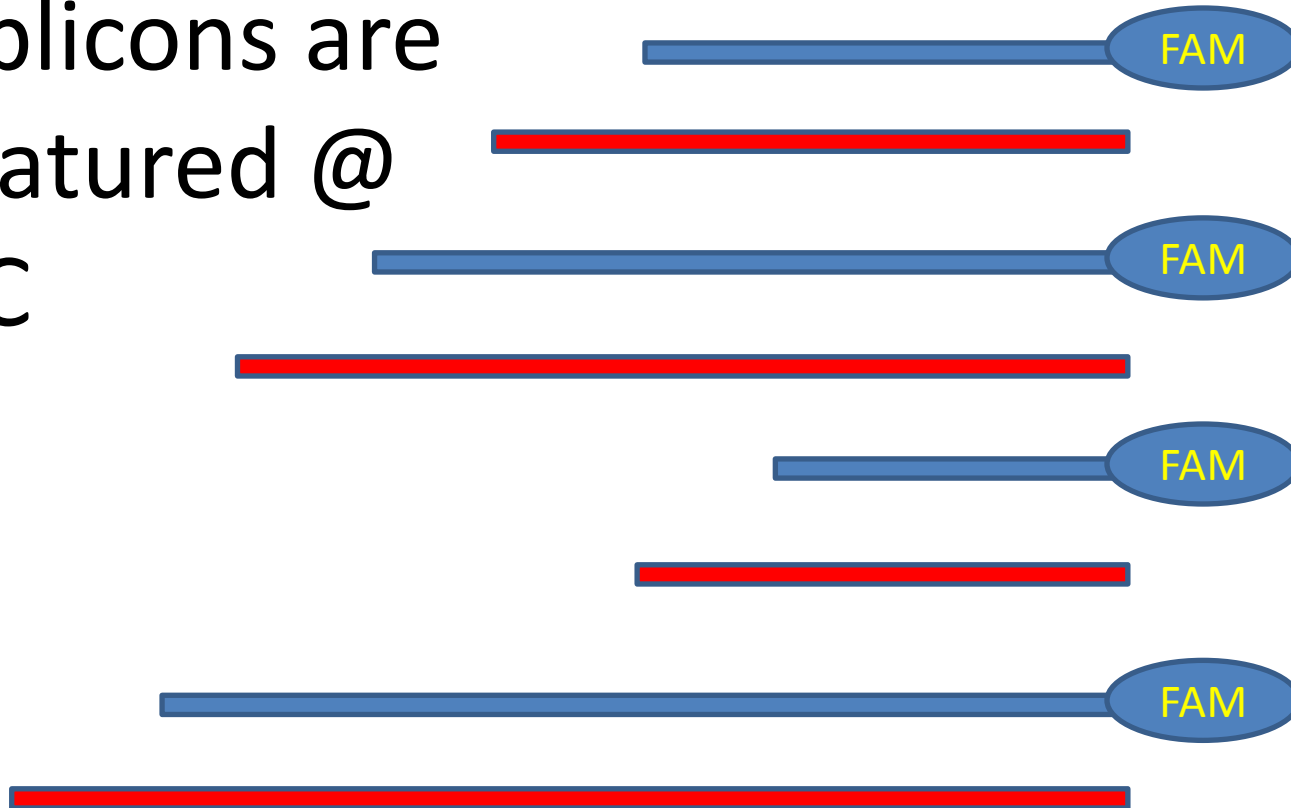
# Capillary electrophoresis

Amplicons are mixed with a size standard and highly deionized formamide



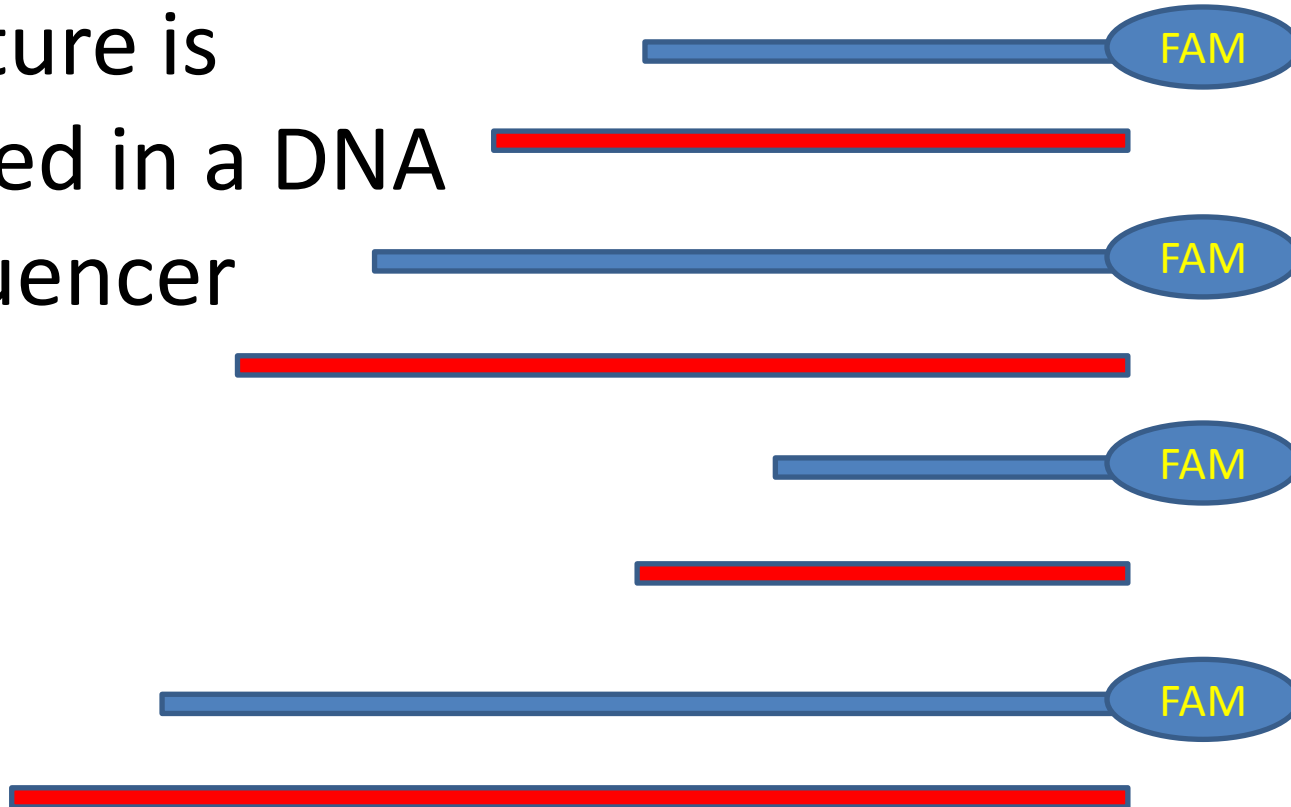
# Capillary electrophoresis

Amplicons are  
denatured @  
95°C

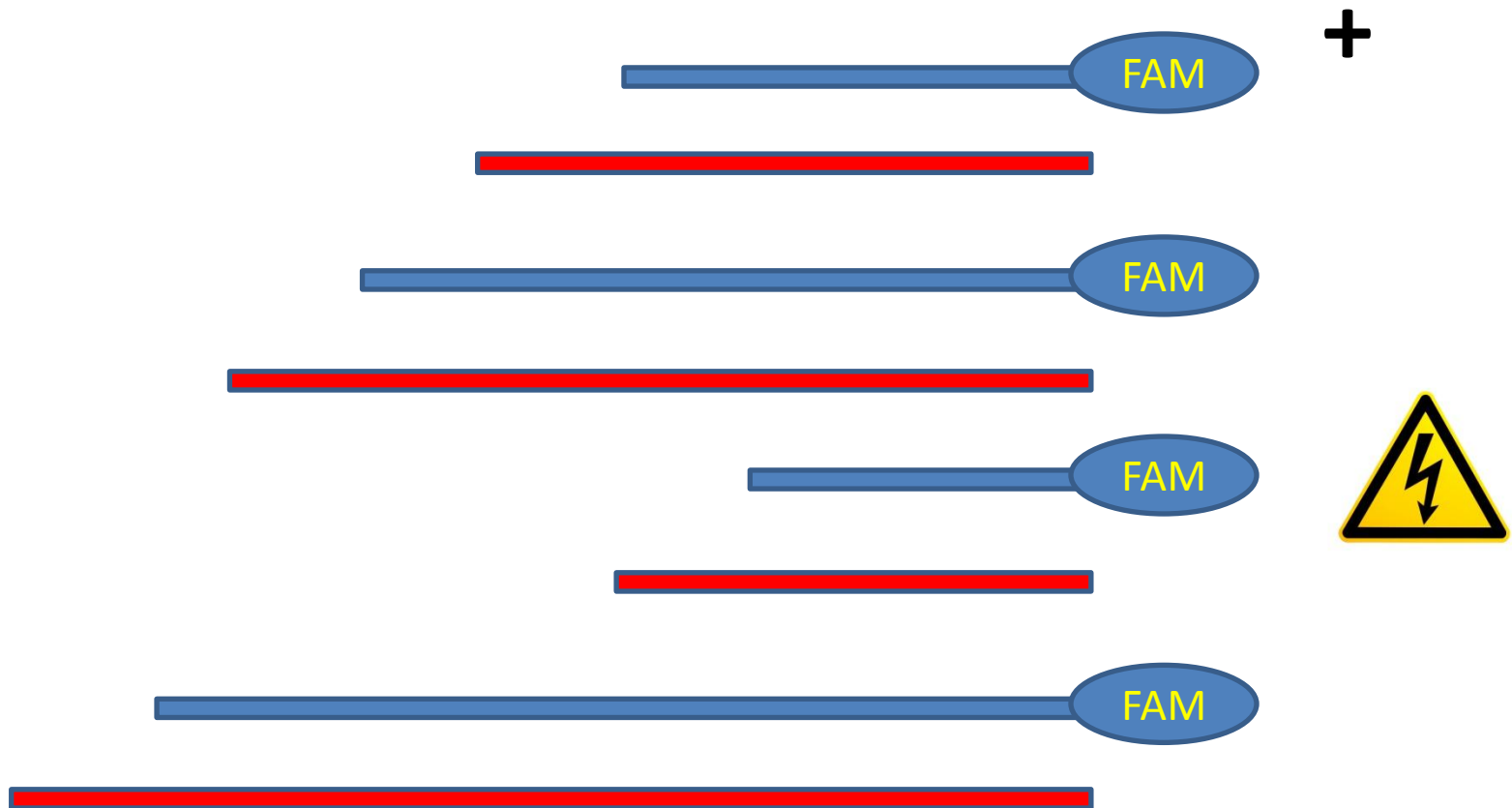


# Capillary electrophoresis

Mixture is  
placed in a DNA  
sequencer



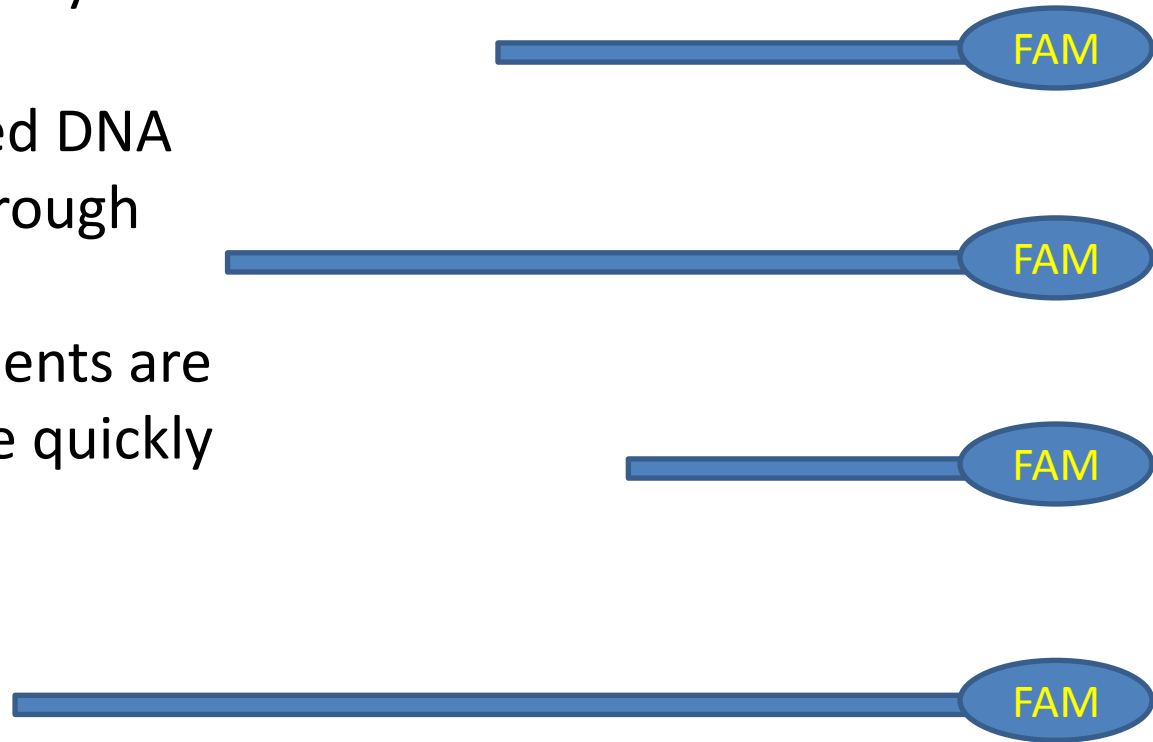
# Capillary electrophoresis



A current is applied

# Capillary electrophoresis

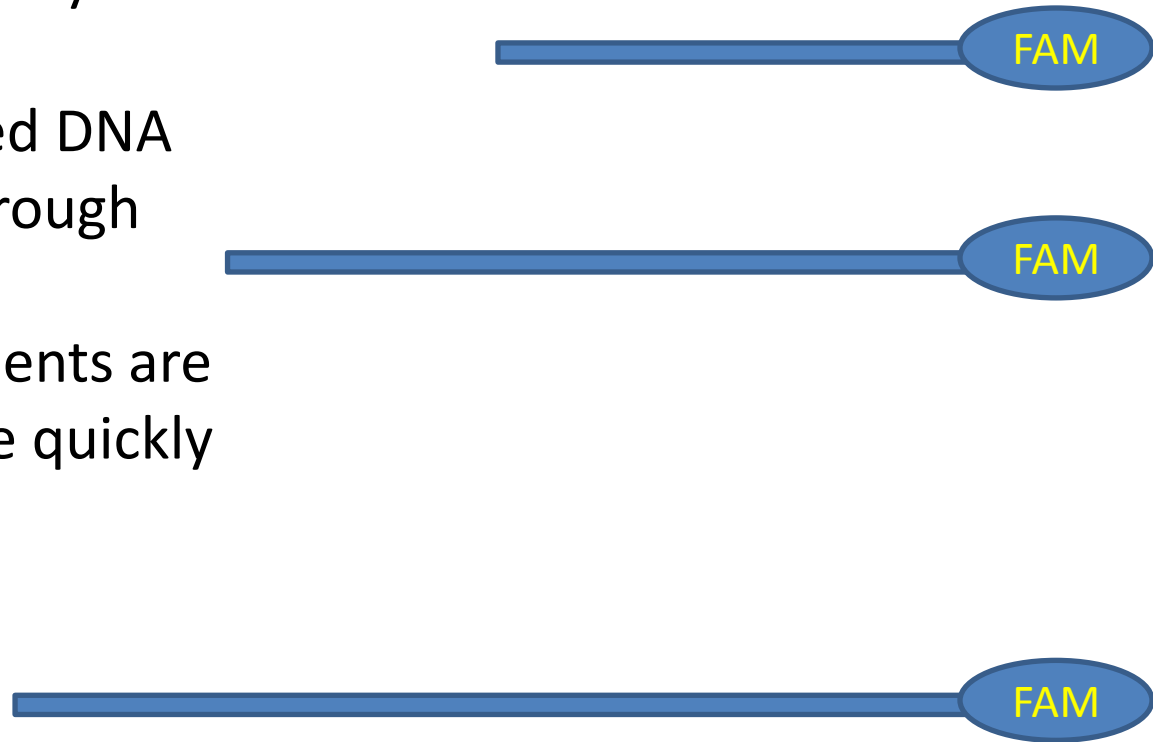
- DNA is negatively charged
- Single stranded DNA is drawn up through capillaries
- Smaller fragments are drawn up more quickly



A current is applied

# Capillary electrophoresis

- DNA is negatively charged
- Single stranded DNA is drawn up through capillaries
- Smaller fragments are drawn up more quickly



A current is applied

# Capillary electrophoresis

- DNA is negatively charged
- Single stranded DNA is drawn up through capillaries
- Smaller fragments are drawn up more quickly

+



-

A current is applied

# Capillary electrophoresis

- DNA is negatively charged
- Single stranded DNA is drawn up through capillaries
- Smaller fragments are drawn up more quickly

+



A current is applied

-

# Capillary electrophoresis

- DNA is negatively charged
- Single stranded DNA is drawn up through capillaries
- Smaller fragments are drawn up more quickly

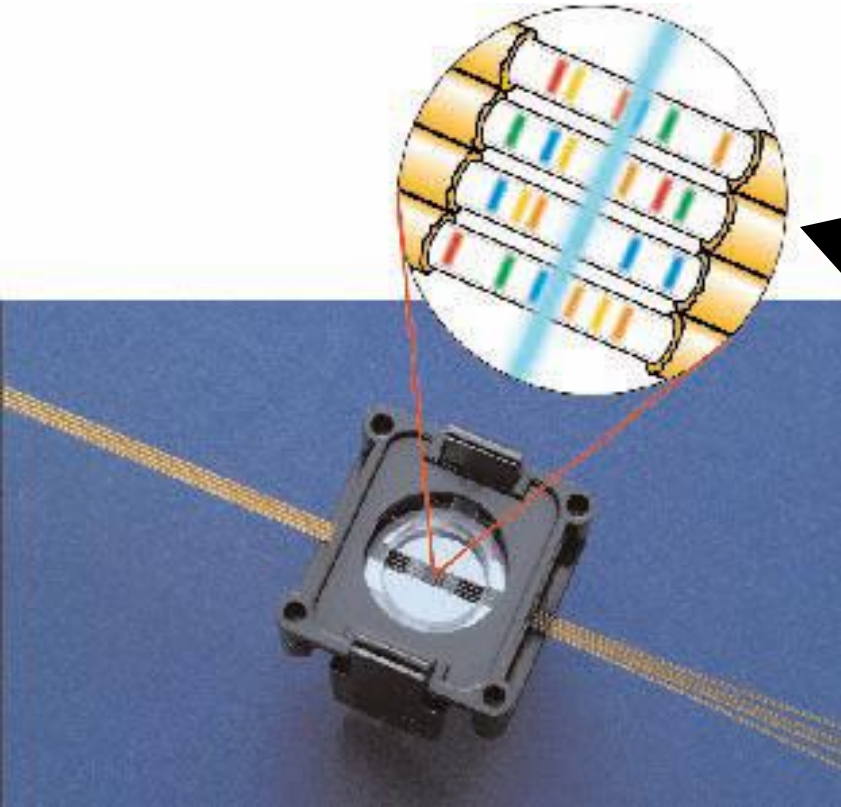
+



A current is applied

-

# Capillary electrophoresis



Flourophores are sensed as DNA passes through capillaries

Image borrowed from Applied Biosystems, 2005

# Capillary electrophoresis

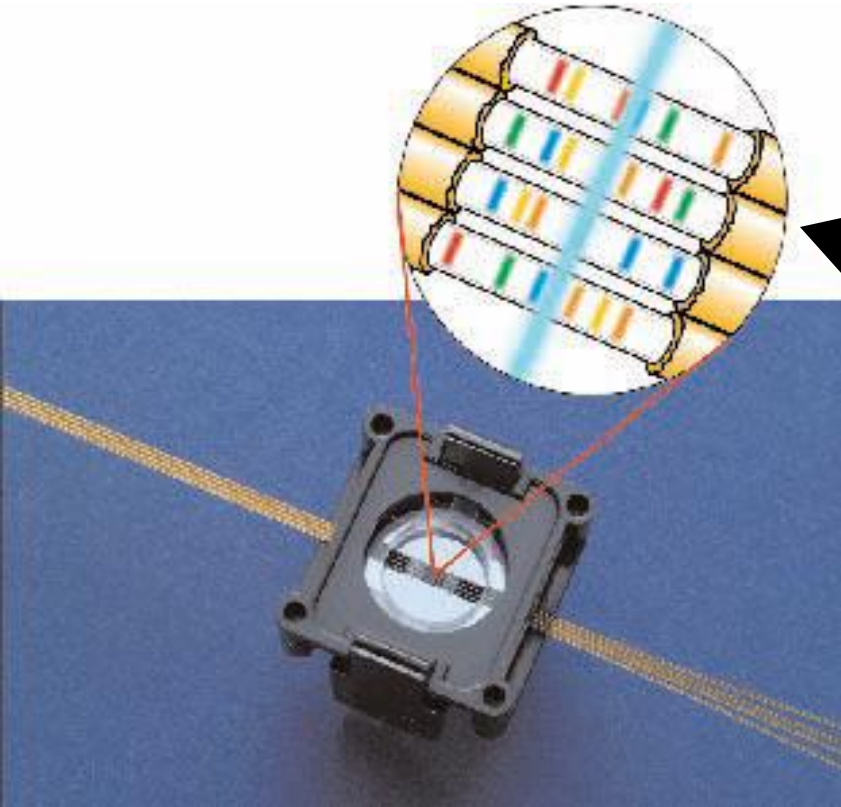


Image borrowed from Applied Biosystems, 2005

- Intensity read in relative fluorescence units (RFU)
  - Corresponds to quantity of DNA of a certain size
- Fragment size is proportional to the time until it is sensed

# T-RFLP analysis

data analysis

sample collection

DNA extraction

PCR amplification

capillary electrophoresis

purification

restriction

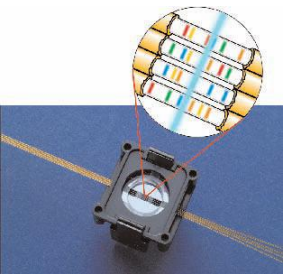
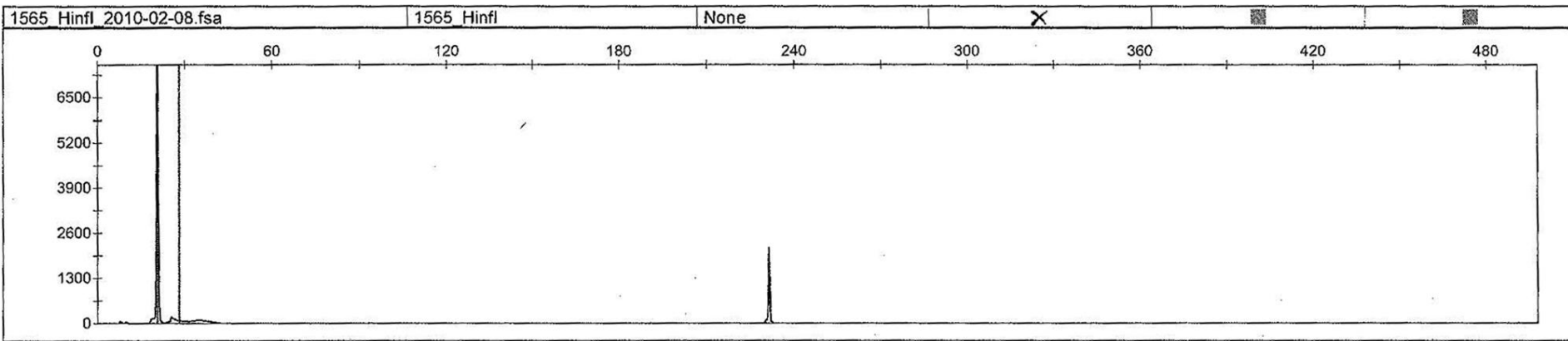


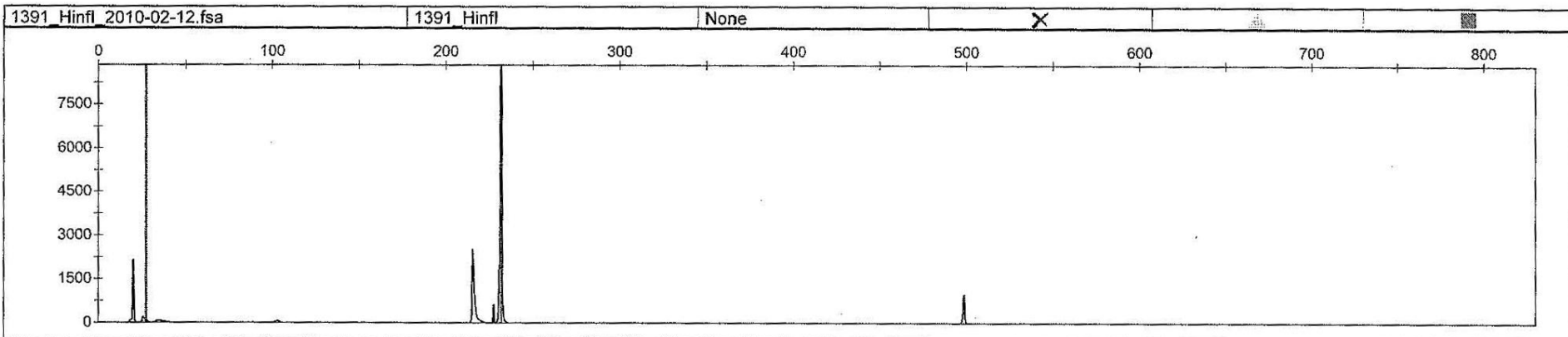
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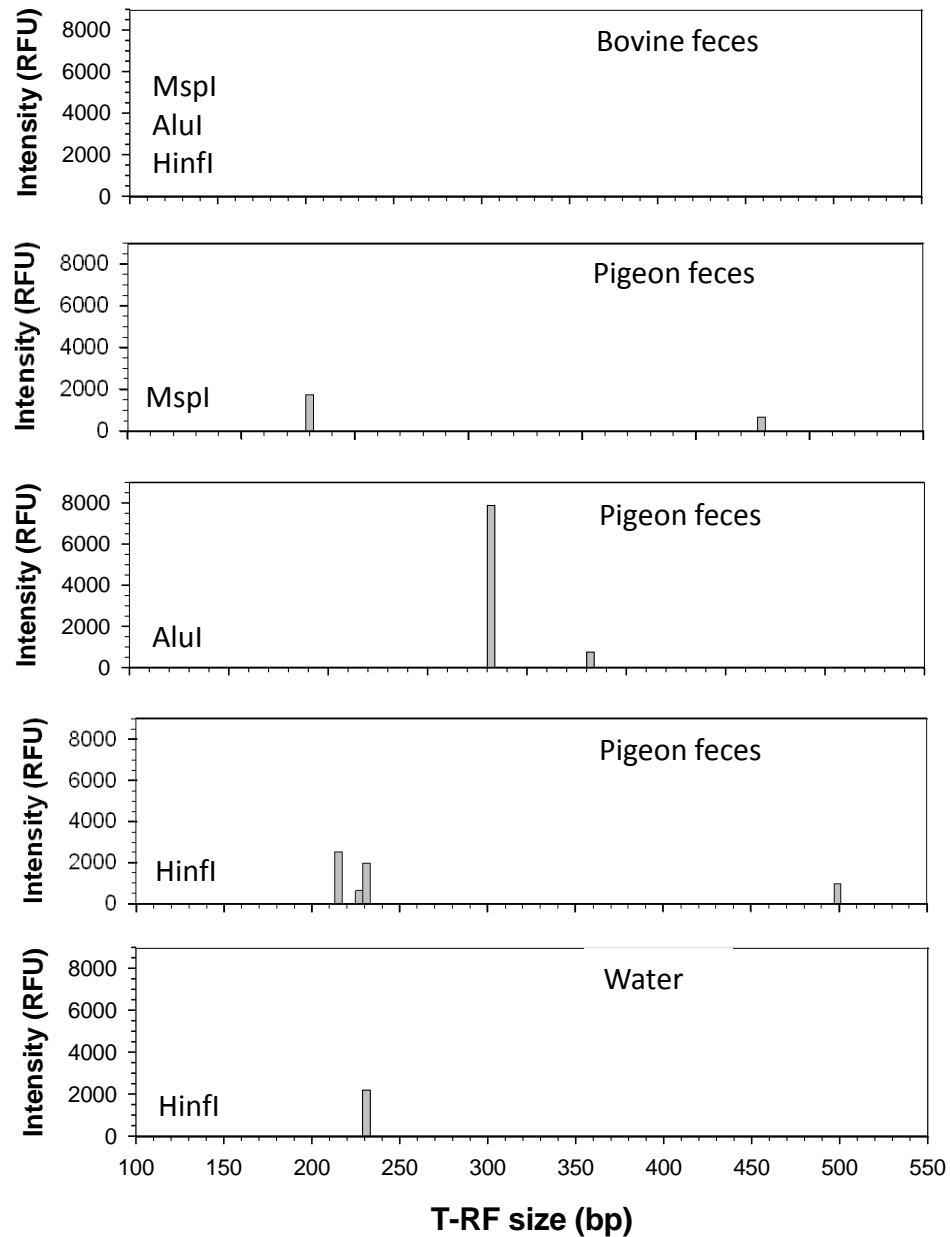
# Electropherograms

*E. coli* from water sample  
digested with HinfI

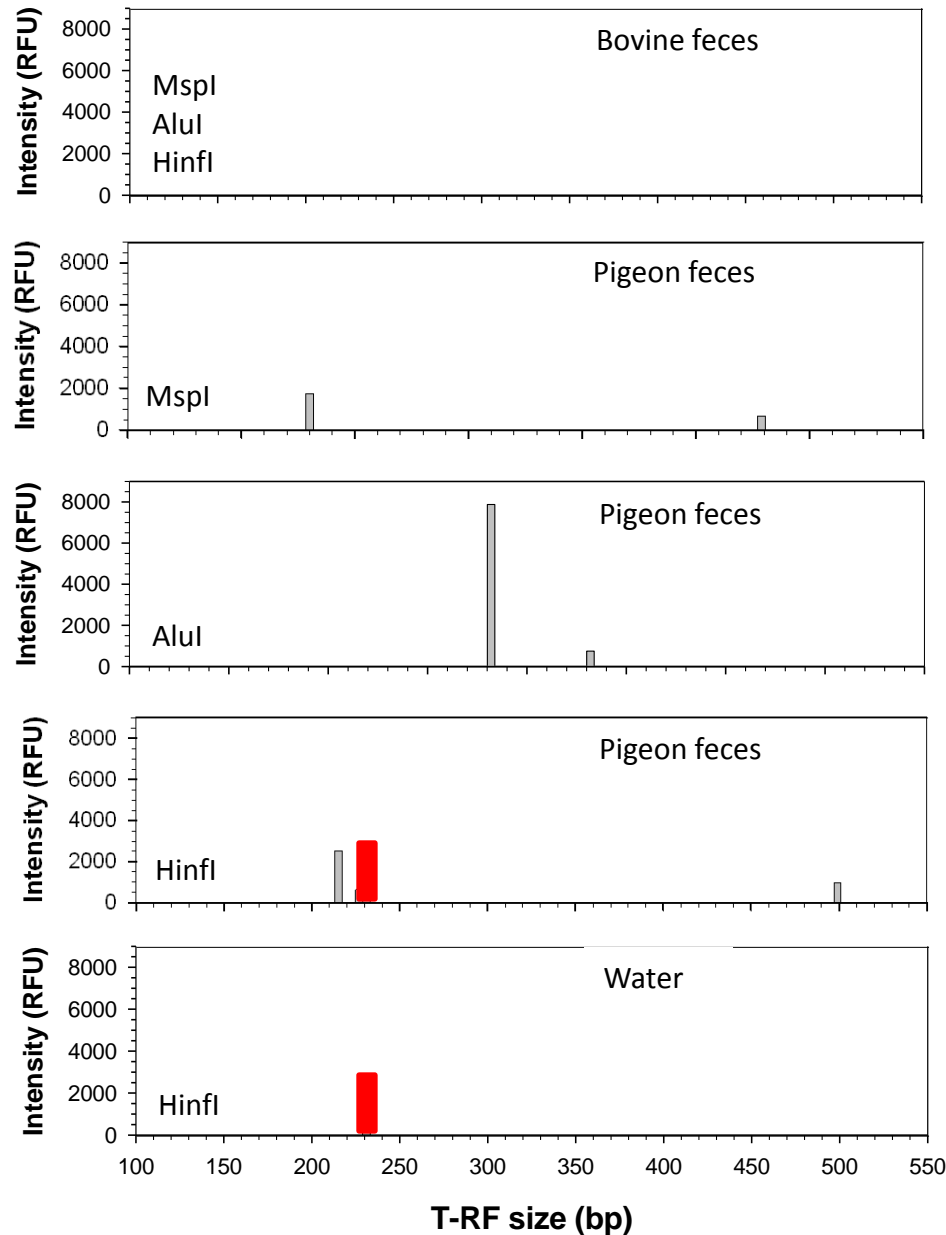


*E. coli* from pigeon feces  
digested with HinfI





**Fig. 1. Unique Terminal Restriction fragments for sample source and digest combinations. No unique T-RFs arose from bovine source material.**



- 231 bp T-RF Produced by HinfI restriction
- Found in H<sub>2</sub>O and pigeon feces
- Not bovine feces
- Indicative of pigeon sourced *E. coli*

**Fig. 1. Unique Terminal Restriction fragments for sample source and digest combinations. No unique T-RFs arose from bovine source material.**

# Future Directions

- Reproduce results
- Develop new primer set
- Size standard > 500 bp
- Include more host species
  - Such as geese, ducks, dogs, humans, rats, groundhogs, rabbits, moles, and other bird species
- Clone and sequence unique strains
  - Pigeon-specific *E. coli* strains have not been published

# Questions?



# Literature Cited

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- Hier, M.B. 2007. The role of vegetation in pollution treatment by a Connecticut stormwater wetland. M.S. thesis. University of Connecticut, Storrs, CT.
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