PCR

- Polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase enzyme which is used to amplify a piece of DNA by *in vitro* enzymatic replication.

- As PCR progresses, the DNA thus generated is itself used as template for next cycle. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

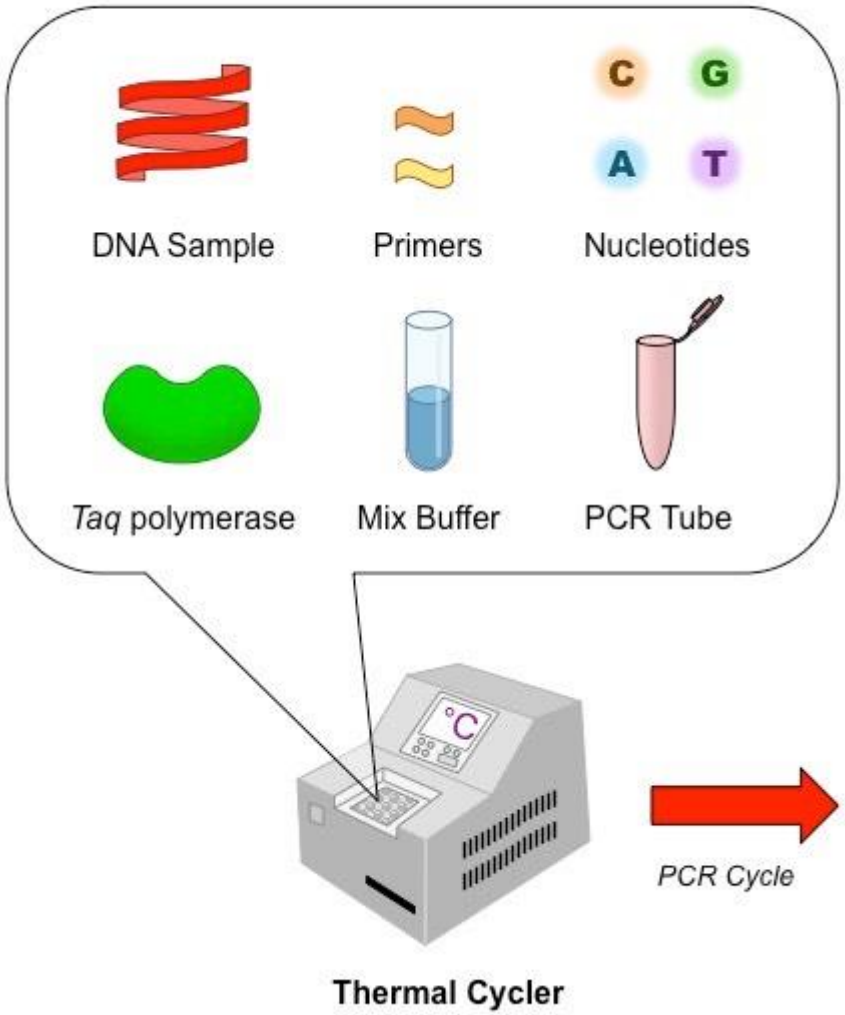
- Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, using single-stranded DNA as template and DNA oligonucleotides (also called DNA primers) for initiation of DNA synthesis.

- The PCR method uses thermal cycling, i.e., alternately heating and cooling of PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the strands (at high temperatures) in a DNA double helix (DNA melting) used as template during DNA synthesis (at lower temperatures) by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

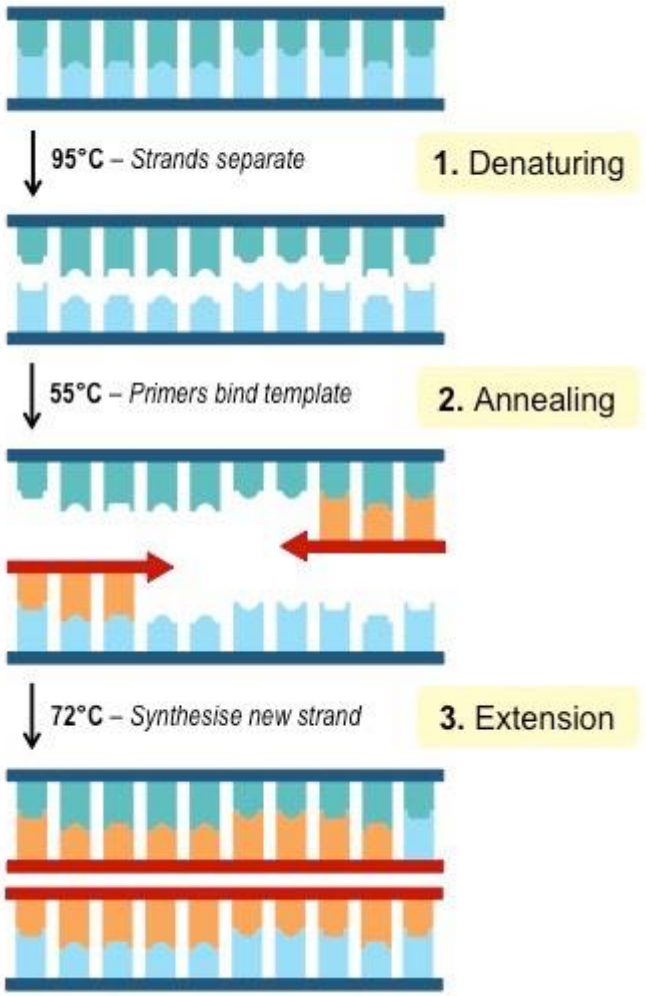
History

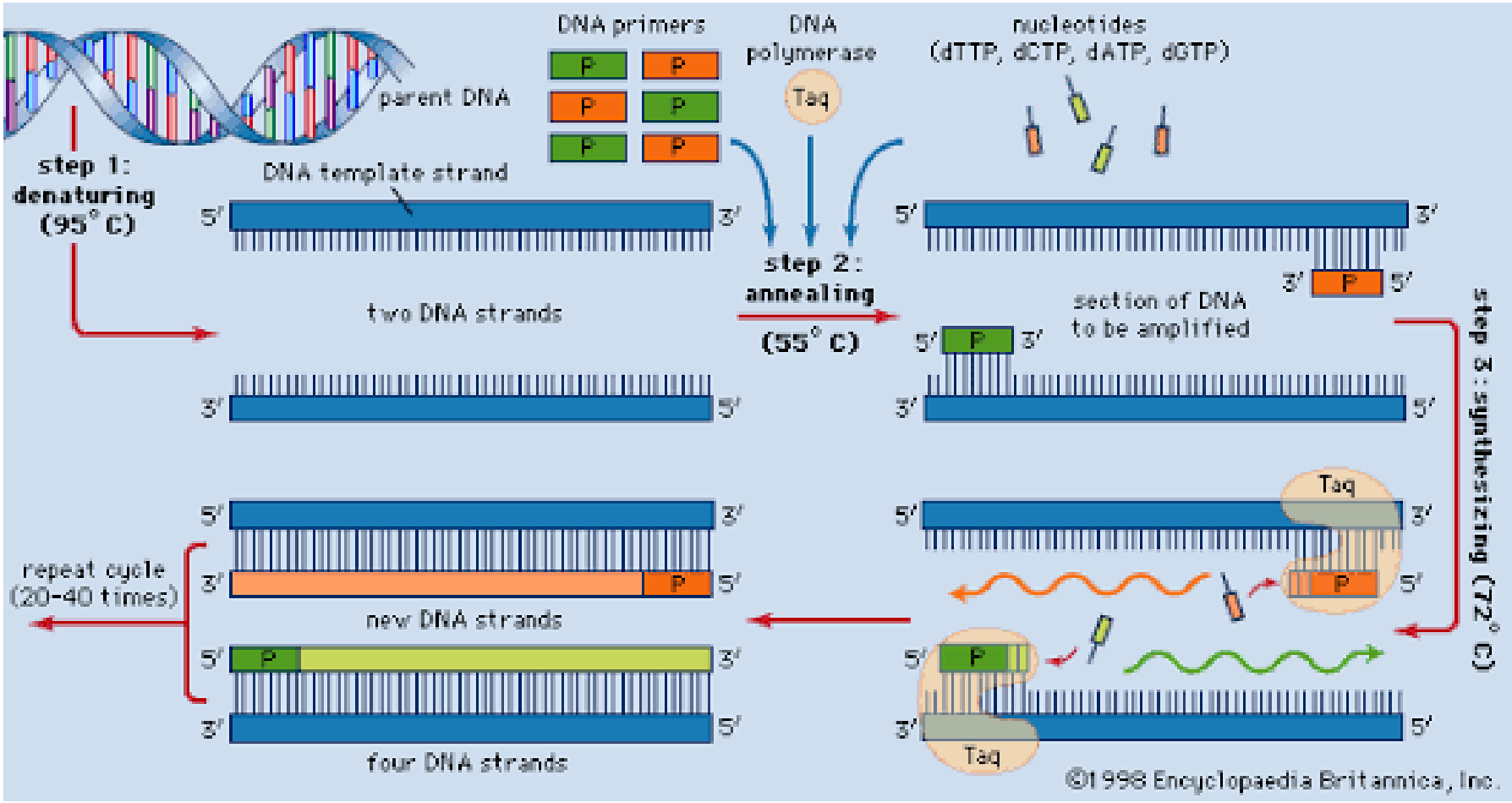
- The polymerase chain reaction was devised by Kary B. Mullis. Mullis was born in North Carolina and grew up in Columbia, S. Carolina. He received Ph.D. from the University of California at Berkeley.
- While working for Cetus Corporation, he invented PCR, which immediately spread to laboratories around the world where DNA chemistry was performed. PCR technology has grown into a several billion dollar industry. For his work, Mullis received the Japan Prize and the Nobel Prize for chemistry, both in year 1993.

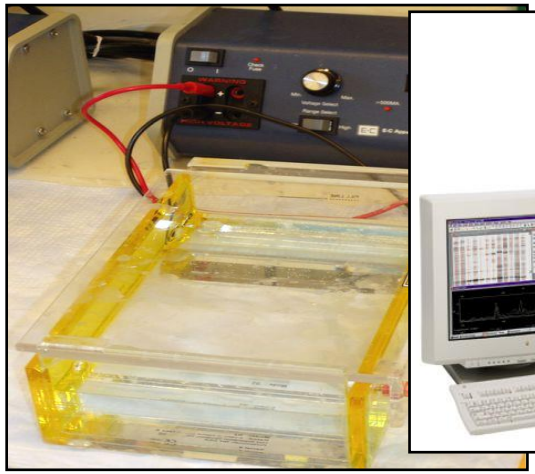
PCR Components

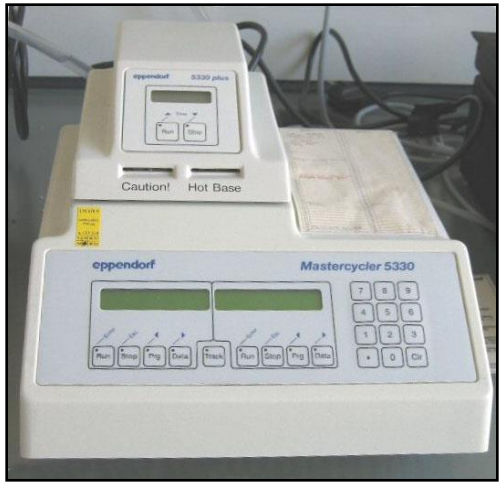


PCR Process (ONE Cycle)



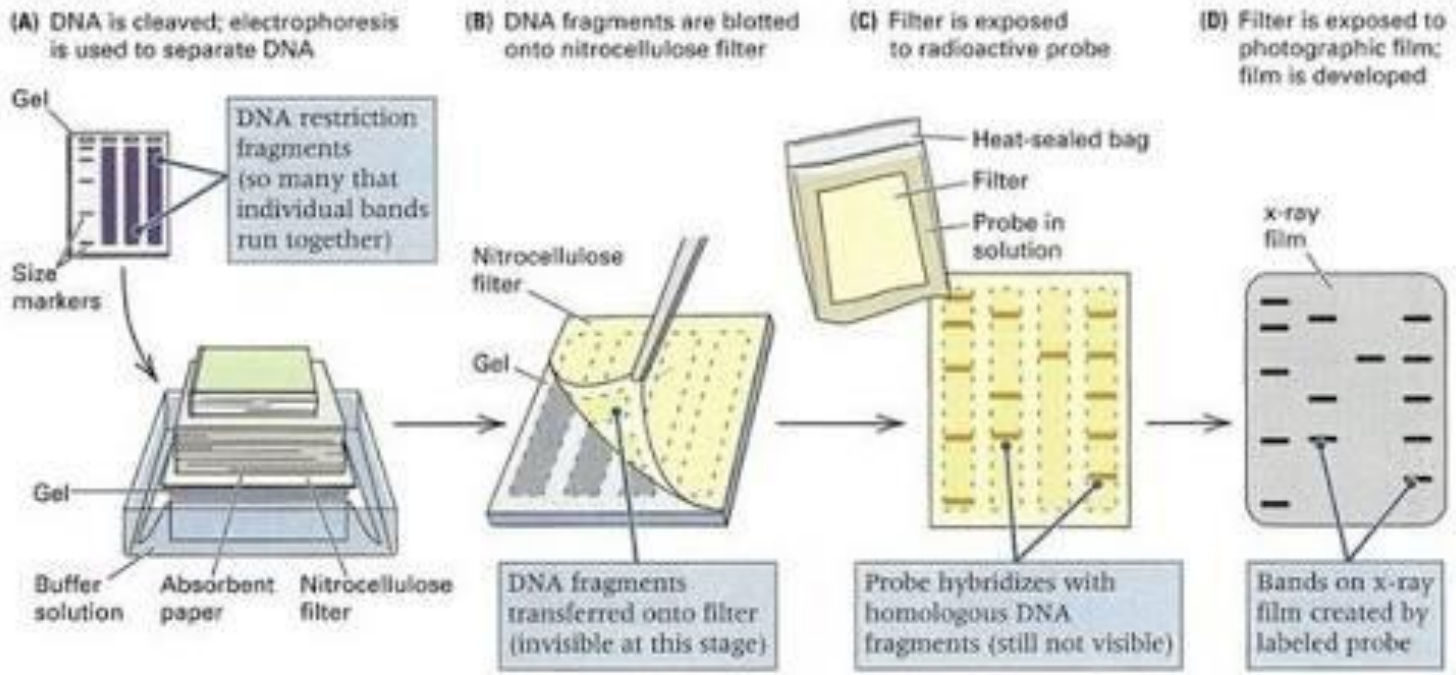

DNA primers
DNA polymerase
Taq
nucleotides
(dTTP, dCTP, dATP, dGTP)
parent DNA
step 1:
denaturing
(95° C)
DNA template strand
5′
3′
P
two DNA strands
step 2:
annealing
(55° C)
section of DNA
to be amplified
step 3: synthesizing (72° C)
repeat cycle
(20-40 times)
new DNA strands
four DNA strands
©1998 Encyclopaedia Britannica, Inc.




eppendorf
5330 plus
Caution!
Hot Base
eppendorf
Mastercycler 5330

Standard Southern Blotting

Southern blot



Procedure

DNA extraction

↓

DNA digestion

↓

Agarose electrophoresis

↓

Gel and membrane preparation

↓

Transfer

↓

Prehybridization

↓

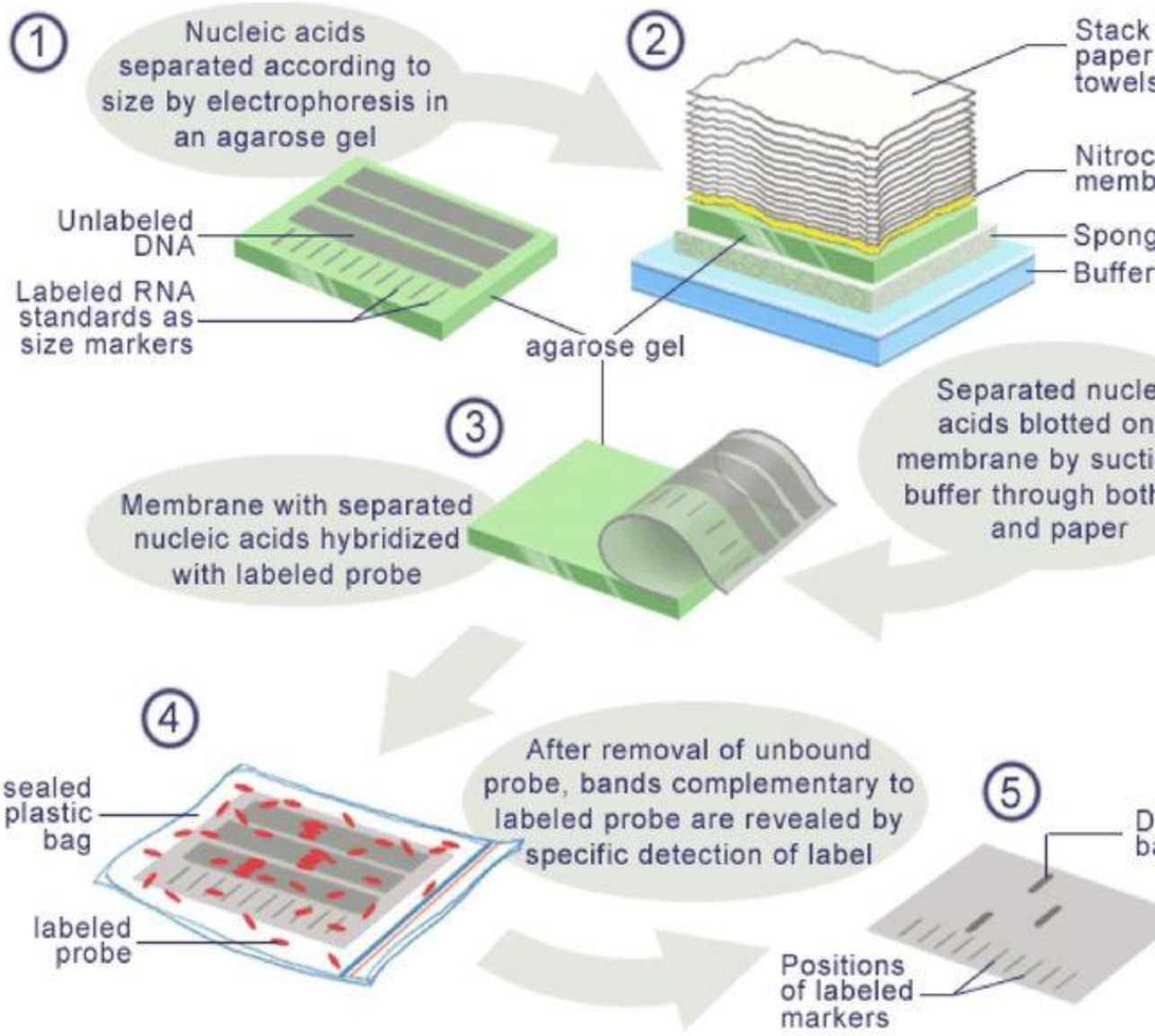
Probe preparation and hybridization

↓

Wash

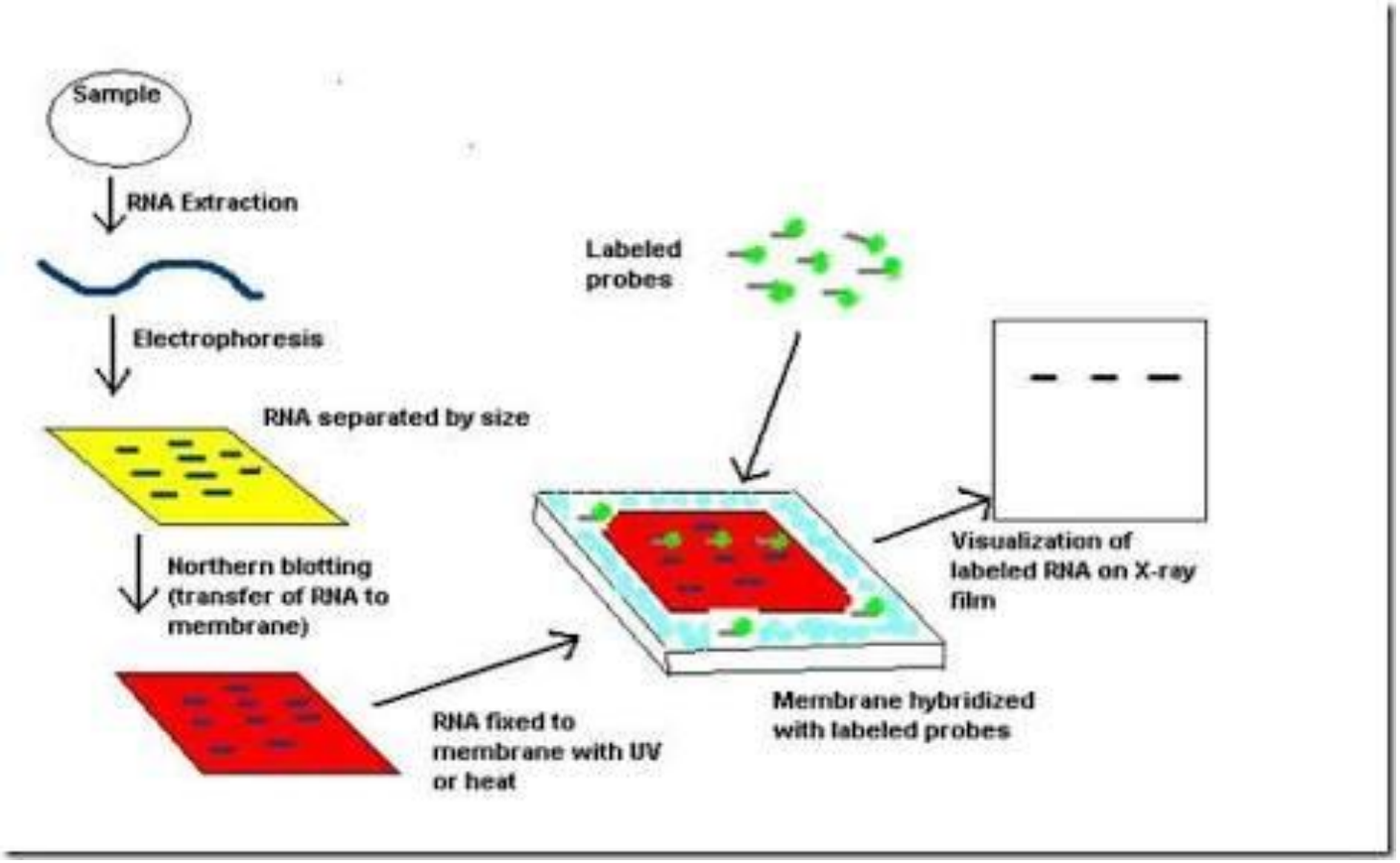
↓

Detection

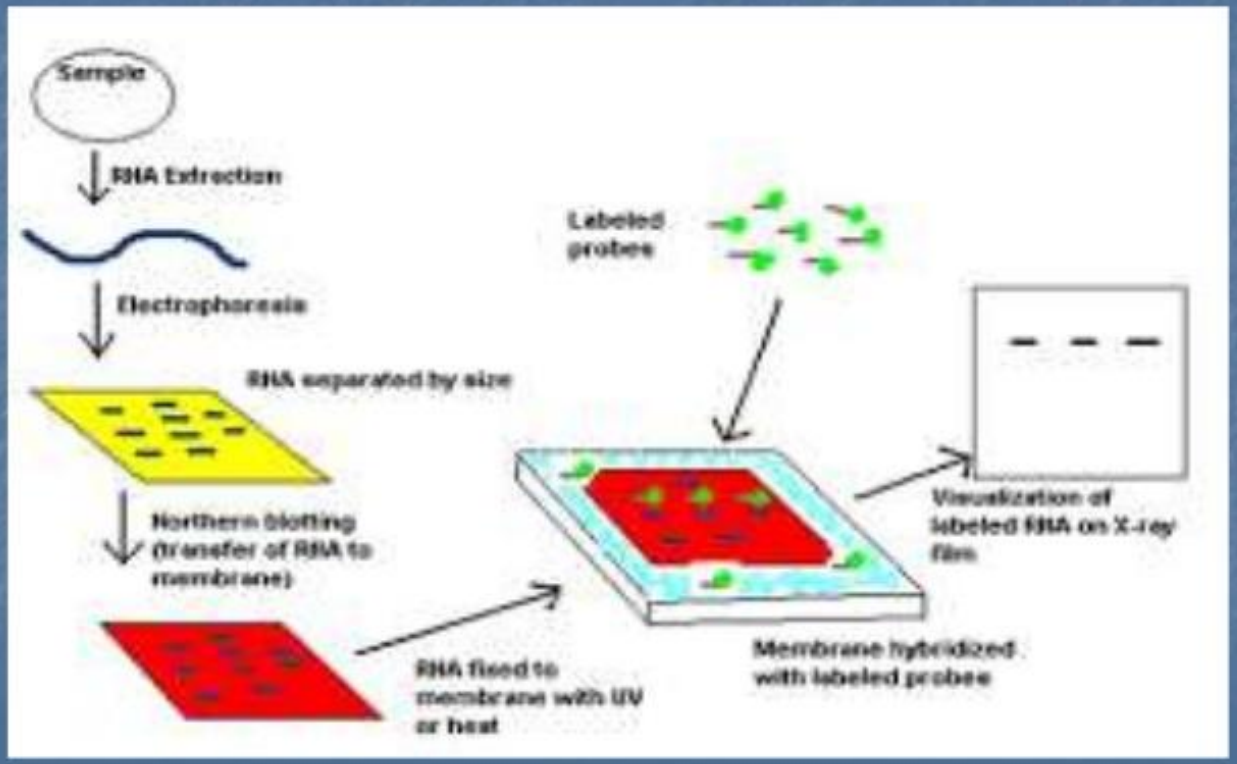


http://www.accessexcellence.org/AB/GG/southblots.html

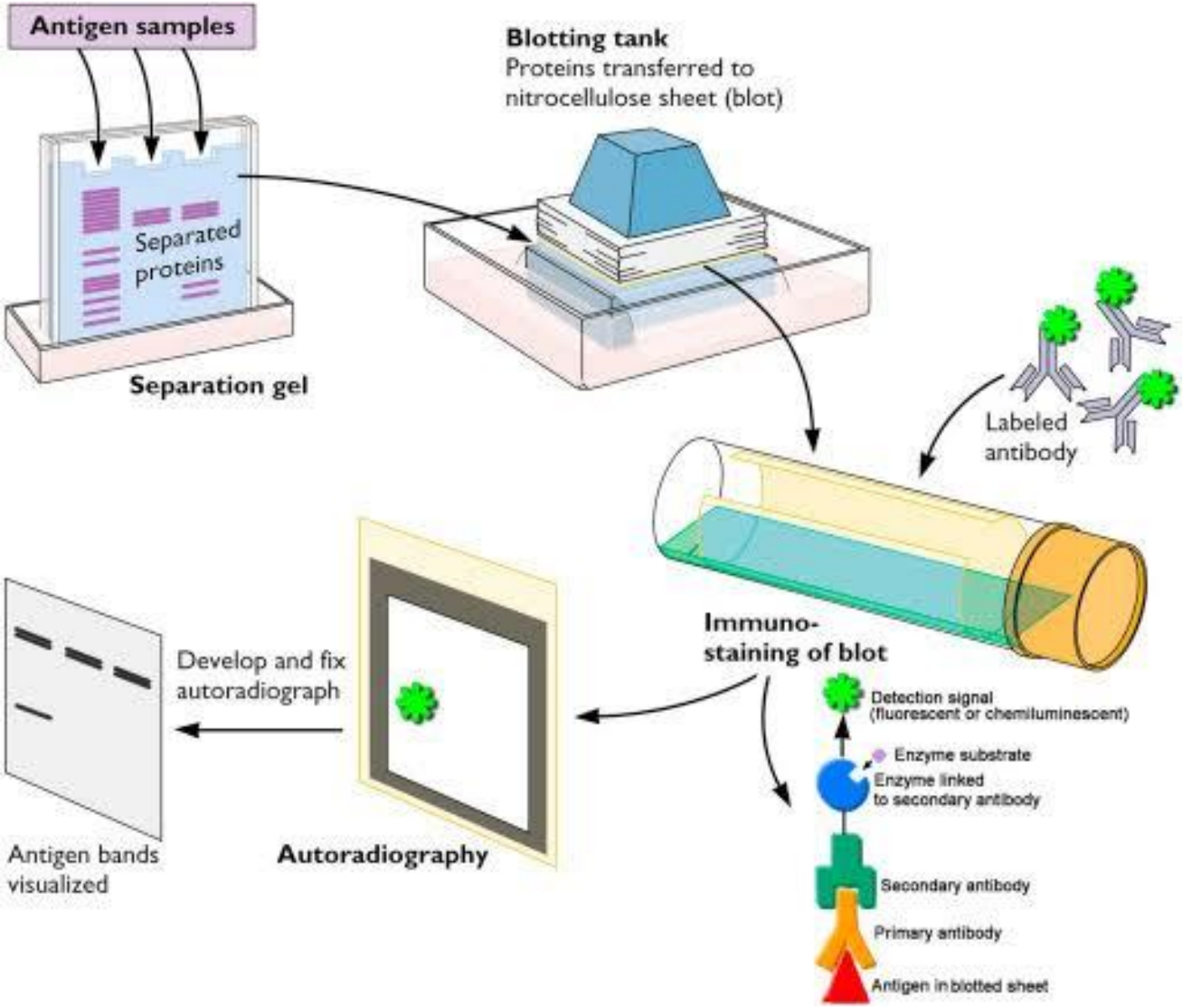
Standard Northern Blotting

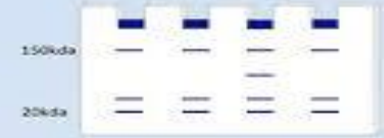

Sample
RNA Extraction
Electrophoresis
RNA separated by size
Northern blotting
(transfer of RNA to
membrane)
RNA fixed to
membrane with UV
or heat
Labeled
probes
Membrane hybridized
with labeled probes
Visualization of
labeled RNA on X-ray
film

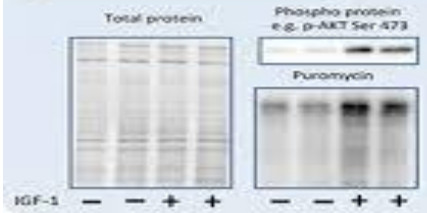
Steps in Northern blotting



WESTERN BLOT


Antigen samples
Separated proteins
Separation gel
Blotting tank
Proteins transferred to nitrocellulose sheet (blot)
Labeled antibody
Immuno-staining of blot
Detection signal (fluorescent or chemiluminescent)
Enzyme substrate
Enzyme linked to secondary antibody
Secondary antibody
Primary antibody
Antigen in blotted sheet
Develop and fix autoradiograph
Autoradiography
Antigen bands visualized





Sample preparation

- Does the buffer require additional salts, detergents or enzymatic inhibitors?
- Subcellular fractionation
- Native or denatured proteins

Gel electrophoresis

- Agarose or polyacrylamide gels
- Gel concentration or gradient, greater resolution of larger/mid sized proteins

Membrane transfer

- Nitrocellulose or PVDF membrane
- Pore size, smaller pores may be required for smaller targets (<20 kda)
- Exclusion of buffer methanol for larger targets

Blocking

- Typically BSA or milk solutions
- Blocking in milk may react with phospho-specific antibodies
- High background may need variations in blocking time or concentration

Primary antibody incubation

- Validation of specificity, positive/negative controls may be required
- Antibody may bind multiple epitopes, protein size must be known
- Phospho-specific antibodies for signalling

Secondary antibody incubation

- Dependent on 1°Ab host species
- Detection method determines conjugation. i.e. chemiluminescent, fluorescent, colourimetric etc.
- Made up in previous blocking conditions

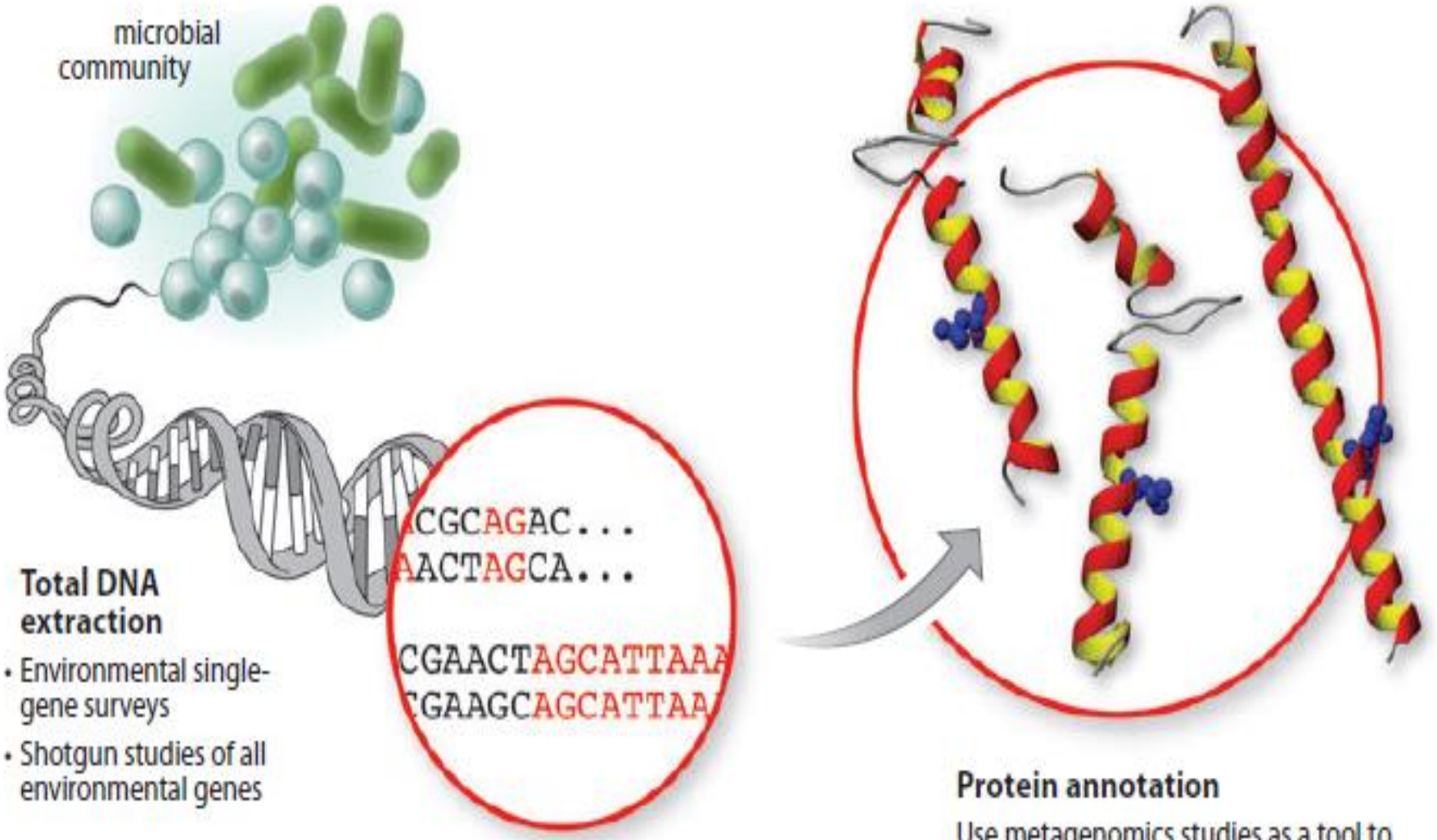
Detection

- Chemiluminescent detection requires incubation with substrate
- Detection within linear range required to prevent over saturation
- Other detection methods may be required for strong signals

Analysis

- Analyse either individual bands or lane profiles
- Bands should be normalised to total protein content

EASTERN BLOT



Total DNA extraction

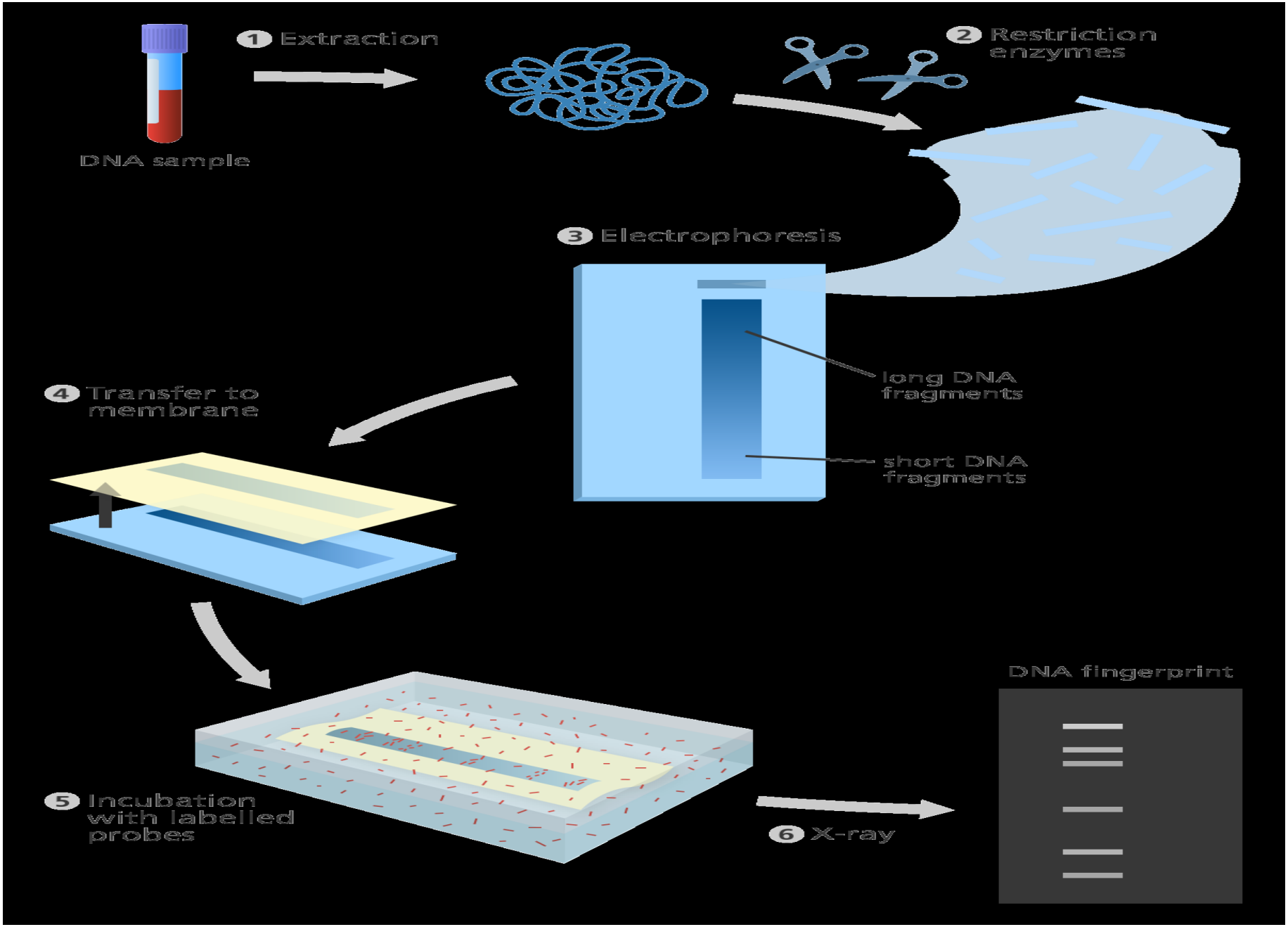
- Environmental single-gene surveys
- Shotgun studies of all environmental genes

DNA sequencing

- Identify common genes within a community
- Identify genome contents favored by current environmental conditions

Protein annotation

Use metagenomics studies as a tool to answer broader ecological or evolutionary questions


1 Extraction
DNA sample
2 Restriction enzymes
3 Electrophoresis
long DNA fragments
short DNA fragments
4 Transfer to membrane
5 Incubation with labelled probes
6 X-ray
DNA fingerprint

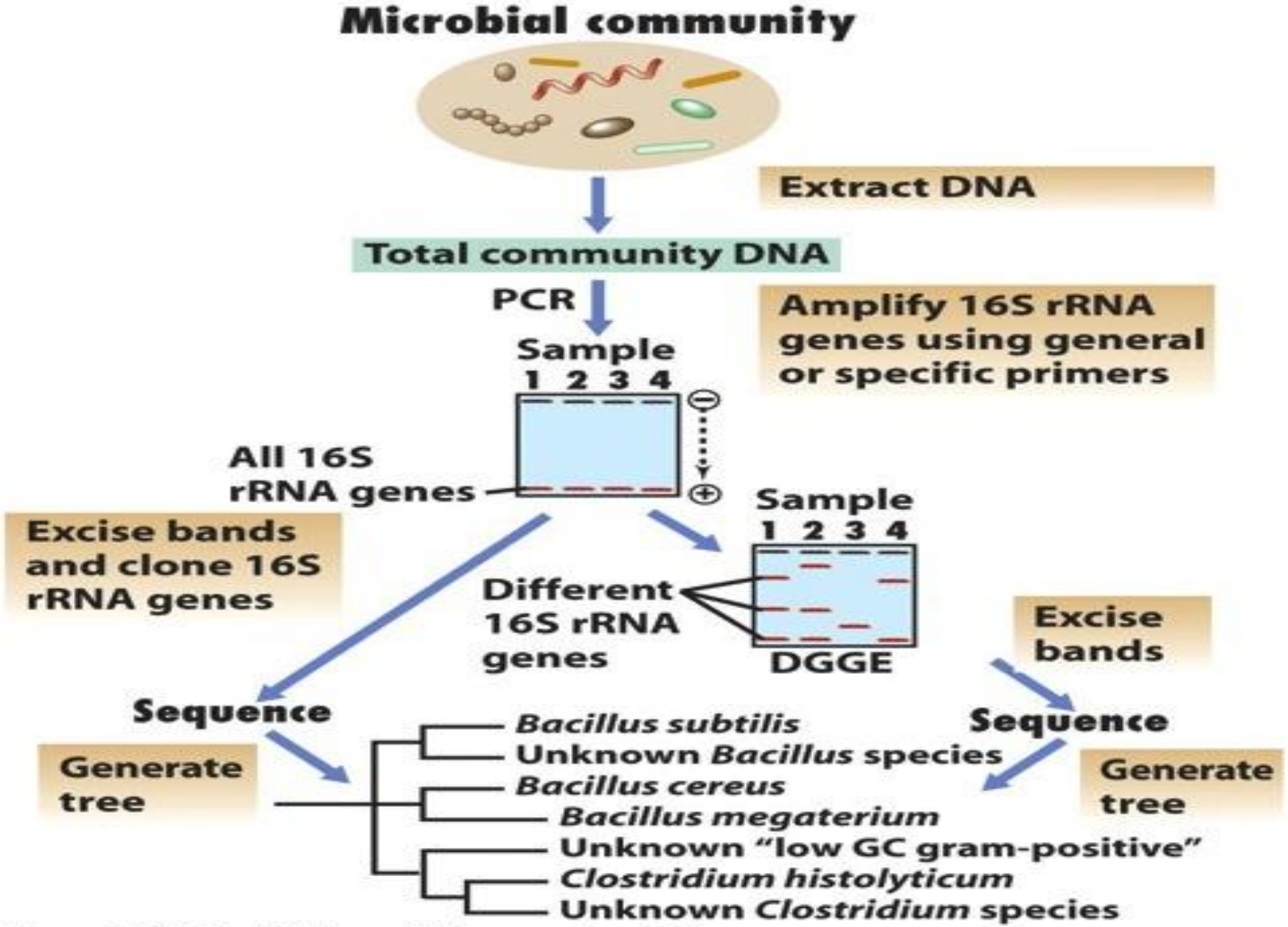
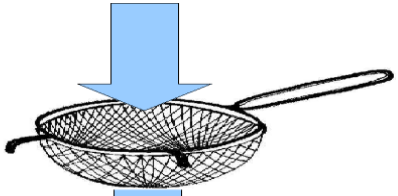
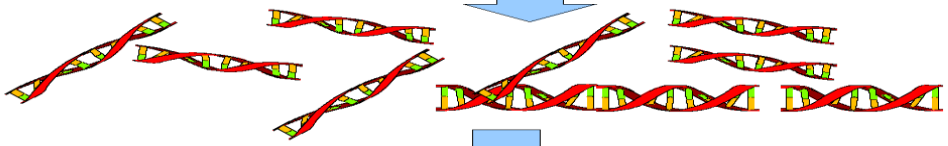


Figure 18-13 Brock Biology of Microorganisms 11/e

A



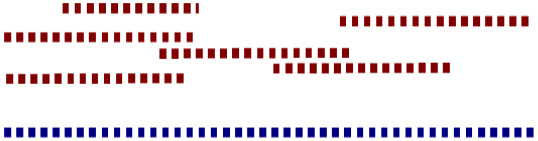
B

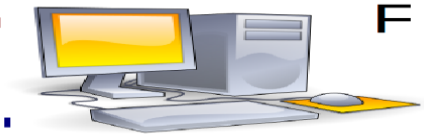


C

D

E

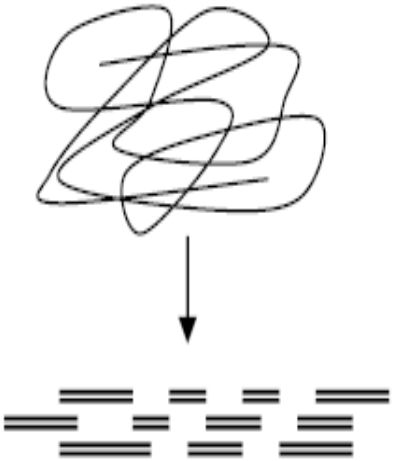




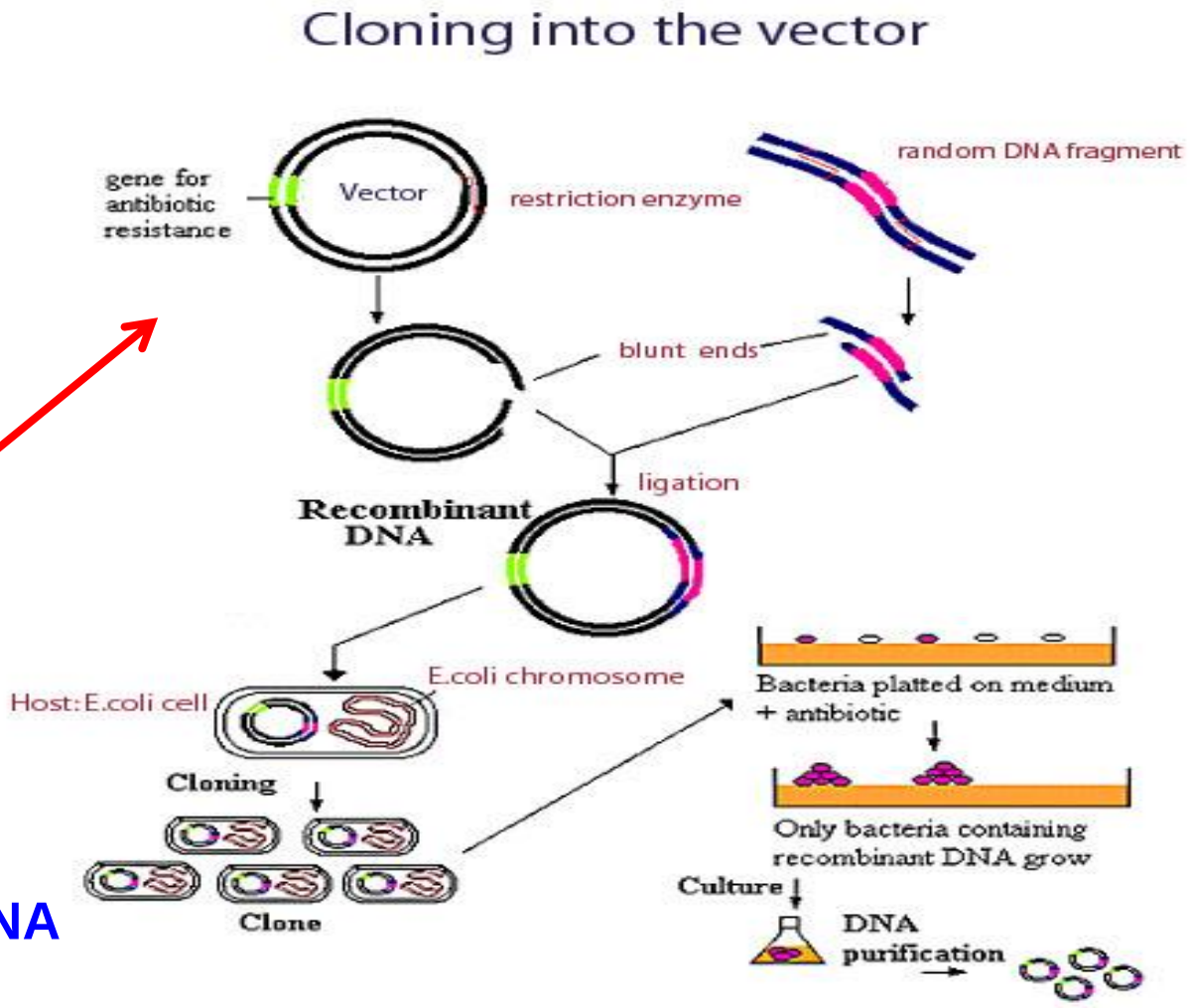
F

Library Preparation

DNA fragmentation

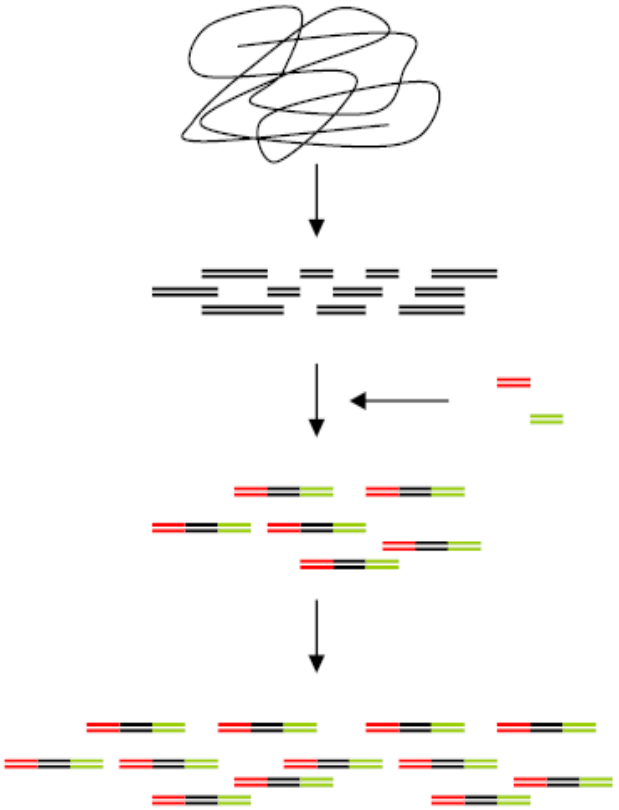


Random fragment DNA

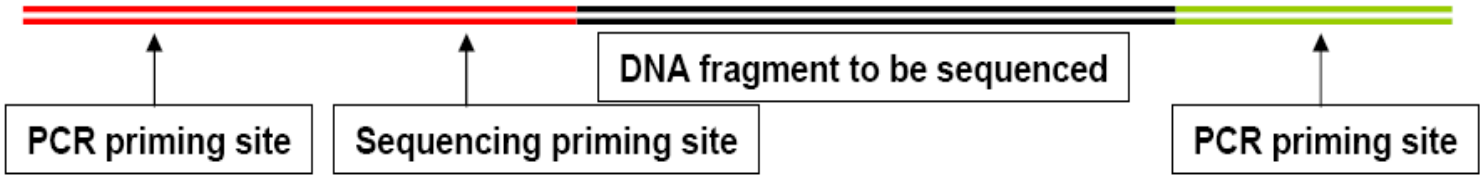


Library Preparation (in NGS)

- **DNA fragmentation using various methods**
  - Different platform requires different DNA size
  - 454: ~600 bp; Solexa: ~250 bp
- **Adaptor (ds) ligation**
  - no conventional cloning vector
- **Size selection**
- **Library amplification by PCR**
  - 454 does no need this
- **Quality assessment and quantification**



Final library fragment structure



Next Generation Sequencing

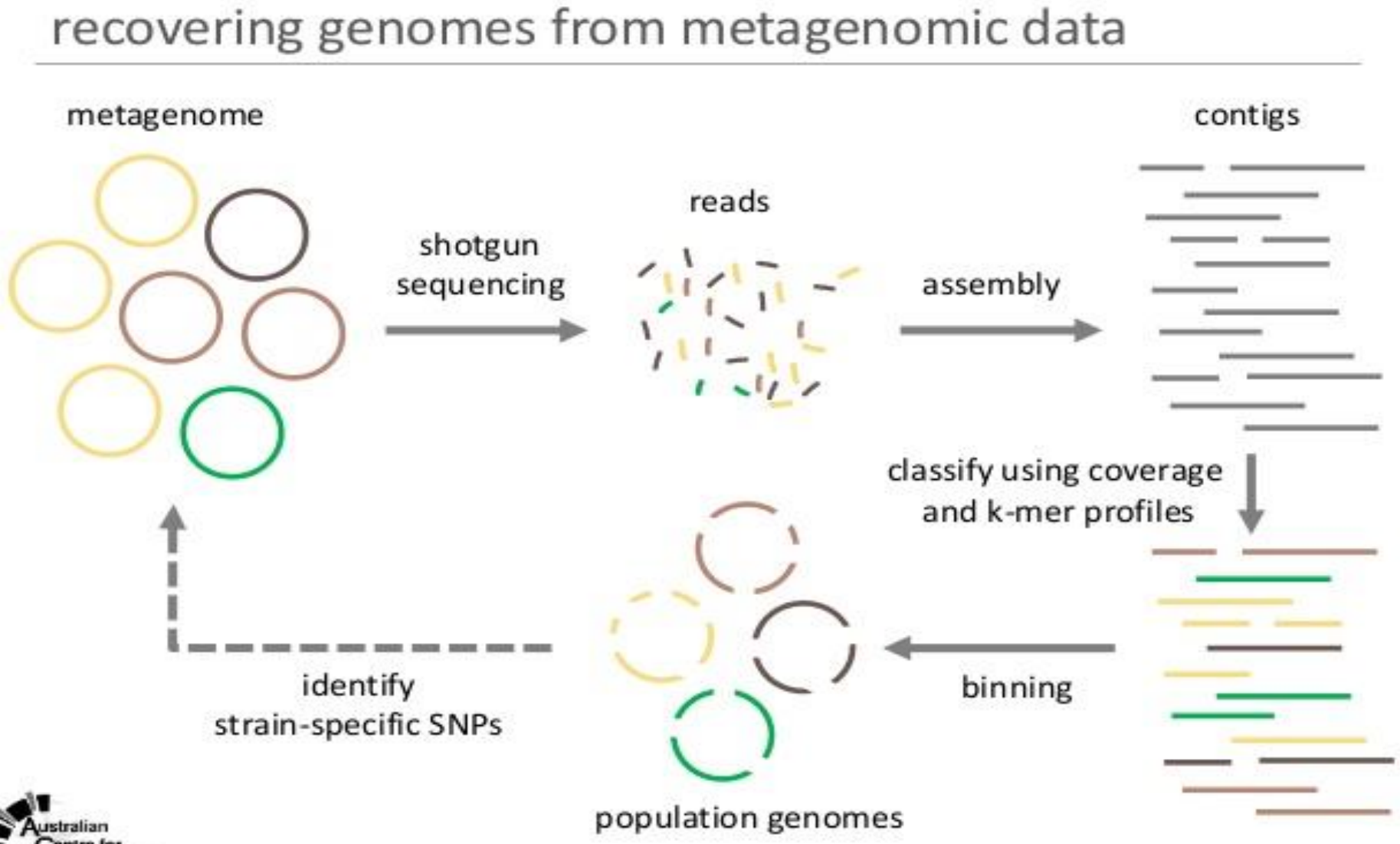
- Thousands of DNA fragments sequenced
- Automated
- All parts of the genome are sequenced multiple times
- Allows overlap to make alignment and assembly easier

Next-generation Sequencing Technologies

Sr. no.	Sequencing	Size of reads	Year
1	454 -Roche	400 bp	2005
2	Illumina /Solexa	400-700bp	2006
3	Applied Biosystem SOLiD	25-75 bp	2007
4	Ion Torrent	400 bp	2012

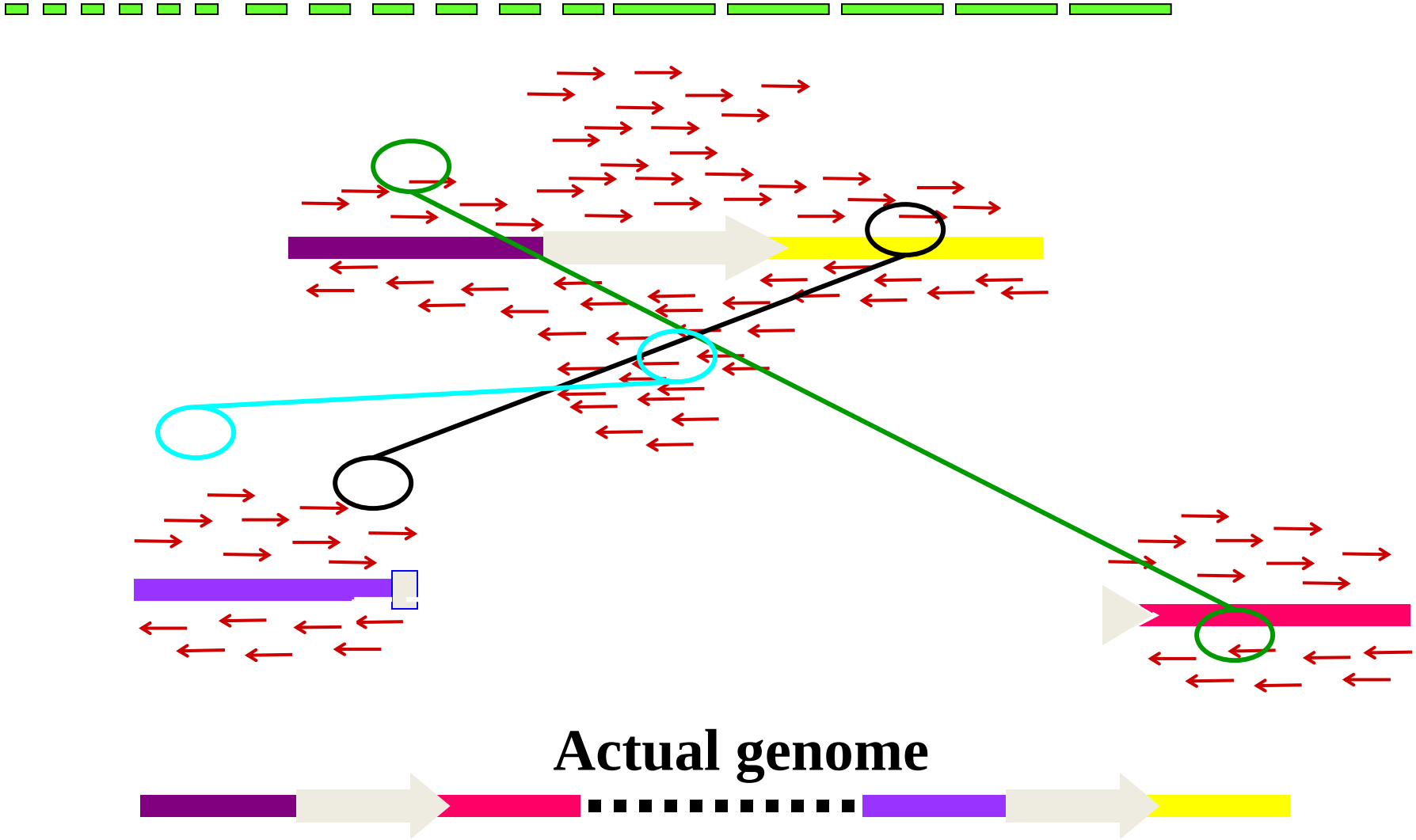
(Schuster, 2007)

Bioinformatics

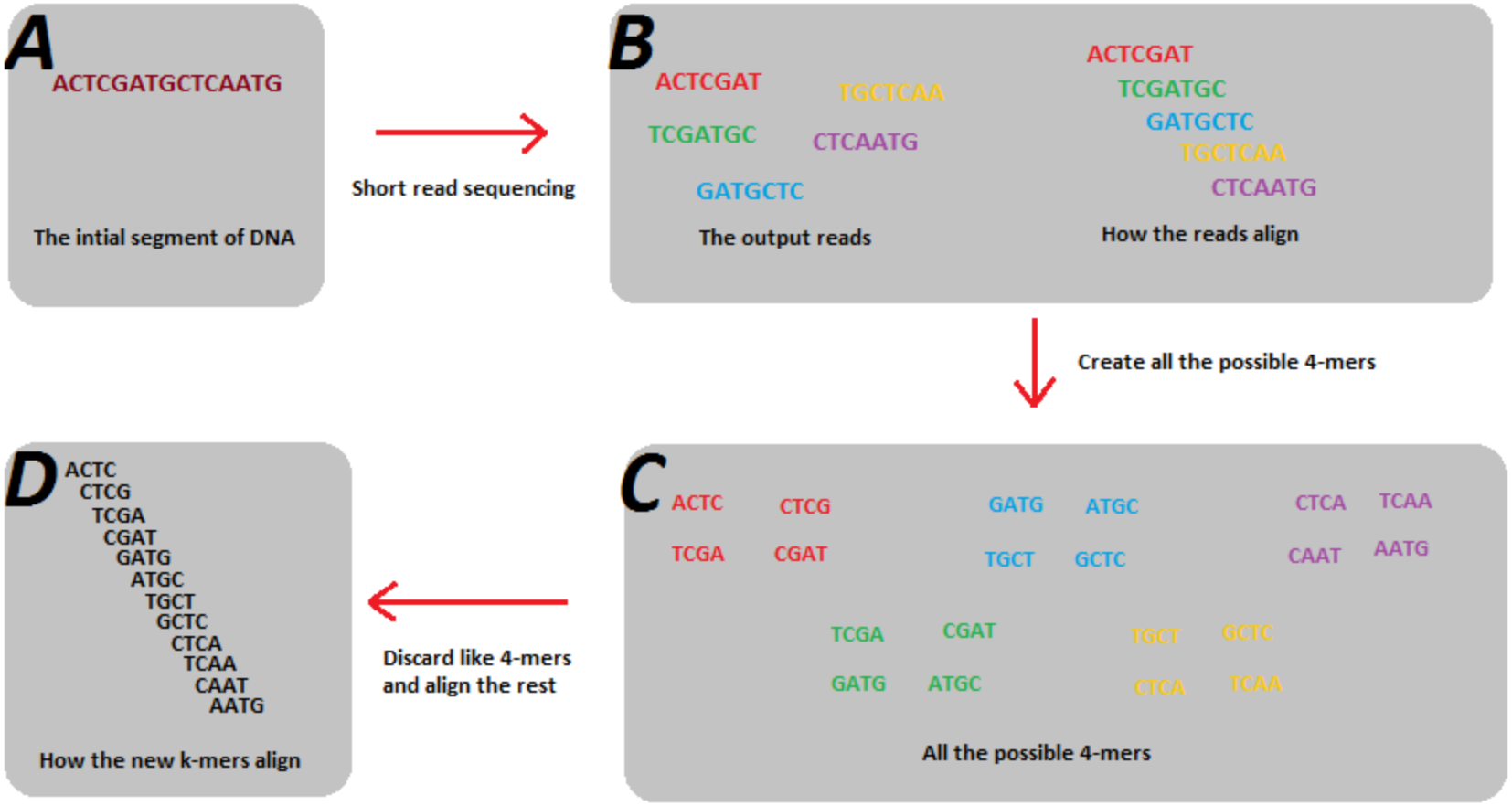


Assembly

Assembly: set of contigs



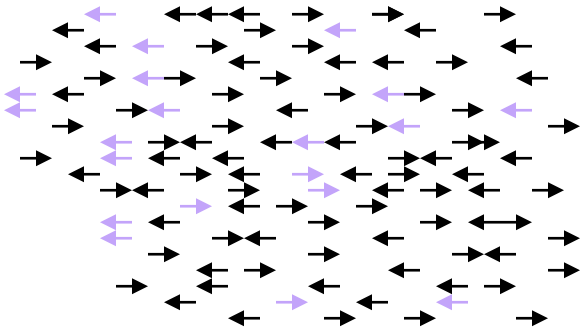
Actual genome


A
ACTCGATGCTCAATG
The intial segment of DNA
Short read sequencing
B
ACTCGAT
TGCTCAA
TCGATGC
CTCAATG
GATGCTC
The output reads
ACTCGAT
TCGATGC
GATGCTC
TGCTCAA
CTCAATG
How the reads align
Create all the possible 4-mers
C
ACTC
CTCG
GATG
ATGC
CTCA
TCAA
TCGA
CGAT
TGCT
GCTC
CAAT
AATG
TCGA
CGAT
TGCT
GCTC
GATG
ATGC
CTCA
TCAA
All the possible 4-mers
Discard like 4-mers
and align the rest
D
ACTC
CTCG
TCGA
CGAT
GATG
ATGC
TGCT
GCTC
CTCA
TCAA
CAAT
AATG
How the new k-mers align

Gene finding and annotation

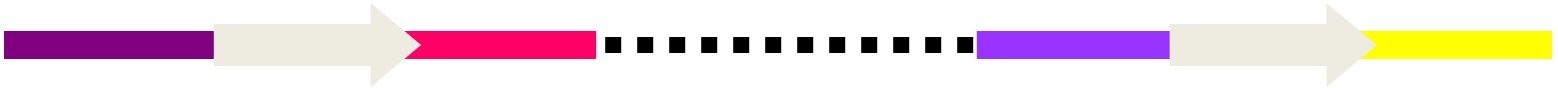
- **Identify Open Reading Frame (ORF) – putative gene**
- **ORFs compared to known genes in databases to identify function of gene.**
- **Identify the protein-coding regions.**

Metagenomic Finishing: Binning



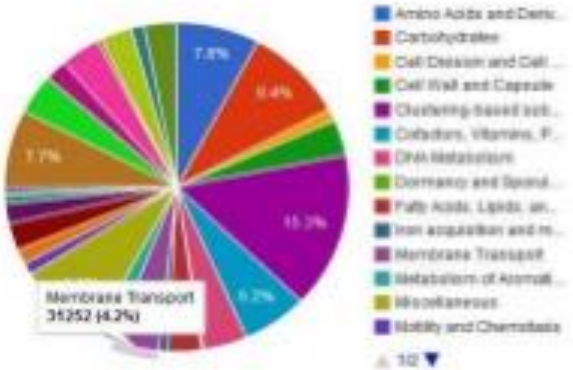
Binning: Which DNA fragment derived from which phylotype?

Complete genome



Assigning information

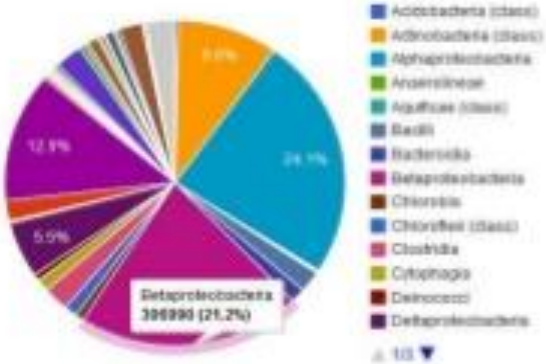
Function



Contigs

Databases

Binning



Taxonomy

Metagenomic Analysis

- **Function-driven screening for an expressed trait. Function-driven analysis seeks to identify clones expressing a desired trait or useful activity.**

- **Sequence-driven screening uses conserved DNA sequences to design PCR primers to screen clones for the sequence of interest**

Metagenomics

Genomic DNA extraction

Heterologous genomic DNA

Restriction-digested vector

Ligation

Vector DNA

E. coli DH10B

Transformation

Metagenomic library

Function-driven Analysis

Heterologous gene expression

Transcription

mRNA

Translation

Protein

Secretion

Sequence-driven Analysis

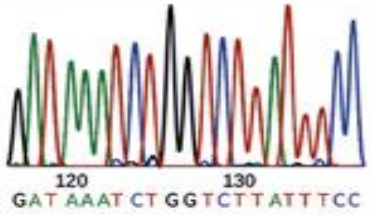
Cloned DNA preparation

ATGACAC ... GATTACA
TGGGCTCCCATCGCTAG

Genomic sequence analysis

Sequence-based screening

Shotgun sequencing of metagenomic DNA



Gene prediction

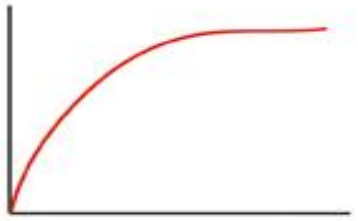
↓

Gene annotation

↓

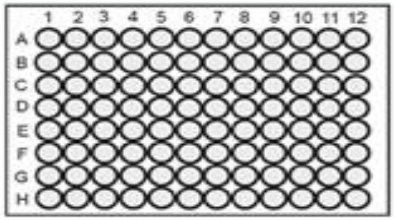
Primer design and amplification

Clonning, enzyme purification and characterization



Function-based screening

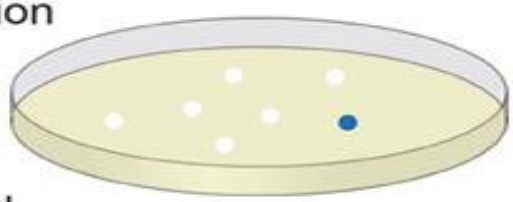
Construction of metagenomic library



↓

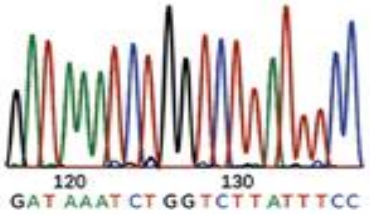
Activity detection

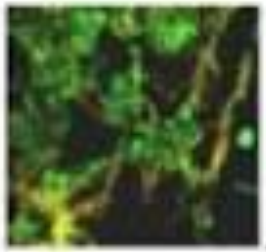
↓



Isolation and sequencing of positive clones

↓





DNA extraction

100++ Abundant species (≈3 Mbp each)

Sequencing

Reads

100-150 bp

Assembly

Contigs

1000+ bp

Search against database

Phylogenetic classification

Who is there?

Bacterium A
Bacterium B
...
Bacterium X

Functional classification

What can they do?

Gene A
Gene B
...
Gene X