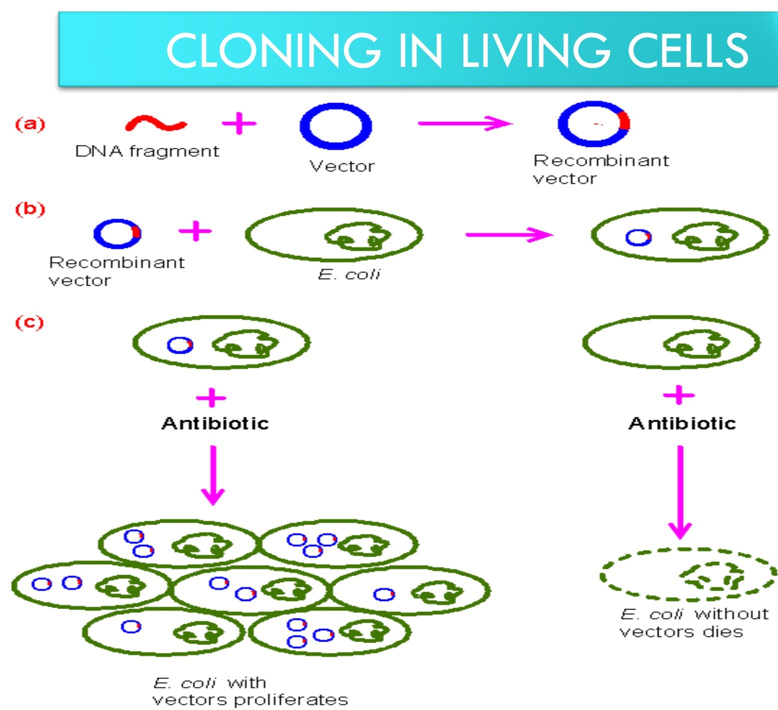
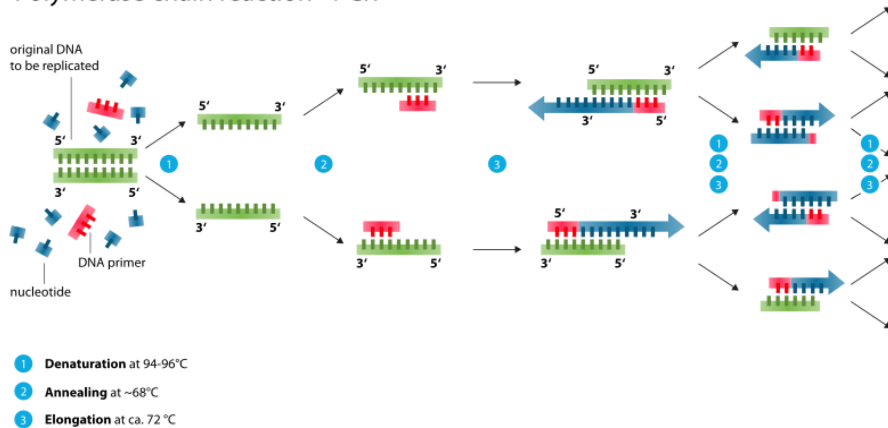
CLONE VERIFICATION

Sompid Samipak

CLONING IN LIVING CELLS



CLONING BY PCR



HOW TO VERIFY

Phenotype analysis

- Color products
- Antibiotic resistance

Presence of the DNA insert

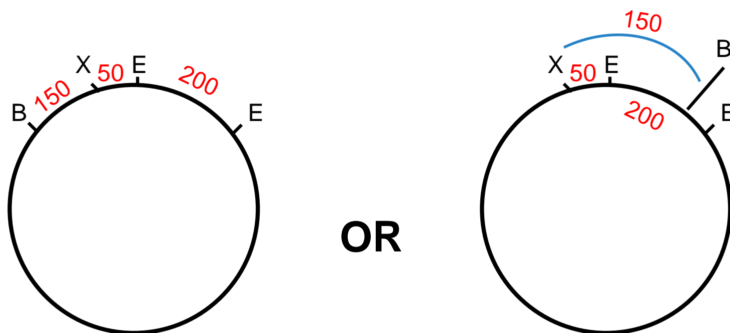
- Check the size of the insert
- For subcloning, need to find the direction of the DNA insert
  - Have to do restriction mapping to verify the insert direction

RESTRICTION MAPPING

When cut 1 kb plasmid with various enzymes, obtained the following fragments

EcoRI:	**200, 800 bp**
XbaI:	**1 kb**
BamHI:	**1 kb**
EcoRI+ XbaI:	**50, 200, 750 bp**
Xba I + BamHI:	**150, 850 bp**

Please draw the restriction map of this plasmid.



Another exercise

Plasmid pRIT450 is 7.0 kb in size and have PstI, EcoRI, and BamHI recognition sites. You have added a 4 kb insert at the PstI site. Then you cut with various enzymes and get the infor below:

Cut with PstI to get 7.0, 4.0 kb

Cut with EcoRI to get 6.0, 5.0 kb

Cut with BamHI to get 8.9, 2.1 kb

Cut with PstI + EcoRI to get 4.3, 3.3, 2.7, 0.7 kb

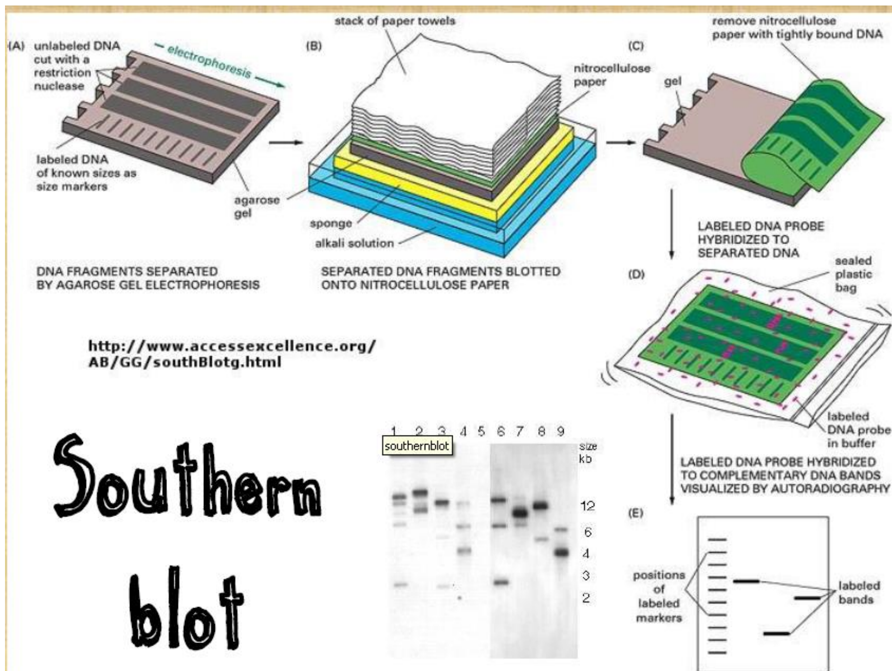
Cut with PstI + BamHI to get 6.1, 2.8, 1.2, 0.9 kb

Cut with EcoRI + BamHI to get 5.0, 2.1, 2.1, 1.8 kb

Please draw the restriction map of this recombinant plasmid.

TO FIND LENGTH OF YOUR INTERESTED GENE

- To locate the approximate length of YFG
- Cut genomic DNA with a restriction enzyme
- Separate on agarose gel electrophoresis
- Perform southern blotting to find which DNA band is your favorite gene.
- Make a replicate gel, cut the gel at the same position as the identified band
- Extract DNA from the cut gel, send for sequencing



SEQUENCING

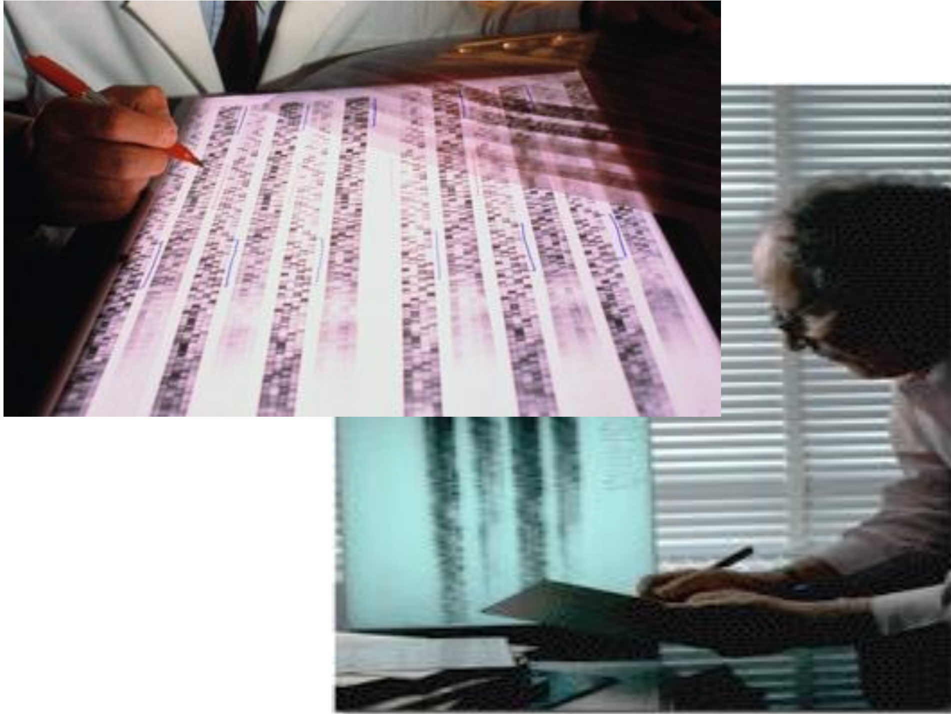
- Chemical sequencing
- Enzymatic sequencing
- Automatic sequencing and High throughput sequencing

TIMELINE

- The invention of sequencing reaction (Maxam and Gilbert, 1977; Sanger, 1977) (Nobel prize in 1980)
- The polymerase chain reaction (PCR) (Karry Mullis et al., 1986) (Nobel prize in 1993) Died August 7, 2019 (aged 74)
- Automated fluorescent DNA sequencer (Smith et al., 1985)

CHEMICAL SEQUENCING

- Developed by Maxam and Gilbert
- Need single stranded-labeled on one end DNA
- In separate tubes, cut by chemical that are specific to different bases to create different in lengths
- Separate by sizes on Polyacrylamide gel electrophoresis
- Check the bands on X-ray film (autoradiograph)



PRINCIPLE

- Label dsDNA, cut with RE and Denature DNA to make single strand
- Add chemicals to each tube to change specific base causing them to fall off
- Cut DNA at the positions that the changes occurred
- Order the DNA fragments by size on sequencing denatured polyacrylamide gel
- Read the band and specify bases from bottom to top of gel

MAXAM-GILBERT

- Dephosphorylate dsDNA at 5′ ends by alkaline phosphatase
- Rephosphorylate by kinase in a solution containing [^{32}P]ATP
- cut the band that want to find sequence out
- Separate on electrophoresis
- denature by heat or treat with NaOH
- Extract DNA and separate INTO 4 TUBES

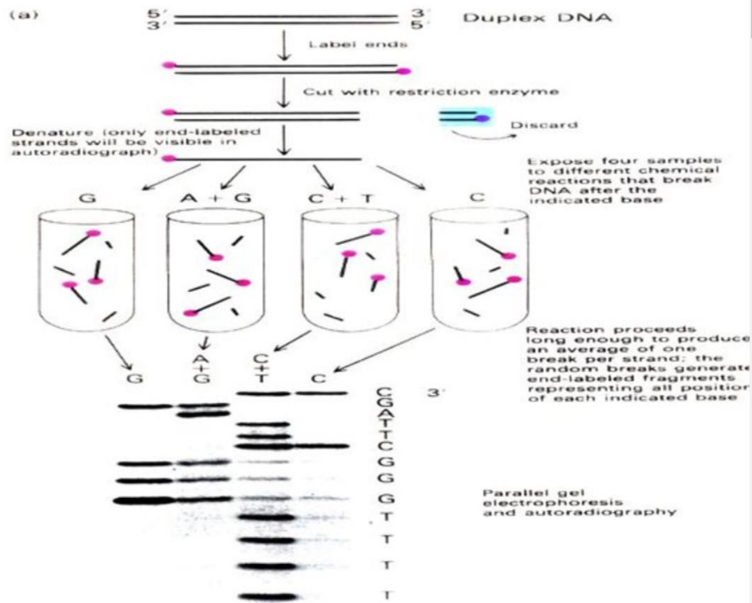
TO EACH TUBES

1. add dimethyl sulphate, attacks G
2. add formic acid to remove A and G
3. add Hydrazine to remove T and C
4. add Hydrazine and NaCl to react with C

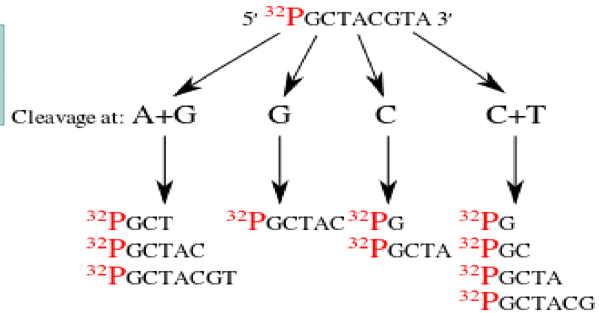
✓add **piperidine** to all tubes

✓the piperidine will cut the phosphodiester bond at the positions in which the bases have reacted and fell off

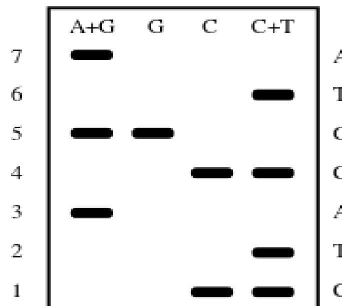
Maxam-Gilbert sequencing - summary



Maxam-Gilbert sequencing

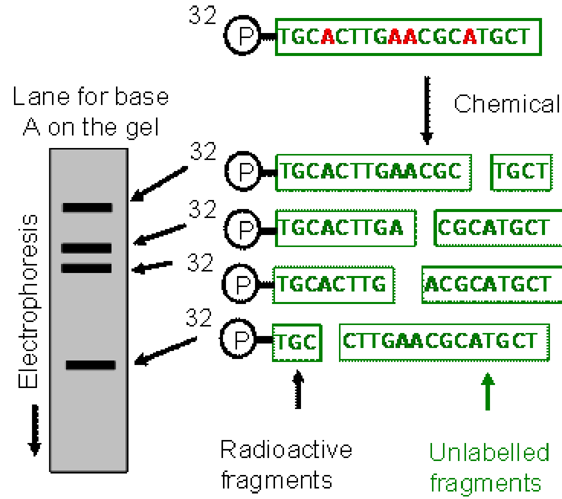


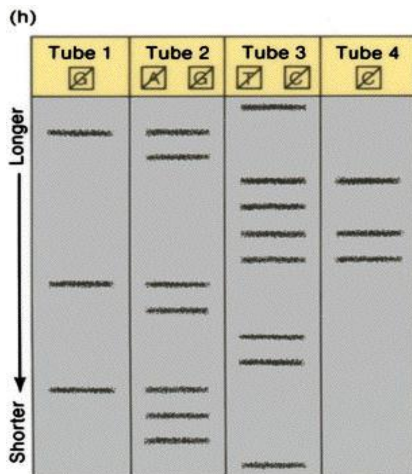
- cut in different tubes
- separate by sizes
- read base from bottom to top to get 5′ to 3′ end

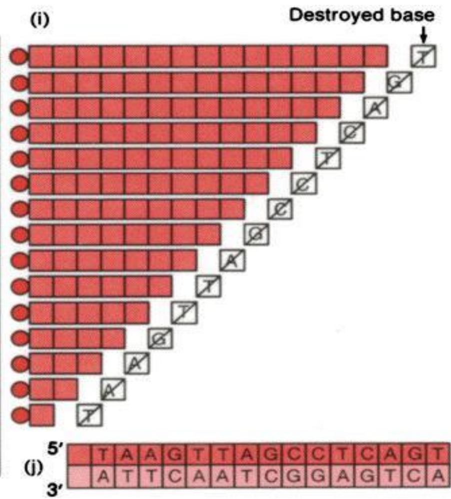


wikimedia.org

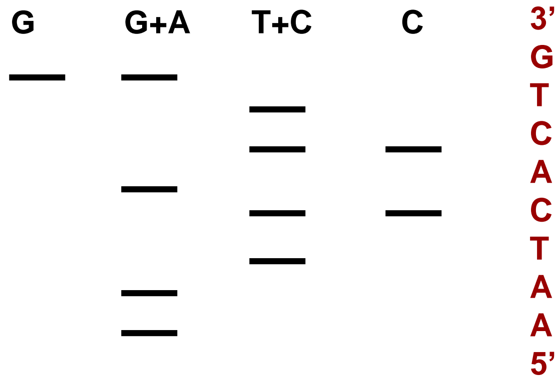
For a test tube that cut at base A







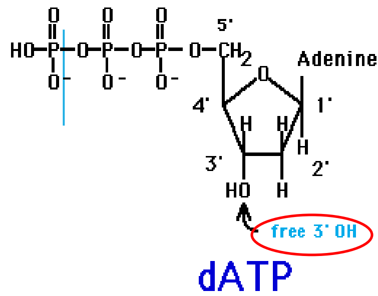
EXAMPLE 1

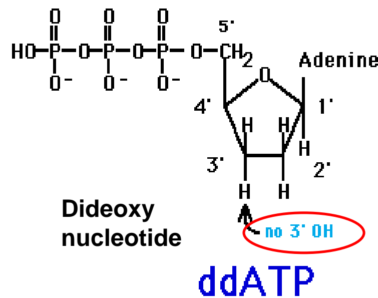


ENZYMATIC SEQUENCING

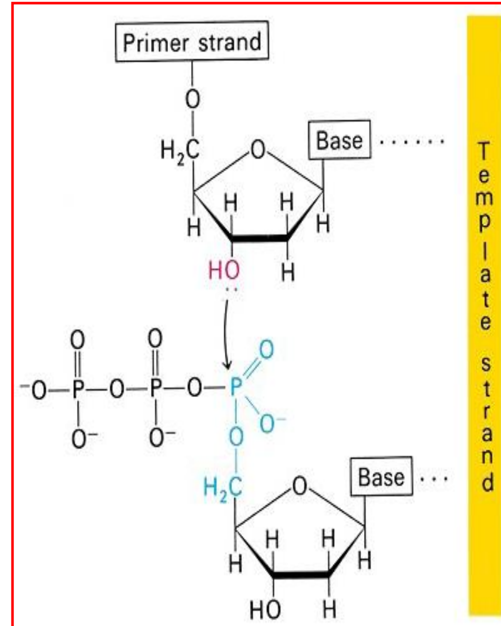
Also known as Dideoxy chain terminating method

- Invented by Alphred Sanger
- Rely on DNA replication using polymerase
- DNA synthesis that will stop once 2',3'-dideoxynucleotide triphospates (ddNTPs) is added to the 3' Hydroxyl group of the newly synthesized strand



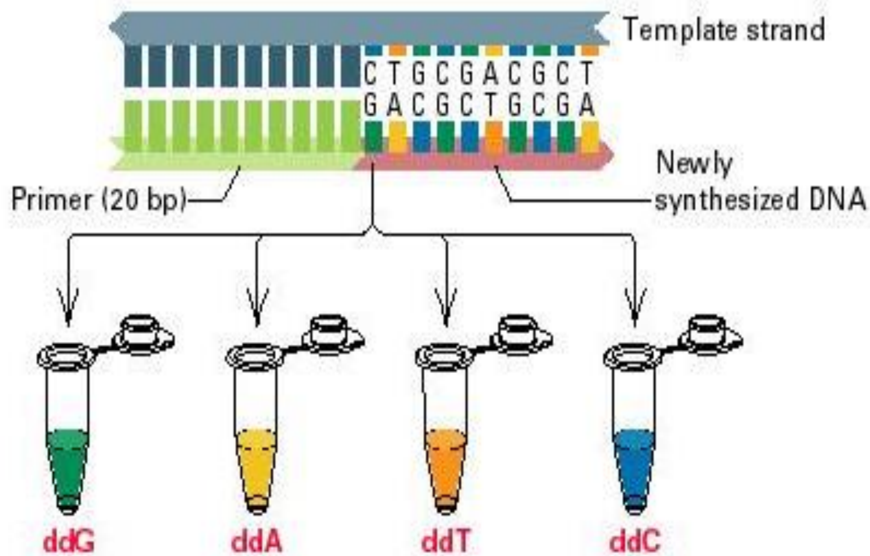


Polymerizing Reaction

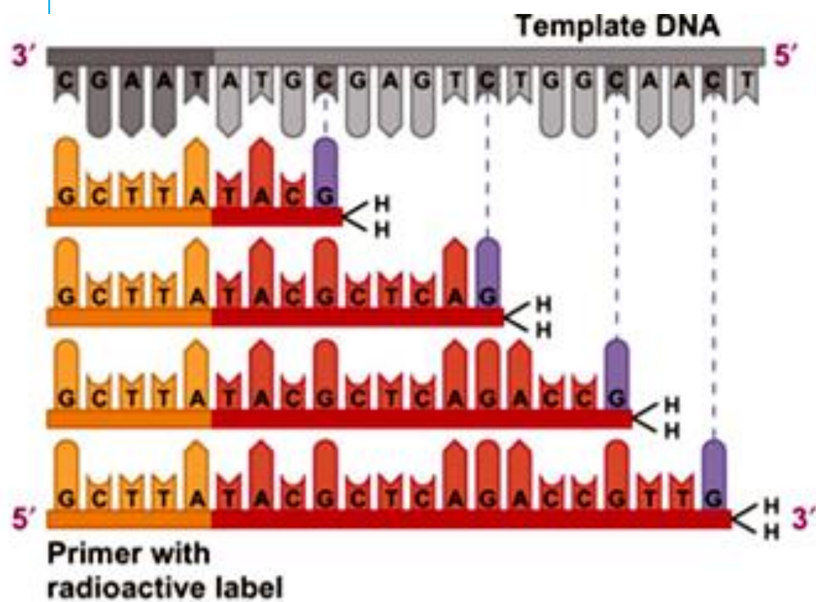


METHOD

- separate DNA into single strand by heat or NaOH
- Label the primer or the ddNTPs
- separate DNA into 4 tubes, add DNA polymerase, all dNTPs, primers, and only one of ddNTPs (to stop the synthesis after being added)
- Separate on polyacrylamide gel



SEQUENCING REACTION



Autoradiograph

Larger Fragment

Smaller Fragment

ddATP	ddCTP	ddTTP	ddGTP

Newly made: 3' A A C T A C G G T C T T 5'

template: 5' T T G A T G C C A G A A 3'

EXAMPLE

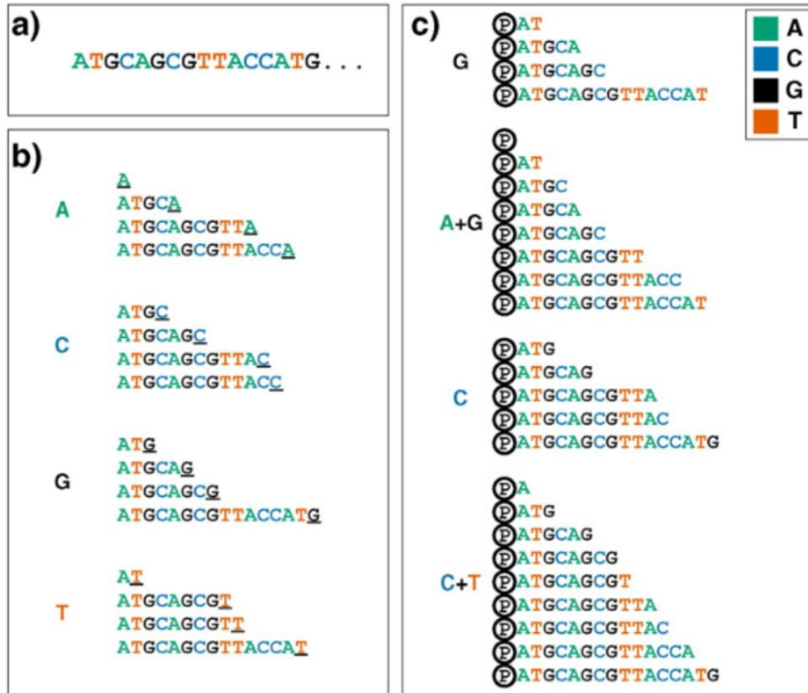
C
T
A
G

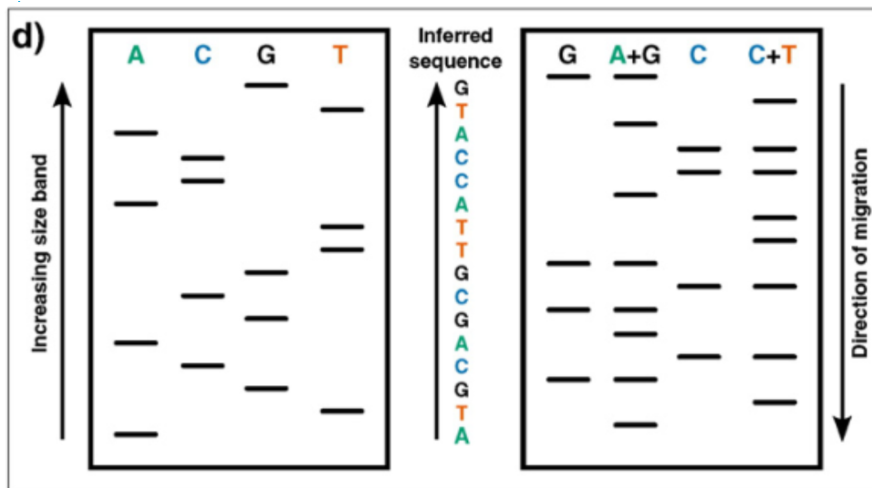
Smaller fragments
5′ end

Larger fragments
3′ end

5' GGAGATCAATTCCGTCAACGACAATCCTTTAATCGACGTATCCAGAG 3'

3' CCTCTAGTTAAGGCAGTTGCTGTTAGGAAATTAGCTGCATAGGTCTC 5'


a)
ATGCAGCGTTACCATG. . .
b)
A
A
ATGCA
ATGCAGCGTTA
ATGCAGCGTTACCA
C
ATGC
ATGCAGC
ATGCAGCGTTAC
ATGCAGCGTTACC
G
ATG
ATGCAG
ATGCAGCG
ATGCAGCGTTACCATG
T
AT
ATGCAGCGT
ATGCAGCGTT
ATGCAGCGTTACCAT
c)
G
ATGCA
ATGCAGC
ATGCAGCGTTACCAT
A+G
AT
ATGC
ATGCA
ATGCAGC
ATGCAGCGTT
ATGCAGCGTTACC
ATGCAGCGTTACCAT
C
ATG
ATGCAG
ATGCAGCGTTA
ATGCAGCGTTAC
ATGCAGCGTTACCATG
C+T
A
ATG
ATGCAG
ATGCAGCG
ATGCAGCGT
ATGCAGCGTTA
ATGCAGCGTTAC
ATGCAGCGTTACCA
ATGCAGCGTTACCATG
A
C
G
T


d)
A
C
G
T
Increasing size band
Inferred sequence
G
T
A
C
C
A
T
T
G
C
G
A
C
G
T
A
G
A+G
C
C+T
Direction of migration

Automated DNA sequencing

- each dideoxynucleotides is labeled with a different fluorescent dyes that will be added in each synthesized fragment
- the dye attached to the ddNTPs will be added to terminate the synthesis of that particular fragment
- The contents of the single tube reaction are loaded onto a single lane of a gel and electrophoresis is done.
- A fluorimeter and computer are hooked up to the gel and they detect and record the dye attached to the fragments as they come off the gel.

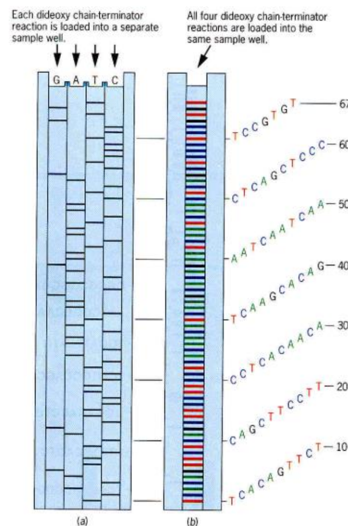
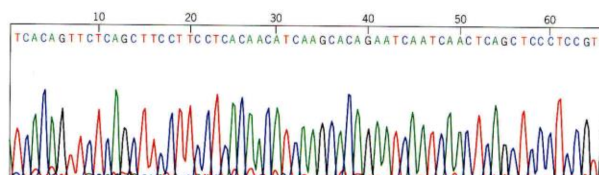
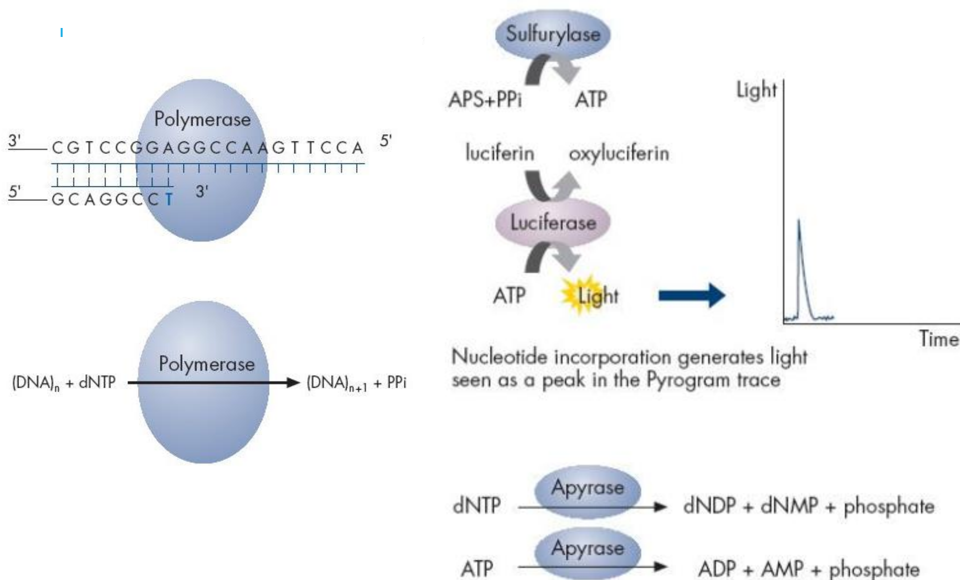


Figure 20.29 Con
the standard slab-gel (
mated (b) DNA seque
dures. (c) Computer p
the results of an auton
sequencing run, showi
nucleotide sequence o
segment of DNA.



PYROSEQUENCING BIOCHEMISTRY

- In DNA synthesis, a dNTP is attached to the 3' end of the growing DNA strand. The two phosphates on the end are released as pyrophosphate (PPi).
- ATP sulfurylase uses PPi and adenosine 5'-phosphosulfate to make ATP.
- Luciferase is the enzyme that causes fireflies to glow. It uses luciferin and ATP as substrates, converting luciferin to oxyluciferin and releasing visible light.
  - The amount of light released is proportional to the number of nucleotides added to the new DNA strand.
- After the reaction, apyrase is added to destroy any leftover dNTPs.



MORE PYROSEQUENCING

The four dNTPs are added one at a time, with apyrase degradation and washing in between.

- The amount of light released is proportional to the number of bases added. Thus, if the sequence has 2 A's in a row, both get added and twice as much light is released as would have happened with only 1 A.
- The pyrosequencing machine cycles between the 4 dNTPs many times, building up the complete sequence. About 300 bp of sequence is possible (as compared to 800-1000 bp with Sanger sequencing).

The light is detected with a charge-coupled device (CCD) camera

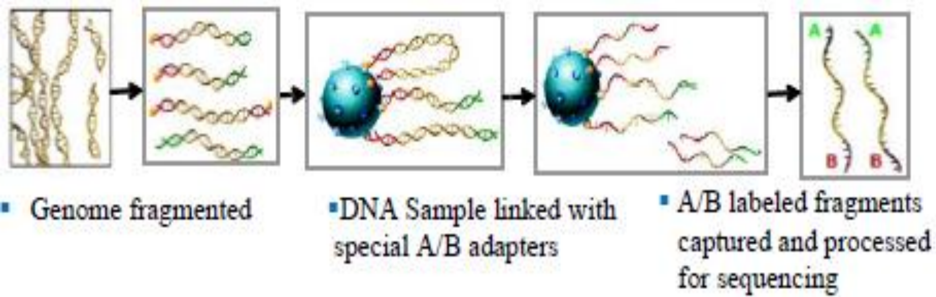
454 TECHNOLOGY

To start, the DNA is sheared into 300-800 bp fragments, and the ends are "polished" by removing any unpaired bases at the ends.

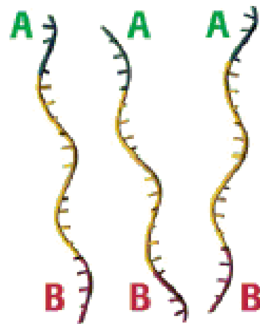
Adapters A,B are added to each end. The DNA is made single stranded at this point.

One adapter (B) contains biotin, which binds to a streptavidin-coated bead. The ratio of beads to DNA molecules is controlled so that most beads get only a single DNA attached to them.

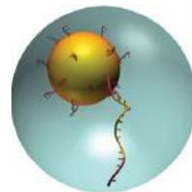
Oil is added to the beads and an emulsion is created. PCR is then performed, with each aqueous droplet forming its own micro-reactor. Each bead ends up coated with about a million identical copies of the original DNA.



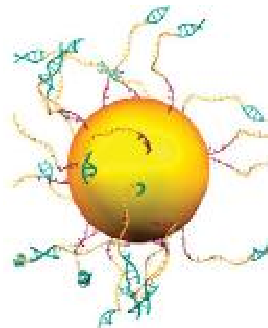
- Genome fragmented
- DNA Sample linked with special A/B adapters
- A/B labeled fragments captured and processed for sequencing



Attach ssDNA library to Beads. Each bead carries a unique single-stranded library fragment. Emulsify beads with amplification reagents in a water-in-oil mixture to trap individual beads in amplification microreactors.

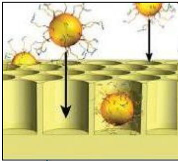


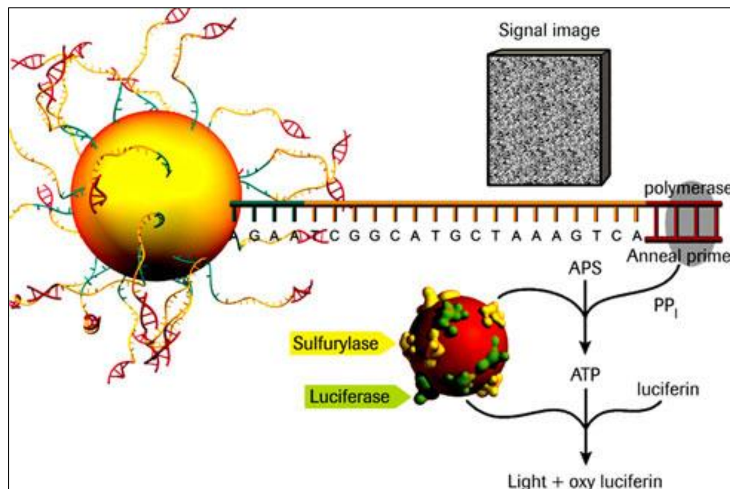
Emulsion PCR



MORE 454 TECHNOLOGY

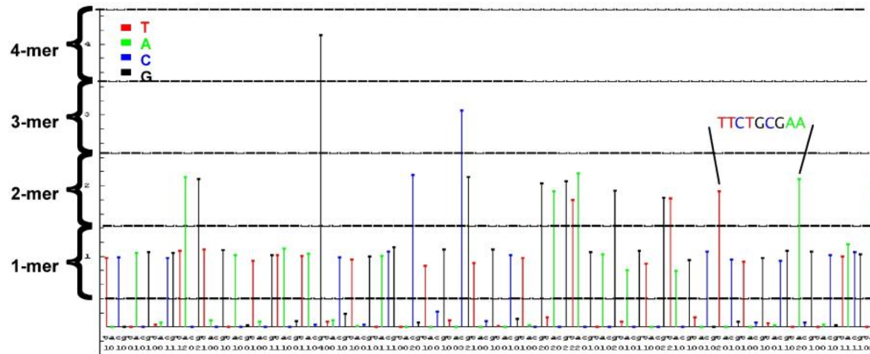
- After the emulsion PCR has been performed, the oil is removed, and the beads are put into a "picotiter" plate. Each well is just big enough to hold a single bead.
- The pyrosequencing enzymes are attached to much smaller beads, which are then added to each well.
- The plate is then repeatedly washed with the each of the four dNTPs, plus other necessary reagents, in a repeating cycle.
- The plate is coupled to a fiber optic chip. A CCD camera records the light flashes from each well.



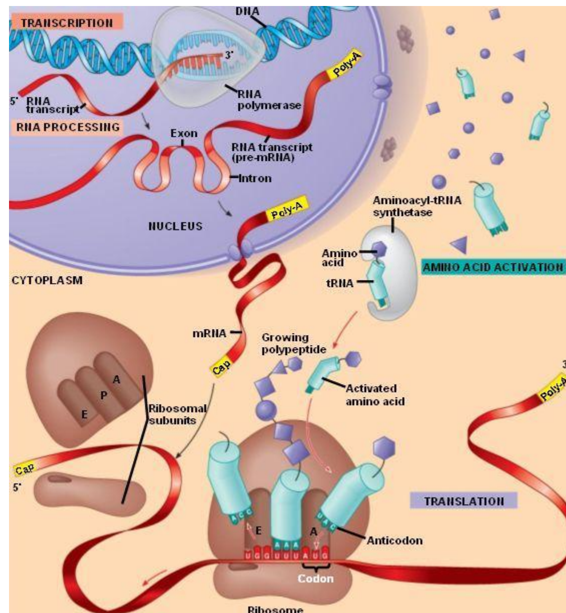


SAMPLE READING

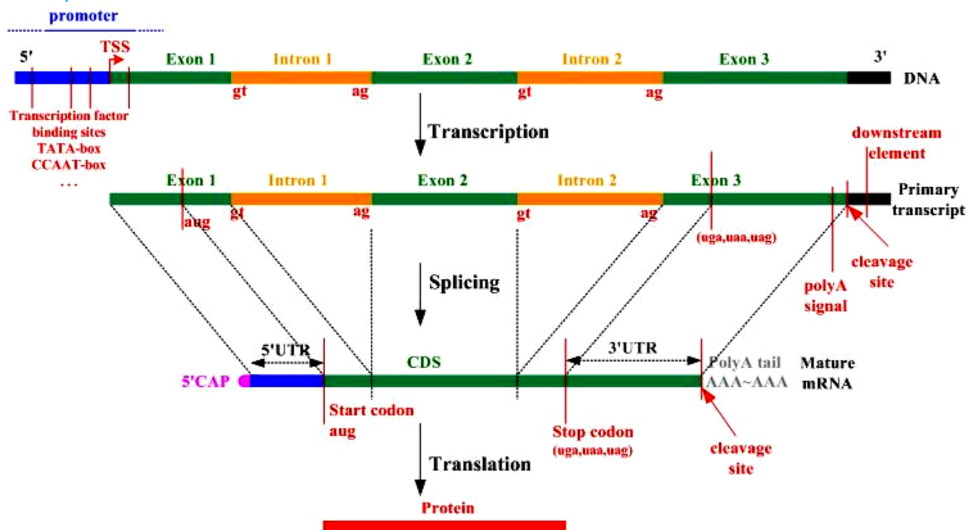
Cycles of addition of T, A, C, G



THE FLOW OF INFORMATION



DNA→RNA→PROTEIN



HOW TO FIND THE 5' END OF MRNA

To find beginning of where transcription start site which is close to the promoter

At 5' of eukaryotic mRNA there is the addition of 7 methyl Guanosine (5' capped site)

At 3'of eukaryotic mRNA there is the addition of …… ?

Two ways to find the 5' of mRNA

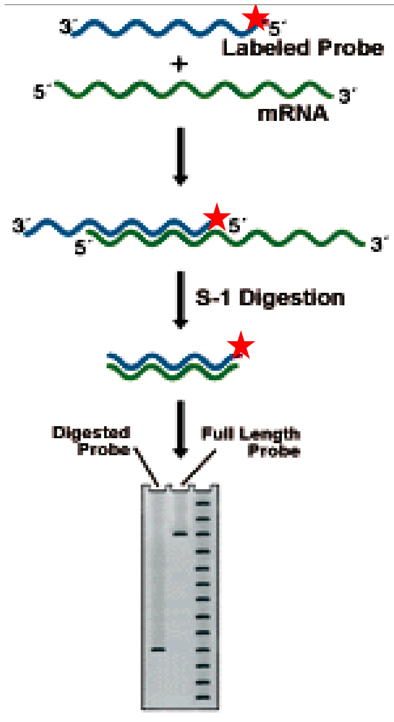
- S1 nuclease mapping
- Primer extension

S1 NUCLEASE MAPPING

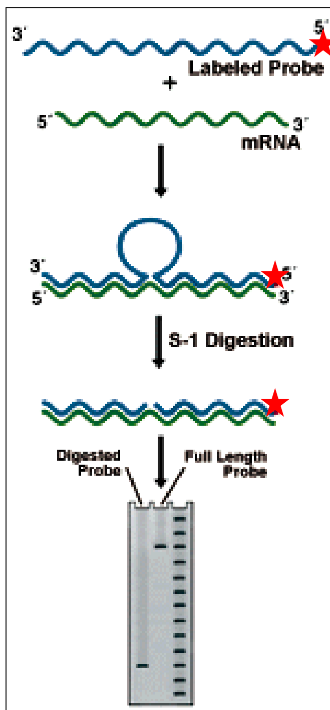
- S1 nuclease
  - Cut only ssDNA and ssRNA
  - Will not cut dsDNA or dsRNA
- Probe is made from your interested gene
  - cut DNA with restrticiton enzyme that will include 5' Capped site in the remaining piece
  - label 5' end with ^{32}P

S1 NUCLEASE MAPPING

- hybridize the labeled DNA pribe with RNA that is isolated from tissues that have expression of the gene
- Cut with S1 nuclease which will cut3' overhang
- Denature to release the ds
- gel electrophoresis to compare the size of probe with DNA ladder
- From the length of the probe, you will know the transcription start site (how far it is from ATG)



S1 NUCLEASE MAPPING FIND ATG STARTSITE

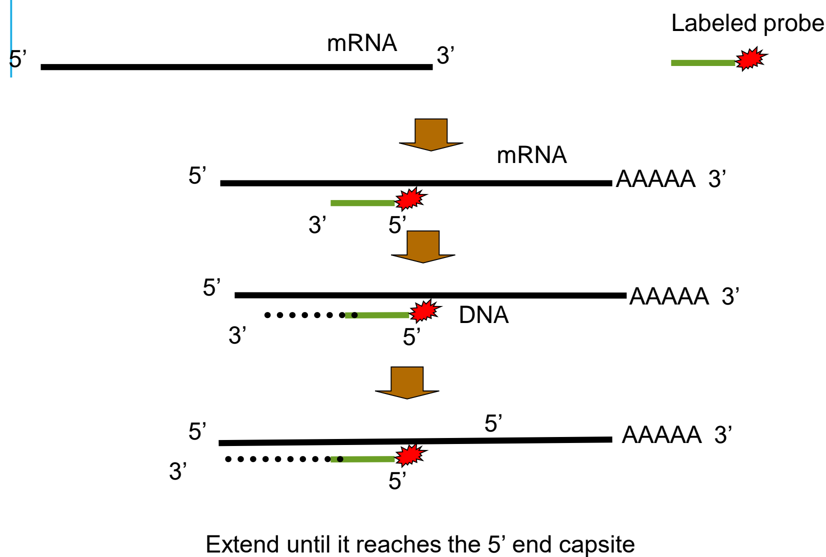


S1 NUCLEASE MAPPING TO FIND ?

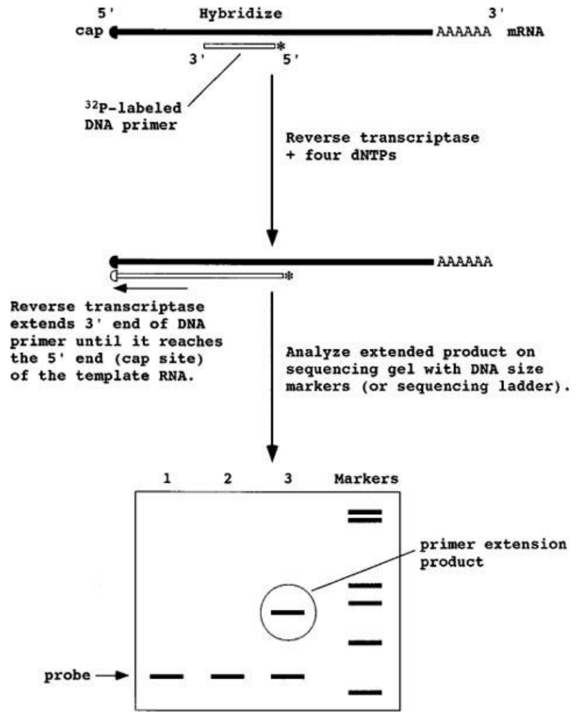
PRIMER EXTENSION

- Probe is made from your interested gene but make it small and use the portion toward the 3’ end and exclude 5’ end
- label 5’ end using ^{32}P
- mix with mRNA from tissues that have expression of that gene
- Use Reverse transcriptase ezyme to synthesize the DNA using the 3’ end of the probe as primer
- Find the size of the synthesized piece on the electrophoresis gel comparing it to the ladder.

PRIMER EXTENSION



Extend until it reaches the 5’ end capsite


5'
Hybridize
3'
cap
AAAAAA
mRNA
3'
5'
32P-labeled
DNA primer
Reverse transcriptase
+ four dNTPs
AAAAAA
Reverse transcriptase
extends 3' end of DNA
primer until it reaches
the 5' end (cap site)
of the template RNA.
Analyze extended product on
sequencing gel with DNA size
markers (or sequencing ladder).
1
2
3
Markers
primer extension
product
probe