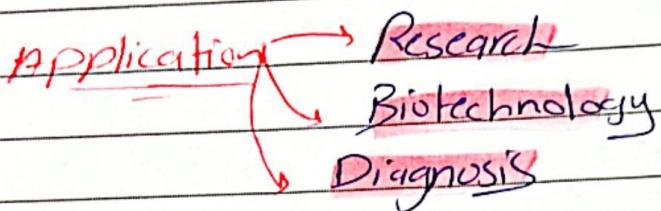


## Part 2

\* Any lab doing experiments on live organism in some point they use PCR



• Chain → Exponential increase in the number of products, so each product used in the reaction could be used as a substrate for the next reaction

- During
  - Denaturation: DNA separate
  - Annealing: primer anneal to their complementary sequences
  - Extension: Bind of polymerases to start the polymerization reaction

### Extension

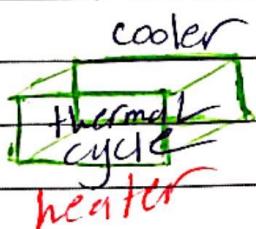
→ DNA poly. will bind & it starts building new strands using one of the strands as a template, but notice that we want to amplify from here to here ~~+~~ ~~+~~

But → polymerase continues beyond the

sequence we want, because DNA polymerase (72-74) as long as the conditions temperature are optimum it continues, what makes it stop is

Going to the next cycle, Because going to the next cycle begins with the denaturation step

- More & more cycle will start getting more of the specific product (predominant)



95°C, 5min → 95-98

Depending on the type  
of enzyme, type/amount of DNA

- Genomic DNA is more clumped

- plasmid smaller than Genomic DNA

- extension temperature depend on the enzyme that is used & the time depend on the enzyme

Taq → Amplify 1 kb Fragment  
(1000 bp) in 30 seconds

\* what would anyone using cloning to make more copy of a gene if PCR is possible?

PCR: Amplify a Gene / PCR product DNA fragment

cloning: Make libraries, sequence Genome, make sure that a sample doesn't contain more than 1 type of amplify PCR product

\* starting from (8-28) cycle exponential increase in the amount of DNA

\* DNA yield → amount = Number of DNA molecules amplified

\* plateau → highly level area, & it's mean that after number of cycles the concentration of DNA doesn't increase anymore

## \* Design Primers

- Primer: short single strand molecule

- Need to know the sequence of the target

- Send it in powder form & we add buffers to powder in order to dissolve the primers & use primer solution

- 3 Hydrogen bond     $G \equiv C$

## Melting temperature

- Temperature that is used to determine the annealing temperature during the PCR

- The temperature at which  $50^\circ$  of dsDNA is changed to single strand DNA

- Estimate of DNA hybrid stability between our primer & the template DNA

• very high will not produce a hybrid DNA or amount of primer-template hybrid

• too low maybe some of the primers bind to the target sequence, but also some primers will bind to other sequences in the genome causing non-specific binding

\* If the G/C is more than 60%, that means the melting temperature is high, that's mean the chance that primers bind to the target sequence is very low

\* If the G/C is less than 10%, melting temperature is very low, which mean that we have a lowing annealing temperature & this might cause non-specific binding of the primer to other non-specific target

- Since we use 2 primers for PCR reaction, the melting temperature of the 2 primers that is used is, because when you use 2 primers they different  
will have different sequence, but the melting temperature differences shouldn't be more than (2-3)

\* General Rule → Annealing temperature about  $5^{\circ}\text{C}$  below the  $T_m$  of your Primers

ex  $\rightarrow T_m$  Forward primer annealing :  $56^{\circ}$   
 $T_m$  Reverse " " ?  $55^{\circ}$

Annealing temperature :  $51^{\circ}$

\* Primers shouldn't contain repeat sequences or self complementary sequences



ex 1  $5'$  GGGAAATTCCGTAC $3'$  → if you put it in PCR, some regions from the primer will anneal to each other

$5'$  GGGAA  
 $3'$  CATGCCCTA

This is called hairpin

ex 2  $5'$  GGGAAAPATTCCAGGTC $3'$  Forward  
 $3'$  CTGGACCTTAAAGGG $5'$

Called self complementarity

ex 3 → 5' TCGTCCATGGTATC 3' Forward  
3' GCAATAGGCCACAGG 5' Reverse  
called primer-primer  
dimer

Self dimer → complementary sequence  
— sometimes between the reverse  
— & forward primer

- It's not easy to do all this so we use software (ex: primer) for primer design

### GC clamp

( ) allow DNA polymerase to bind to your primer & start the amplification or making a new strand

\* primers → negatively charge

Doesn't bind to Double strand DNA

\* 2 Major DNA poly. → Taq  
Phu

one of the important factors of the activity of DNA poly. is  $Mg^{+2}$

- Imagine if we have high concentration of negatively charge primers, they will start binding to the  $Mg^{+2}$ , & there will be not  $Mg^{+2}$  for DNA poly. & it won't work

\* Typically in PCR reaction, we add buffer & other additive that will stabilize the structure of polymerase & enhancer their activity.

- Some companies they genetically engineered some polymerases to increase their extension rate

\* Fidelity → Ability of polymerase to see its mistakes & correct them

so → Taq poly. in general make mistakes, it might incorporate to incorrect nucleotide

Typically polymerization  $5' \rightarrow 3'$   
If they want to go back & correct the mistake they go  $3' \rightarrow 5'$  & we call this proofreading

Typically they are used for cloning Gene, if we want to express the Genes & we don't want the Gene sequence to be altered or mutant during amplification

\* processivity → probability that poly. will attach to the DNA, start polymerizing, keeps attaching & moving for number of nucleotides

high processivity is important if we want to amplify long amplicons (amplified target sequence)

DNA poly. → large protein complex (more than 1 protein more than 1 gene)

consist of several subunits:

- one subunit might be important for  $5' \rightarrow 3'$  polymerase activity

- " " " " " for  $3' \rightarrow 5'$  proofreading activity

- " " " " " for  $5' \rightarrow 3'$  exonuclease

### \* Thermo stable DNA poly.

Polymerases that we use for routine PCR, if we want to check if there is a specific DNA sequence in Genome or we just want to amplify a product to see if it's there or not

We use standard Tag poly. & the unique about it that it add an extra (T) to  $3'$

~~+F PR~~  $\xrightarrow{5'}$   $\text{A}^3$   $\xrightarrow{5'}$  sticky end. The

Tag poly. isn't used for amplification of long DNA fragment, not highly labeled, don't possess high processivity

\* Hot-start → polymerases inactive when you add it to the PCR reaction but they become active after initial denaturation, so these poly. will be active during PCR

Minimize the non-specific PCR amplification that could happen while mixing all the component of the PCR reaction

H DNA dNTP primer DNP poly

\* Hi-Fi → used for cloning & gene expression

Express of a protein with the correct amino acid sequence, when you do cloning you want to use (Hi-Fi) poly. To make sure that the enzyme doesn't make mistakes & your cloning the correct sequence of your Gene

long amplicons → high processivity, which mean they can be used for long DNA fragment

\* After we do PCR typically we check if our PCR successful by running some PCR reaction on Gel elect.

- Agarose → powder mixed with buffer solution
- buffer solution → specific pH that maintain negative charge on the DNA that will be separated on the Gel
- Buffer → TBE buffer : Tris Boric acid EDTA, pH = 8.3  
TAE buffer

→ pH will ensure that all DNA molecule will have a negative charge

- before we pour the melted gel we have to make holes wells & after the Gel solidify we take it & add it to electrophoresis chamber.

- These electro. are connected to power by wires & the power can provide the Gel with electricity & the DNA will start moving

• we add SDS to the Gel → ethidium bromide

push it's self  
between DNA  
bases

Give  
fluorescent light  
UV → violet

- The Gel is colorless & also the DNA sample, so in order to see it (DNA) we need to take the Gel out of the electro. chamber & we have to expose it to UV light

- 2 Types of adding ethidium bromide

→ into agarose + buffer before making & pouring the Gel

" " " " after "

\* bands closer to wells have largest DNA fragment

\* If you zoom in to any band, each band will contain millions of DNA fragments of the same size

- When we put the Gel inside a chamber filled with a buffer & want to put our DNA

( colorless solution that should be loaded into the middle of colorless gel & colorless buffer )

If you loaded your DNA by itself your DNA will go out because you are loading a solution (liquid to liquid), that will diffuse with each other

So before we add DNA to wells, while the wells are submerged into a buffer we have to mix our DNA with loading buffers

### \* loading buffer

has loading dyes & glycerol, sucrose  
will give a color  
to your sample & make  
sure that our DNA remain  
inside the Gel  
Increase the  
density of our  
DNA sample, so  
when we load our  
DNA sample it will  
stay in the well

### \* marker DNA we use it as a ruler

- looking at the number of bands doesn't give you an idea of how many restriction site might be present

### • Why do we have smear ?

Because the size of the DNA in E.coli is very large which means the probability of finding hundreds of restriction site for this enzyme is possible

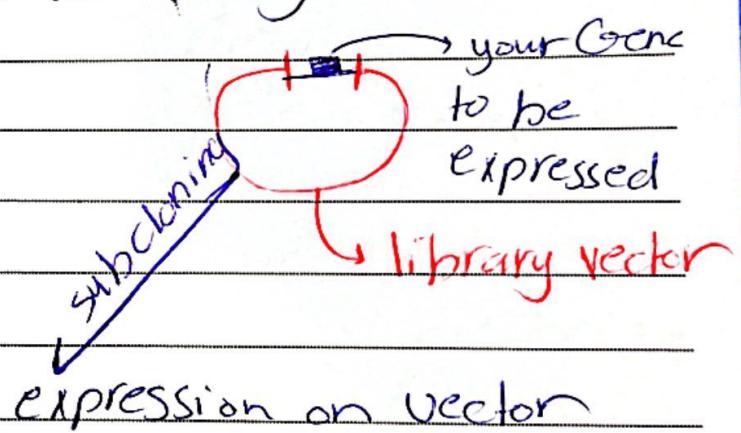
## \* Restriction mapping

→ Cutting DNA fragment with different restriction enzymes to map the location of restriction site

→ Do it when we want to do Subcloning

→ DNA fragment in a library & we want a certain gene

- we do Restriction mapping to make sure that restriction enzyme won't cut in the middle



- We could detect the presence of mutation

- 1) PCR amplification
- 2) Restriction digestion
- 3) Gel electro.

## \* isothermal amplification of DNA

Amplify DNA at constant temperature (25-65)

we don't need thermocycler

some of it use specific type of DNA poly.

poly. these poly. can operate moderate temperatures (25-40) (50-65)

• IAMP → size product less 25bp

detection of amplification using this technique by colorimetric & fluorescent

As more DNA products are being formed, it will accumulate & it could be seen by eye

At the end you will have a large amount of amplified DNA

- Colorimetric → After the end of the reaction we add a dye & it will change its color to indicate if the amplification happen or not

luffer  
Primer  
nucleotides

sample

60 min

65°

development of color because accumulation  $Mg^{+2}$  pyrophosphate

BST (Polymerase)

\* dNTP → nitrogen bases & 3 phosphate but when they added to DNA by poly. they will lose 2 phosphate

→ pyrophosphate (will bind to Mg)

\* RPA → sensitive technique

detect specific sequence of DNA

we need primer, DNA poly., recombinase (protein that facilitate recombination)

\* FISH → involves use of fluorescent dye to detect certain sequence on chromosome

Fluorescence → Fluorescently labelled probes

in situ → probes will be added to cells

Hybridization → involves the use of probes

← Fish is easy \*

- it starts with cells that is dividing & it's in metaphase in cell division

Chromosomes, short  
visible, thick

• cell will be added to glass light, then chemical will fix the cell

& the chromosome in the cell, & we add a fluorescently labeled probe that will be able to find specific sequence on chromosome or a whole chromosome

- probe that is used to track telomeres (probe have a sequence complementary to telomeres)

Find at the end of the chromosome

\* CGH

based on using the Fluorescent dye to track the presence of DNA

We don't use a probe

Genomes will hybridize to each other

Comparitive : comparing 2 genomes & how they will hybridize to labeled DNA

use to compare how the chromosomes in normal cells will be different in tumoral cell

To Compair

1. Isolate DNA

2. Labeled with Fluorescent dye with different colors

3. Mix & spread them into a normal metaphasic chromosome

\* southern

Running DNA fragments on  
Gel electrophoresis

DNA won't visualize using  
UV light or ethidium bromide

In order to track a specific sequence  
of DNA, we use a probe

After running the Gel the DNA will  
be transferred into a membrane

The Gel will be treated with  
NaOH to denature DNA, then DNA  
transferred into a membrane

nylon  
nitrocellulose

Put on top  
of the Gel

\* paper towels up the membrane then we have  
to put weight & the chamber is filled with  
salt solution.

\* So DNA in Gel will be separated using electricity but after that in order to transfer cell into membrane we rely on capital reaction

- filter paper beneath the Gel & it's immersed in the buffer

↓

will be moving & it will carry the DNA into the membrane  
& it will continue moving but DNA stop on the surface of the membrane  
(bottom of chamber → filterpaper → Gel ...)

- The surface of the membrane now have the DNA & we need to make sure that DNA remain attached to the membrane

So → we have to do sth called membrane backing : backed on high temperature to make DNA stuck or UV

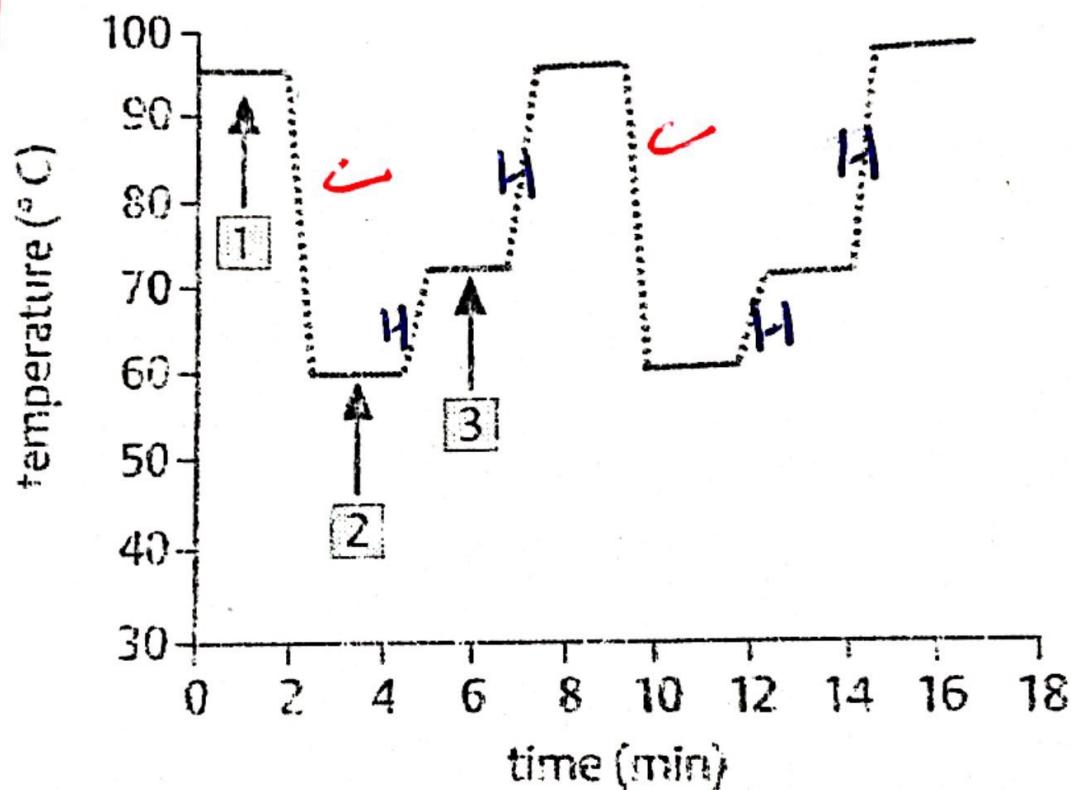
then → I use a probe (radiolabel or fluorescent) to look for the DNA i want to track

\* tracking of the DNA using the probe could be done by autoradiography, chemiluminescence

\* the purpose of filter paper, paper towels & the buffer is to transfer DNA from the Gel into the membrane

Summary → separating DNA on a gel & tracking DNA using probe

5 min 95°C  
 1 min 95°C D ] 25-35 cycles  
 30 sec 60° A ] n-fold repetition  
 30 sec 72° E of cycle  
 10 m 72° C  
 hold 40°C



- [1] step 1: denaturation
- [2] step 2: annealing of primers
- [3] step 3: extension of primers

Shows the  
 cycling/steps within  
PCR & the temperature  
changing

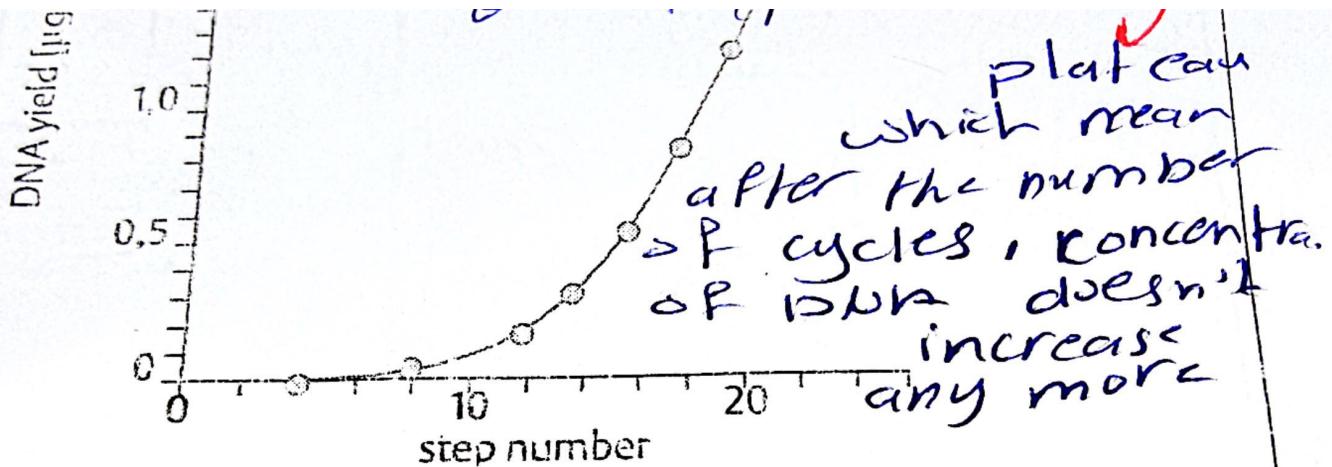
assuming  
100%  
efficiency

$$N = N_0 \times 2^c$$

Number of DNA molecules

No of DNA template molecules

number of cycle



by incorporation of ethidium bromide or SYBR Green  
the PCR yield can be determined in real time (Light  
Cycler™)

This graph shows  
how the concentration  
of DNA increases  
exponentially as

The number of cycle increase  
during PCR

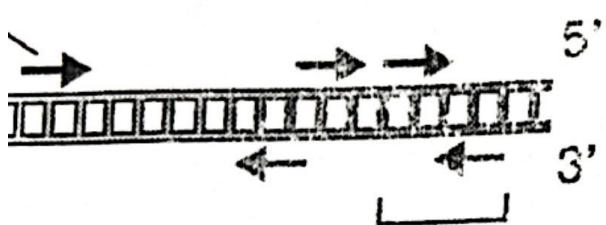
Schmid, Schmidt-Conrath and Hammelohle  
(2010) Biotechnology. An Illustrated Primer

rs

ucleotides

primers

s



real primer pairs can  
amplification products  
- specific  
products

\* less than 18  
we might get  
sth called  
non-specific binding

\* the shorter  
the primers  
the higher  
probability  
that primers  
will bind to  
more than 1  
location

probability =  $(\frac{1}{n})^n$

to have  
primers

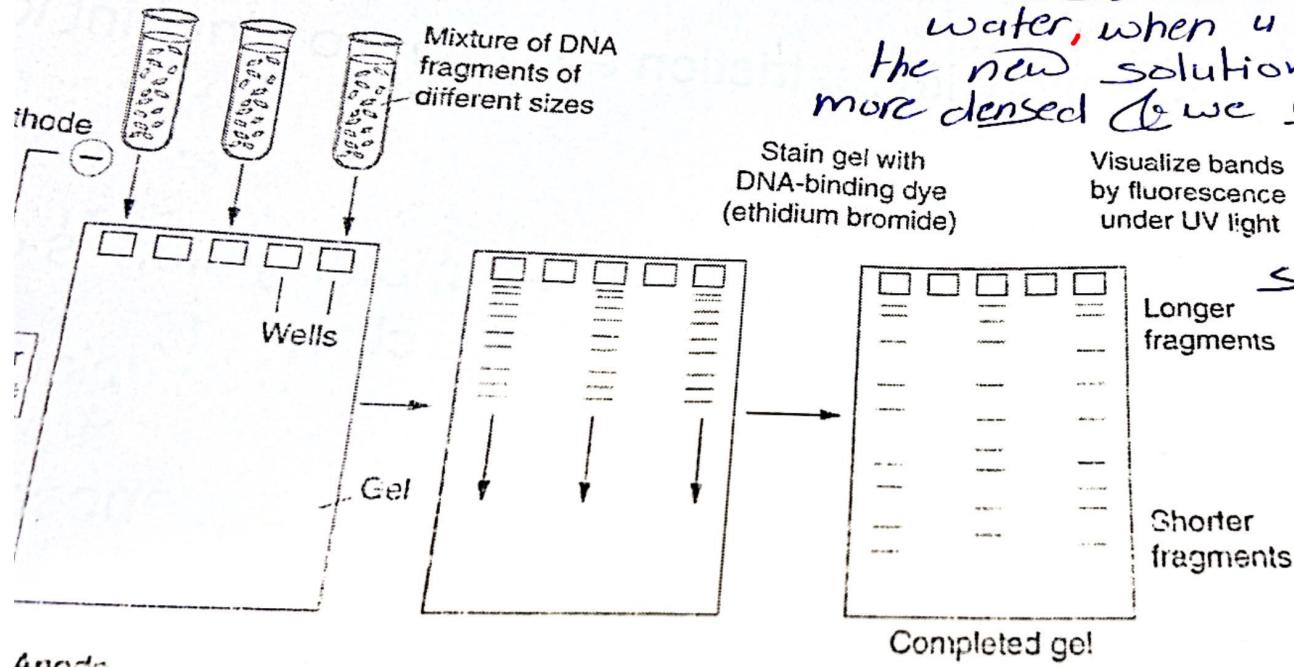
*↓ higher, ↓*

T. A. Brown (2010) Gene Cloning  
A DNA Analysis, An Introduction

## Laboratory Techniques

### Agarose Gel Electrophoresis

a)



\* Glycerol or sucrose are more dense than water, when u mix them the new solution will be more dense & we use it to make sure that the DNA will stay in the well

Anode

\* The loading dye is always ahead of the DNA Fragments & it's used to know when to stop the running of the Gel

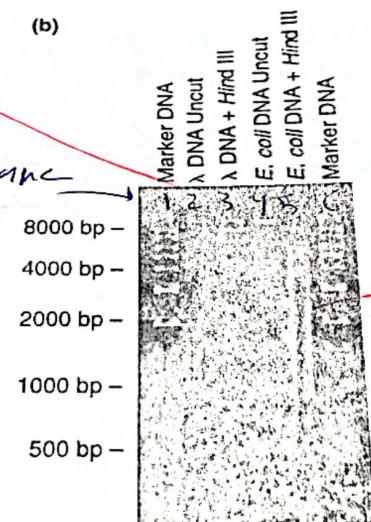
## Laboratory Techniques

### Agarose Gel Electrophoresis

One band →  
Fluorescent  
Cut by a Restriction  
enzyme

Genome

(b)



It Fluorescent line  
appears here which is  
called smear, a smear  
is a very low difference  
in sizes so it appears  
like a 1 line but actually  
it is a lot of bands that  
are very close to each  
other in size decreasing  
from largest to  
smallest

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tracking Gene exp. (tracking if a particular organ or tissue is expressing a particular mRNA)

**Fluorescence in situ hybridization (FISH)**

Identify which chromosome contains a gene of interest abnormality

DNA or RNA probe for gene of interest is labeled with fluorescent nucleotides

FISH used to analyze genetic disorders caused by numerical & structural chromosomal abnormalities

FISH used to determine which cells in a particular organ are expressing the particular mRNA

numerical → missing or extra chromosome

structural → A segment of chromosome is deleted or chromosome duplicated or chromosome exchange DNA segments