Biochem 717
Nucleic acid and gene isolation

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DNA isolation

- Sample homogenization in liquid nitrogen
- CTAB buffer (for plants)
- Phenol/chloroform/isoamyl alcohol extraction
- Ethanol precipitation
- 70% ethanol wash
- Resuspend in TE buffer or water
- Quantification (Spectrophotometric) A260 nm; 1A= 50 ug/mL for DNA
- A260/A280 nm ratio for DNA purification

DNA isolation from plants

- **Grind the leaf sample (0.2-0.5 g) in liquid nitrogen in a mortar and pestle until it converts into fine powder.**
- **Transfer the powder into a microfuge tube.**
- **Add hot (65^{o}C) 2X CTAB buffer (0.7-1.5 mL) and incubate in water bath for 30 min.**
- **Add one volume (V) of chloroform/isoamyl alcohol (24:1). Mix thoroughly to form an emulsion.**
- **Centrifuge in a microfuge for one minute at 13,000 rpm.**
- **Transfer the supernatant solution from the top phase to a new microfuge tube. Discard lower phase.**

- **Add 1/10 V of 10% CTAB solution and mix.**
- **Perform another chloroform/isoamyl alcohol extraction as in steps 4-6.**
- **Add an 0.6V of cold isopropanol/ precipitation buffer or double volume of 100% ethanol and mix gently.**
- **Centrifuge for 5 min., discard the supernatant.**
- **Add equal V of cold 70% (stored in freezer) ethanol and spin for 5 min. Discard the supernatant.**
- **Dry till evaporation.**

- **Rehydrate the pellet of DNA in 1X TE or dH_2O.**
- **Treat with RNase.**
- **Perform chloroform/isoamyl alcohol extraction as in steps 4-6.**
- **Perform DNA precipitation as in steps 9-13.**

DNA isolation from *Bacillus subtilis*

- Take 10 mL fresh Inoculum of *Bacillus subtilis*; centrifuge it on 10,000 rpm for 10 min. at 4^{o}C.
- Wash pellet with 5 mL of 0.1xSSC by centrifuging it on 10,000 rpm for 5 min. at 4^{o}C.
- Suspend solution in 0.01 M sodium phosphate buffer in 20% sucrose, with 0.025 g of lysozyme
- Incubate at 37^{o}C for 45 min.
- Add 9 mL of lysis buffer along with 40 uL of proteinase K.
- Incubate at 37^{o}C for 30 min.
- Add equal volume of phenol/chloroform/isoamyl alcohol.
- Invert carefully few times and centrifuge on 10,000 rpm for 5 min. at 4^{o}C.
- Transfer upper layer to another tube.
- Repeat phenol/chloroform/isoamyl alcohol procedure.

DNA isolation from *Bacillus subtilis*

(Continued)

- Add equal volume of chloroform.
- Invert carefully few time and centrifuge on 10,000 rpm for 5 min. at 4^{o}C.
- Transfer upper layer to another tube.
- Add 1/10 vol. of 3 *M* Sodium acetate and 2.5 vol. of cold 100% ethanol.
- Incubate tubes at –20oC over night OR 60 min. in liquid nitrogen.
- Centrifuge on 10,000 rpm for 5 min. at 4^{o}C.
- Discard supernatant, add 500 uL of 75% ethanol to the pellet, swirl gently and Centrifuge on 10,000 rpm for 5 min. at 4^{o}C.
- Suspend pellet in 100 uL of T.E buffer.

SOLUTIONS

- **1xSSC: 0.15 *M* NaCl, (0.876 g/100 mL) 0.015 *M* sodium acetate (0.441 g/ 100 mL).**
- **0.01 *M* sodium phosphate buffer in 20% sucrose: dissolve 0.095 g of acid (NaH_2PO_4), 0.054 g of salt (Na_2HPO_4), and 20 g of sucrose and make volume up to 100 mL.**
- **Lysis buffer: 10 m*M* Tris-hydrochloride (pH 8.0) 1 m*M* EDTA, 1% sodium dodecyle sulphate.**
- **Phenol/chloroform/isoamyl alcohol: equilibrated phenol with 25/24/1 ratio respectively.**
- **T.E buffer: 10 m*M* Tris: Cl, 1 m*M* EDTA prepare from stock solution of 100 m*M* Tris: Cl (diluted from 1 *M* stock) and 100 m*M* EDTA (diluted from 0.5 *M* stock)**

- **Sodium acetate solution, 3 *M*: dissolve 24.61 g of sodium acetate (anhydrous) in 40 mL dH_2O adjust pH to 5.2 by 3 *M* acetic acid (8.6 mL glacial acetic acid to 50 mL with dH_2O). Make volume up to 100 mL by dH_2O.**

- **EDTA 0.5 M stock: dissolve 18.61 g of Na_2.EDTA.2H2O in 70 mL dH_2O. Adjust pH to 8 with 10 *M* NaOH. Make final volume to 100 mL.**

- **Tris.Cl 1 *M* stock: dissolve 12.1 g of Tris base in 70 mL dH_2O, adjust pH to 8 with conc. HCl and make final volume to 100 mL.**

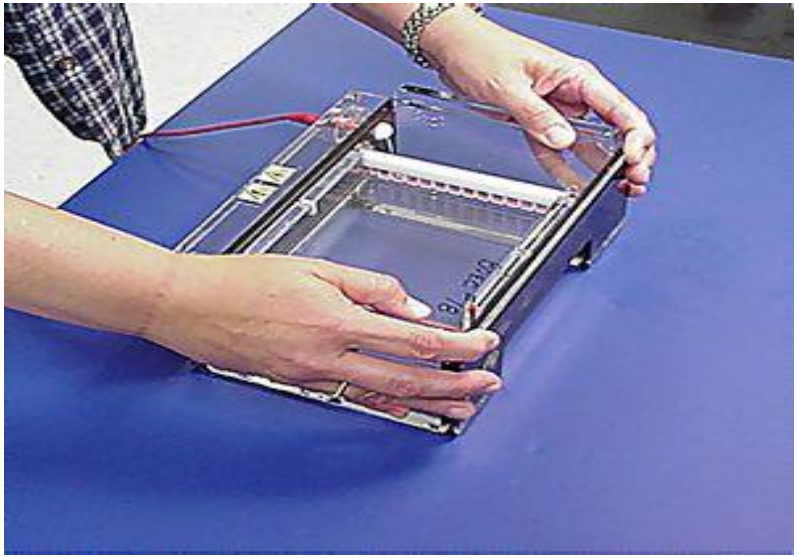
Nucleic Acid Quantification

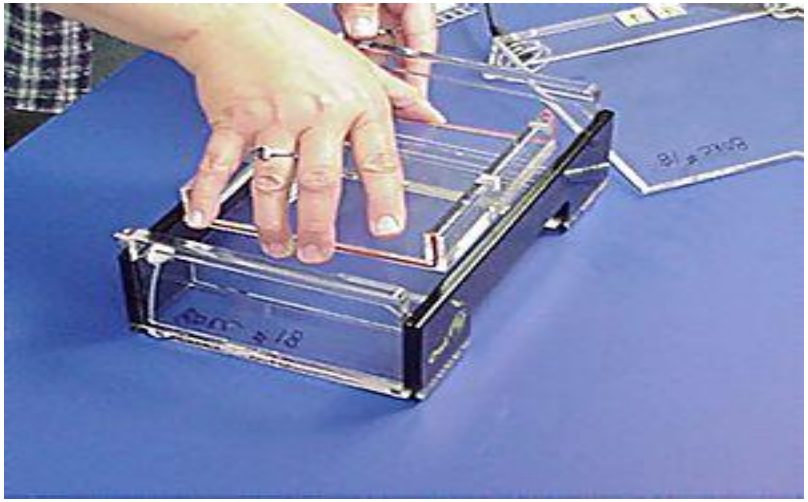
- Absorbance at 260 nm
- 1 Absorbance at 260 nm= 50 ug/mL double stranded DNA; for RNA 40 ug/mL
- Absorbance at 280 nm and A260/A280 ratio

DNA Quantification

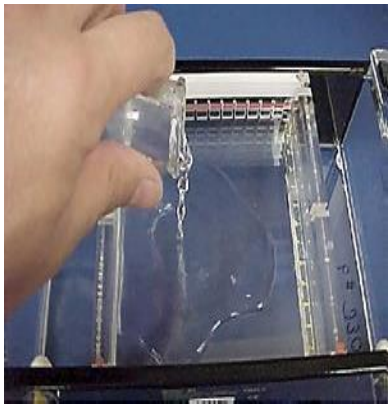
Sample	A_{260}	A_{280}	Conc. mg/mL	Mean $A_{260/280}$
G1a	1.055	0.602	1.3187	1.75
Glb	0.833	0.475	1.0412	1.75
G2a	0.8	0.435	1.0	1.8
G2b	0.755	0.425	0.9437	1.78

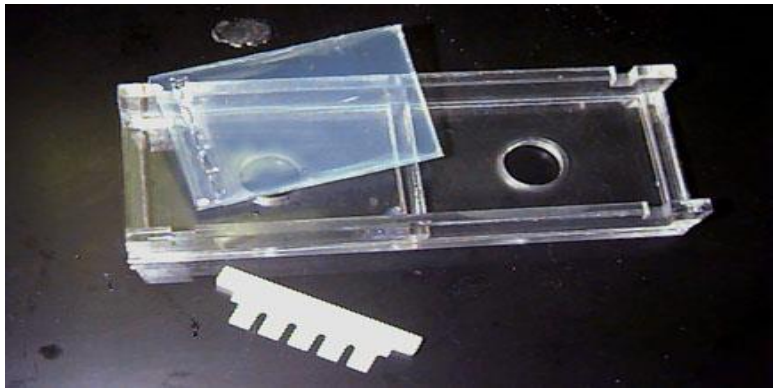
Agarose Gel Electrophoresis



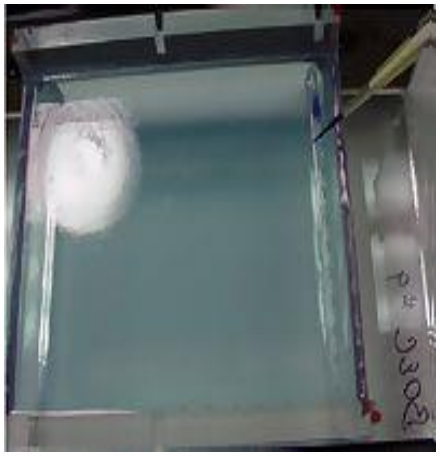


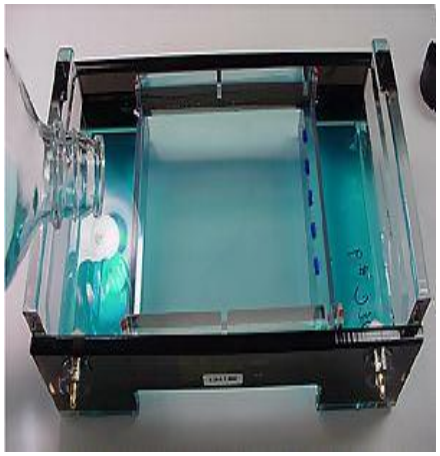

250 mL

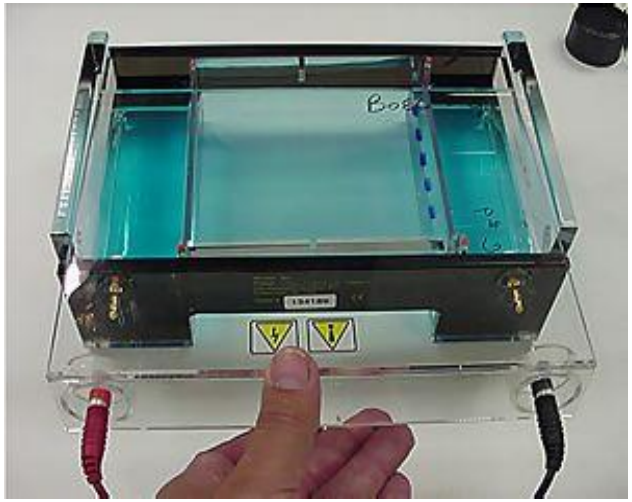


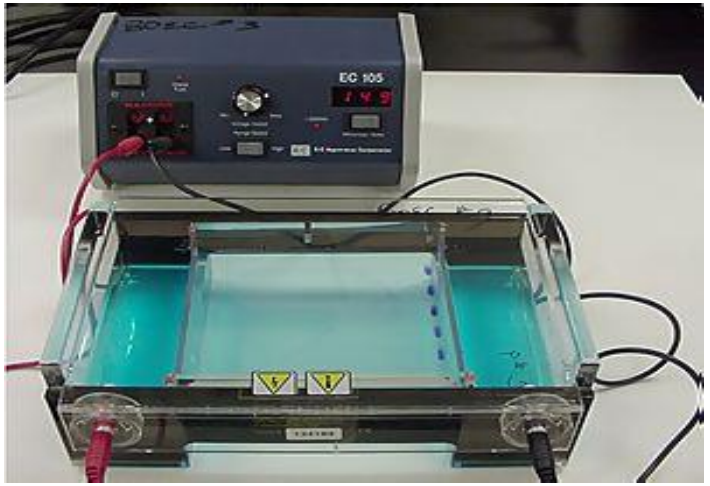


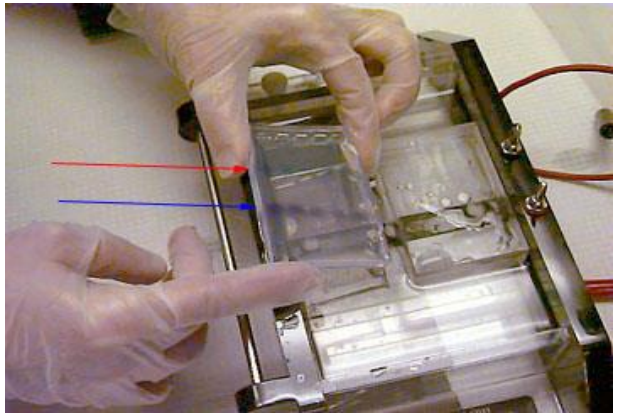

Shown below is the AH 68-0444, Maxi-Plus Horizontal Gel Unit. The AH 68-0455 Unit includes a cooling base.



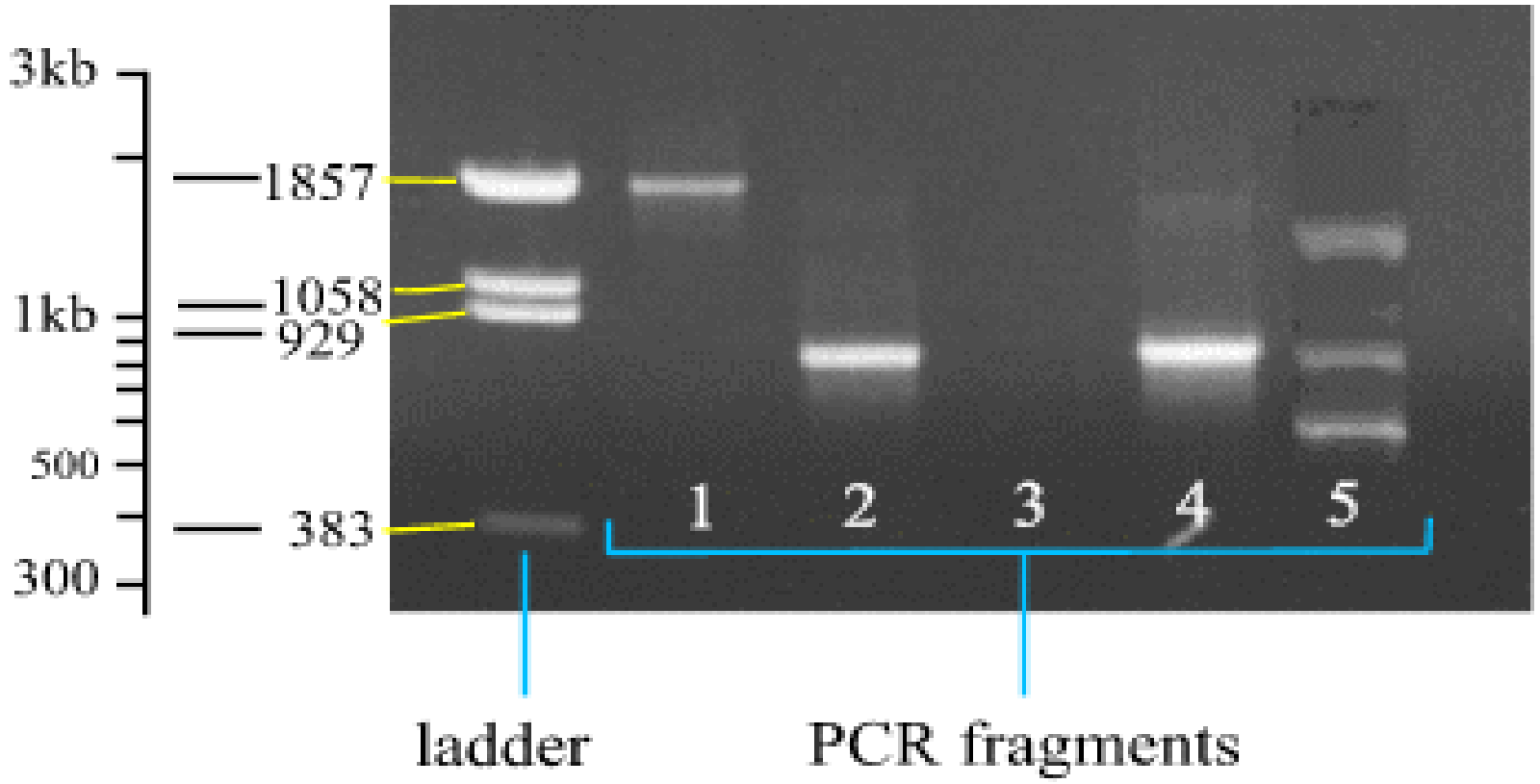





EC 105



DNA fragments can be resolved through agarose gel electrophoresis.



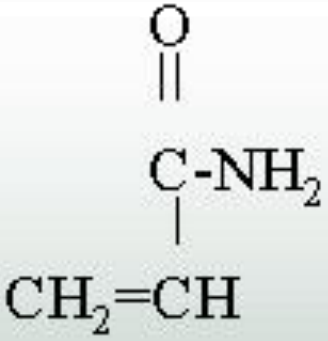
Gel Formation

- Acrylamide
- Bis-acrylamide
- Ammonium per sulphate (APS, initiator)
- TEMED (Tetramethylnediamine, catalyst).
- Photochemical polymerization.

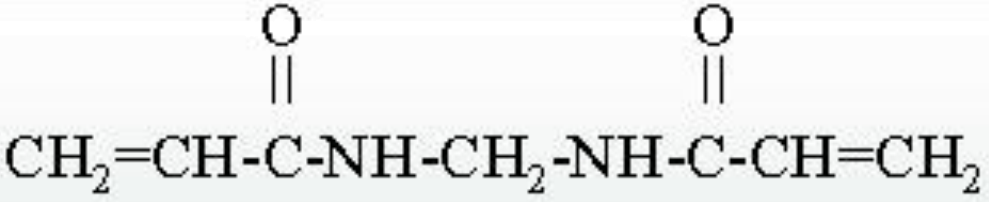
Riboflavin exposed to UV radiation.

Polyacrylamide gel electrophoresis

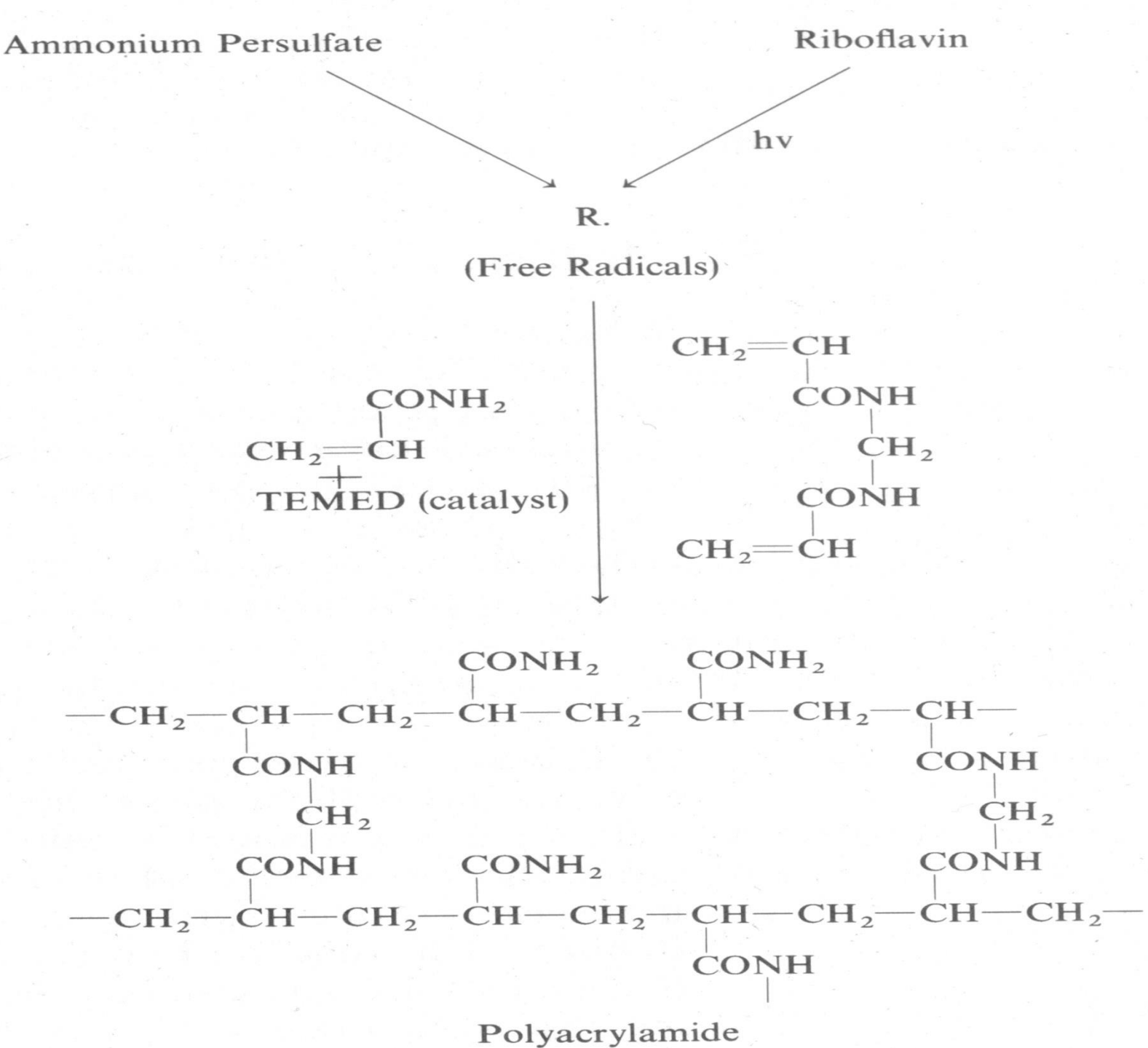
- Gel is made by the polymerization of acrylamide and bis-acrylamide.



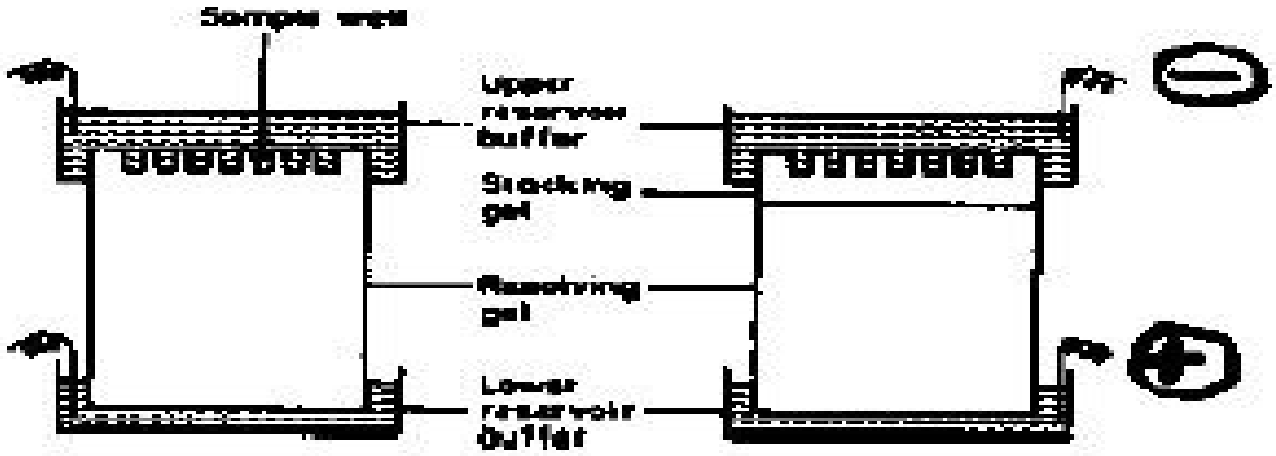
acrylamide

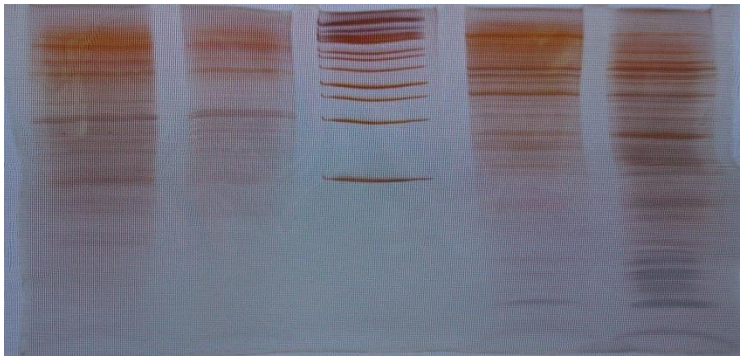


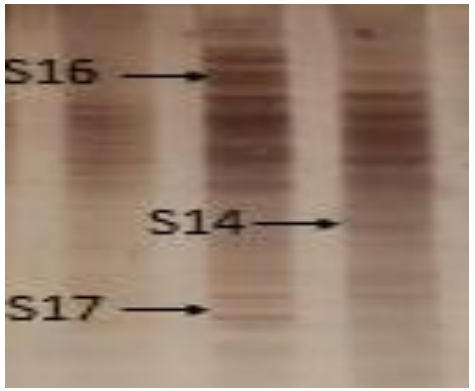
Bis-acrylamide


Ammonium Persulfate
Riboflavin
hv
R.
(Free Radicals)
CONH2
CH2=CH
+
TEMED (catalyst)
CH2=CH
CONH
CH2
CONH
CH2=CH
CONH2
CONH2
—CH2—CH—CH2—CH—CH2—CH—CH2—CH—
CONH
CH2
CONH
CONH2
—CH2—CH—CH2—CH—CH2—CH—CH2—CH—CH2—
CONH
Polyacrylamide

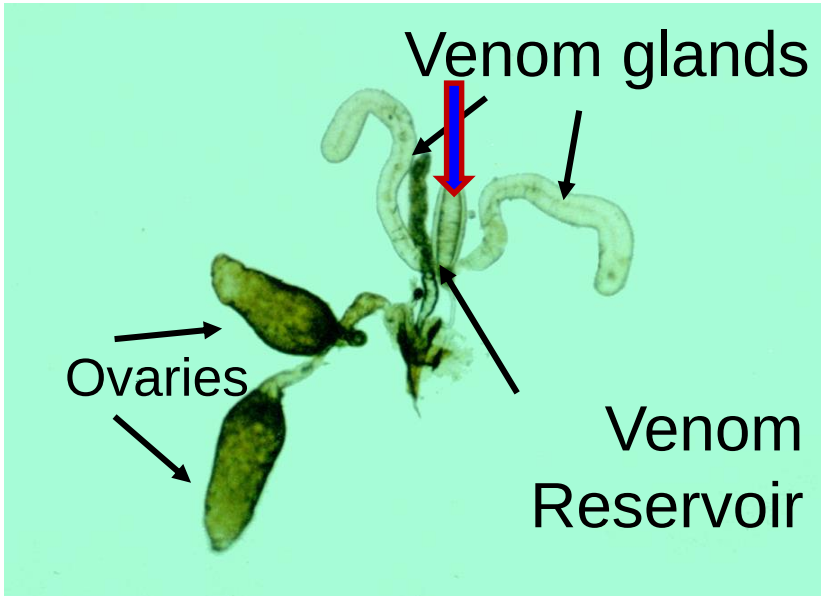
Models of PAGE and Setups







Collection of venom glands with Tri-Reagent (Sigma)



20 venom glands in 1.ml Tri/Trizol Reagent

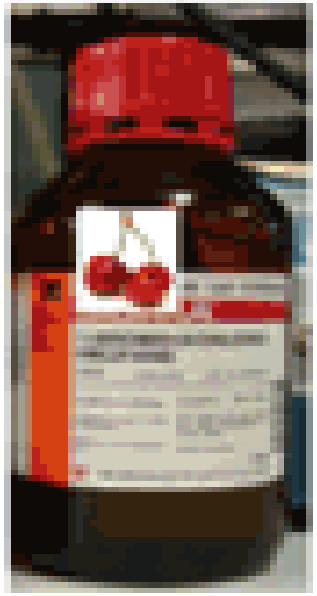
Crushing of venom glands with pestle inside eppendorf tube

Storage at -80°C

RNA EXTRACTION FOR THE STUDY OF GENE EXPRESSION

Cell lysis and homogenization

- Total RNA of males, females devoid of venom glands and venom glands will be extracted with TRI Reagent® (Sigma-Aldrich Catalog #T9424).



Phase separation with chloroform

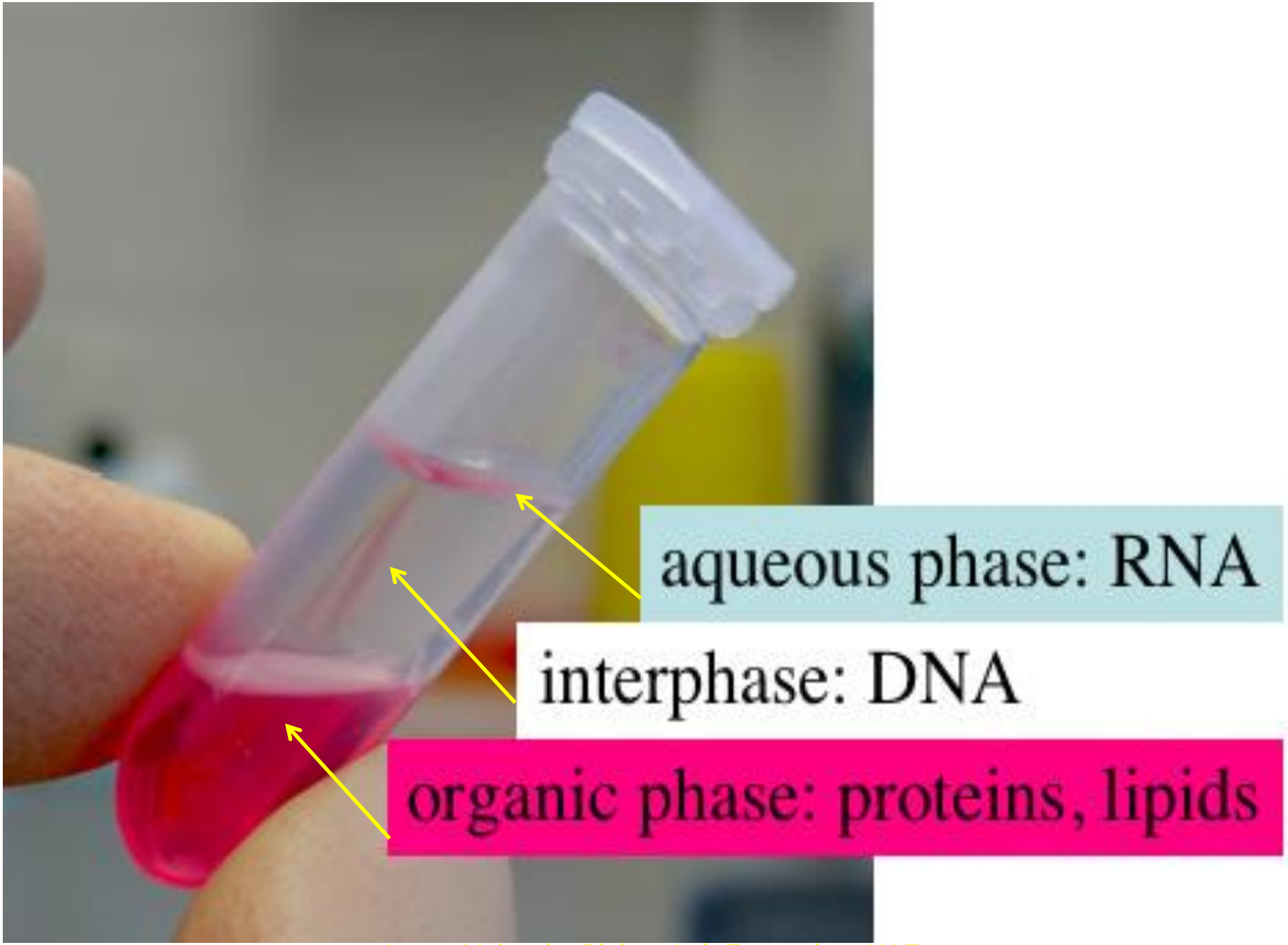
0.2 ml chloroform per 1 ml of TRI Reagent®

Vortexing

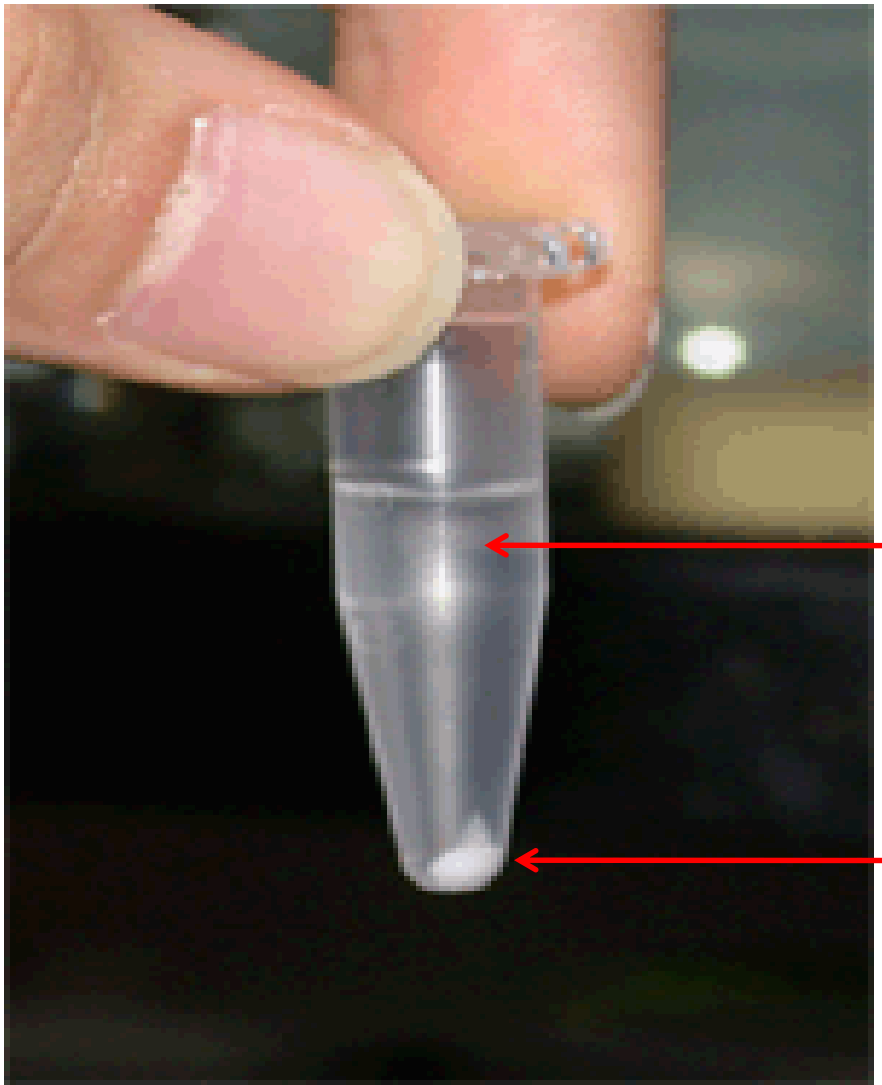
Incubation for 15 min.at RT

Centrifugation at 12000 g for 15 min.at 4 °C

Phase separation


aqueous phase: RNA
interphase: DNA
organic phase: proteins, lipids

RNA precipitation by Isopropanol



Washing of RNA pellets

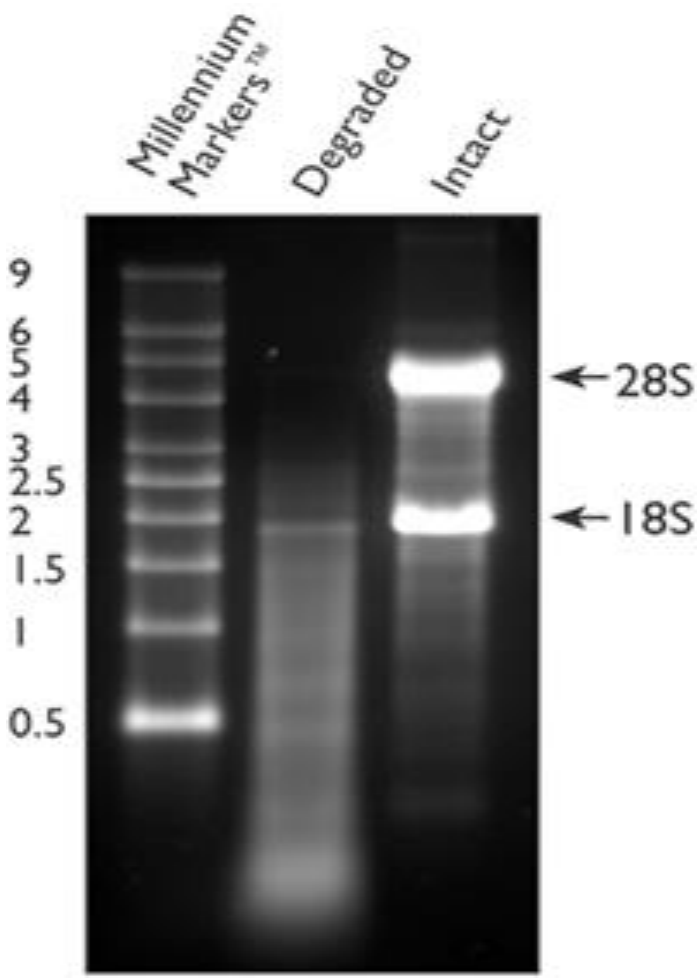
75-80% Ethanol in 0.1% H_2O DEPC

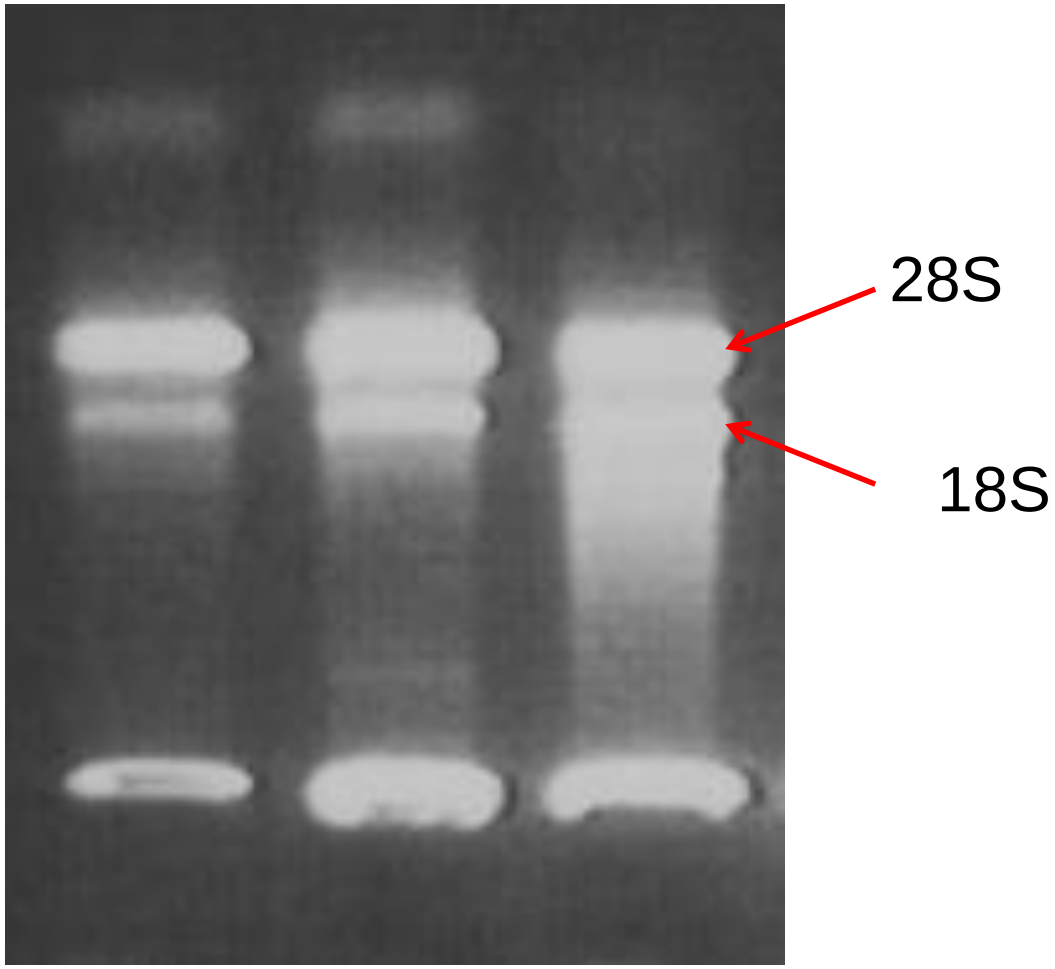
Resuspension of RNA pellets

0.1% H_2O DEPC

Qualitative analysis of RNA

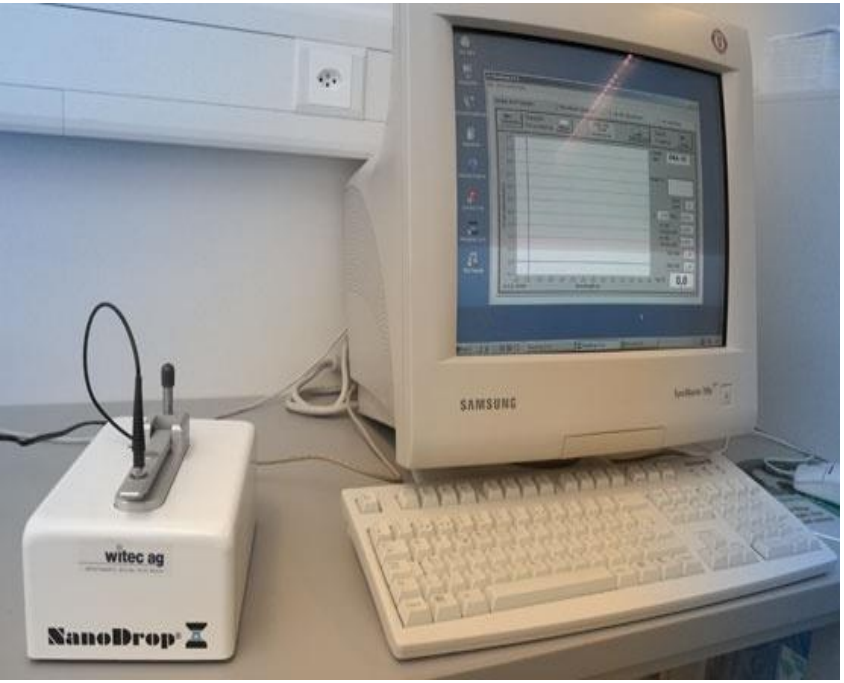
0.8% Agarose gel

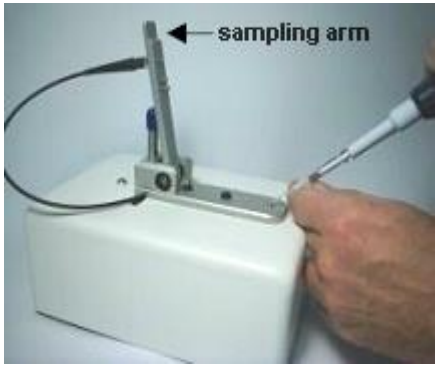


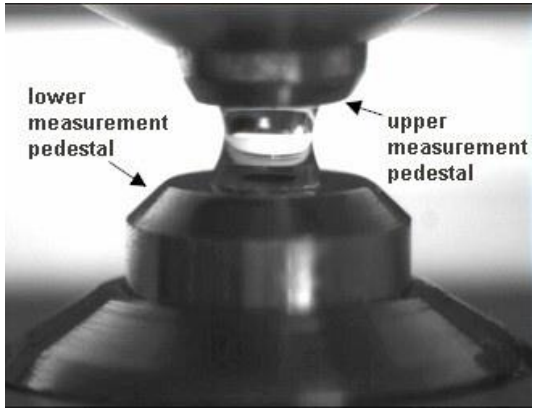


Quantitative analysis of RNA

ng/µl with Nano drop/double beam spectrophotometer (ND-1000 Spectrophotometer V3.2 Applied biosystem)







Polymerase Chain Reaction

Kary B Mullis

Thermocycler

The polymerase chain reaction (PCR)
is a technique to amplify a piece of DNA very rapidly outside of a cell

The PCR is *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonulcotide primers that hybridize to opposite strand and flank the region of interest in the target DNA

PURPOSE OF PCR

- Amplify specific nucleic acids in vitro (“Xeroxing” DNA)
- PCR will allow a short stretch of DNA (usually fewer than 3000 base pairs) to be amplified to about a million fold within a few hours
- This amplified sample then allows for size determination and nucleotide sequencing
- When routine diagnostic methods fail to a diagnose a specific disease
- When rapid results are needed with more reliability

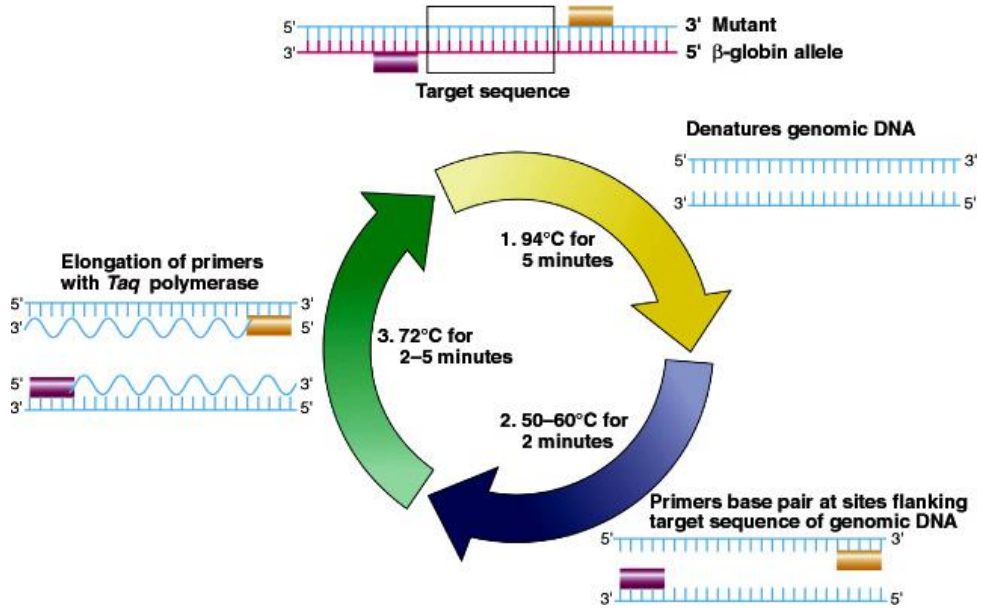
Some applications of PCR

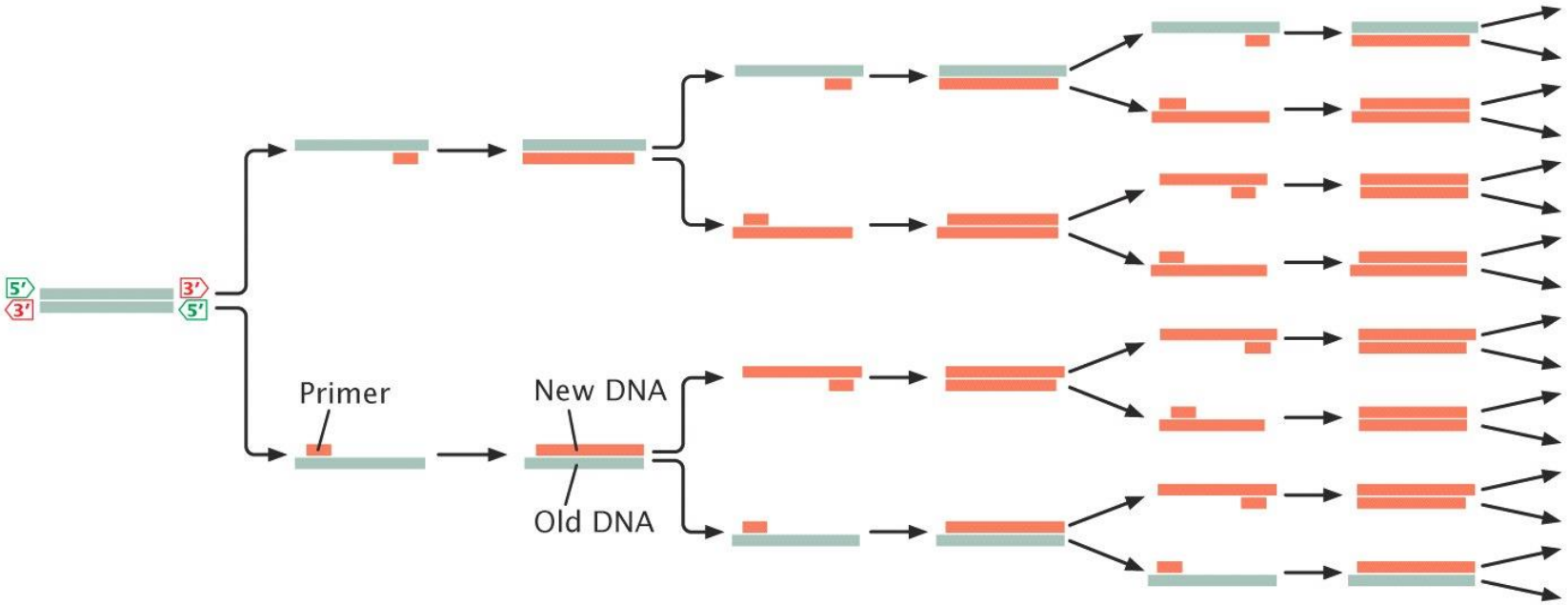
- Forensic medicine
- DNA Fingerprinting
- Detection of variations and mutations in genes
- Molecular biology
- Archeology
- Medical diagnosis
- Paternity testing

Steps Involved in PCR

- **Step 1: Denaturation (95^{o}C-98^{o}C).**
- **Step 2: Annealing (45^{o}C-65^{o}C)**
- **Step 3: Extension (70^{o}C-75^{o}C)**

Polymerase Chain Reaction





Three Steps

- Separation: Double Stranded DNA is denatured by heat into single strands.
- Short Primers for DNA replication are added to the mixture.
- DNA polymerase catalyzes the production of complementary new strands.
- Copying the process is repeated for each new strand created
- All three steps are carried out in the same vial but at different temperatures

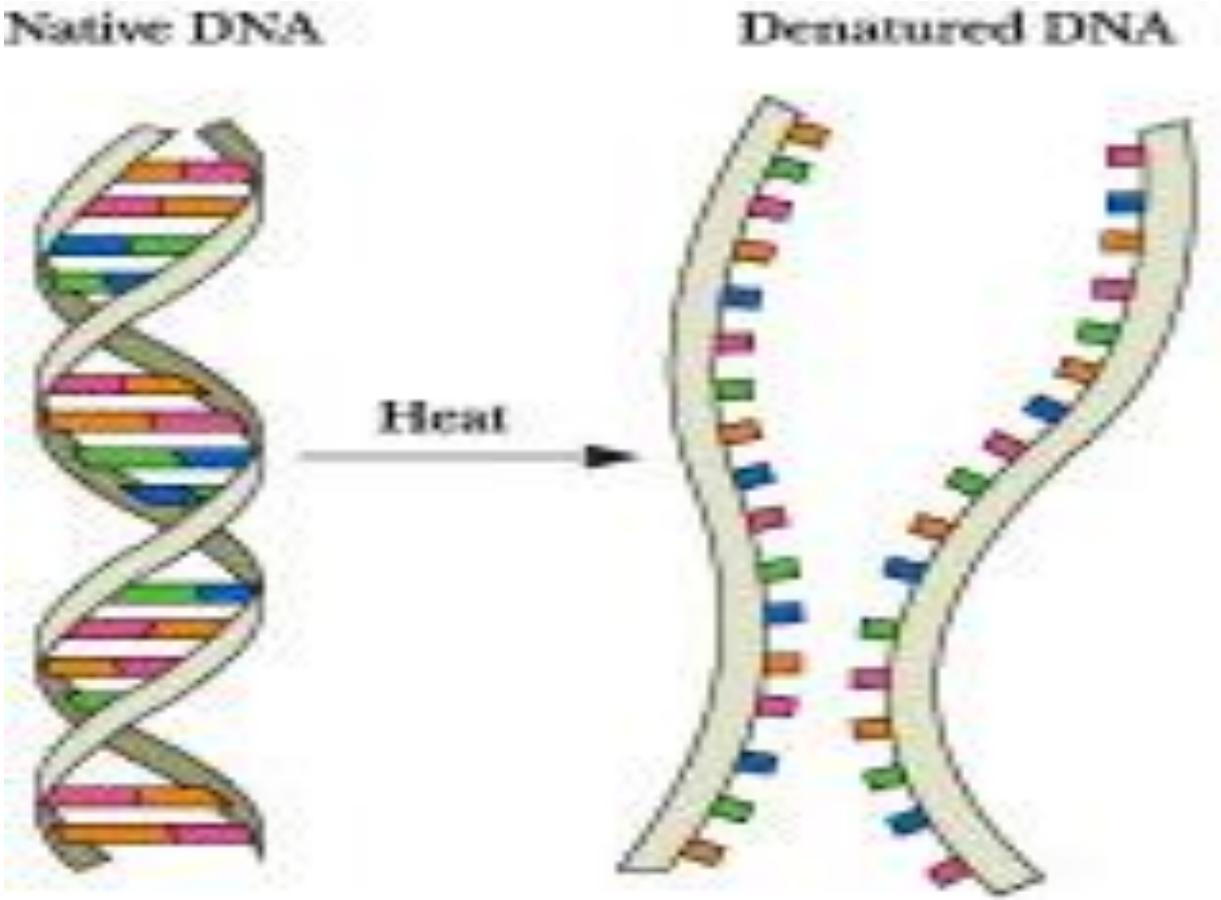
Step 1: ***Denaturation***

- Combine Target Sequence, DNA primers template, dNTPs, Taq Polymerase
- Target Sequence: Usually fewer than 3000 bp
  - Identified by a specific pair of DNA primers- usually oligonucleotides that are about 20 nucleotides
- Heat to 95^{o}C to separate strands (for 0.5-2 minutes)
  - Longer times increase denaturation but decrease enzyme and template

Magnesium as a Cofactor

- **Stabilizes the reaction between:**
  - **oligonucleotides and template DNA**
  - **DNA Polymerase and template DNA**

Heat Denatures DNA by uncoiling the Double Helix strands



Step 2: *Annealing*

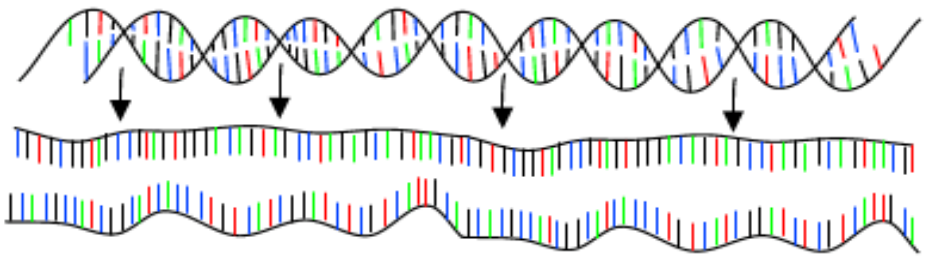
- Decrease temperature by 15-25 degrees
- Primers anneal to the end of the strand
- 0.5-2 minutes
- Shorter time increases specificity but decreases yield

Step 3: *Extension*

- Increase temperature by 70-75 degrees
- The DNA polymerase recognizes the primer and makes a complementary copy of the template which is now single stranded.
- For 2 minutes
- Approximately 150 nucleotides/sec

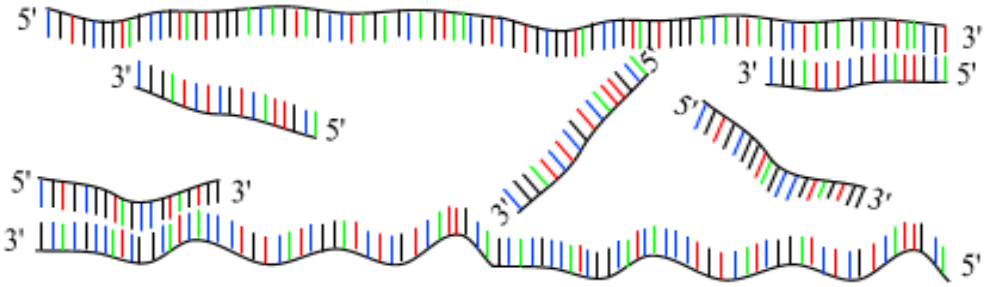
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

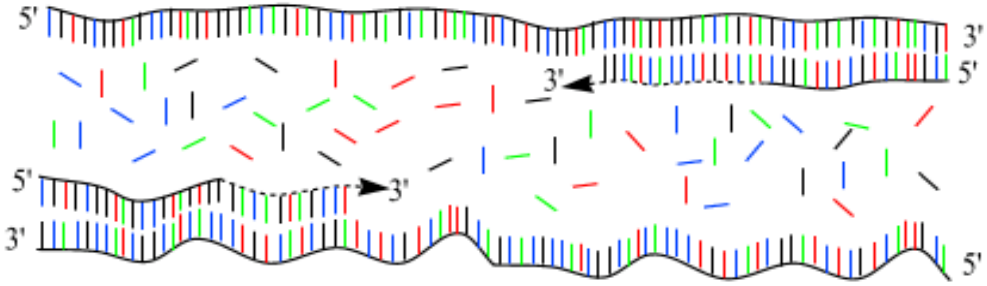
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



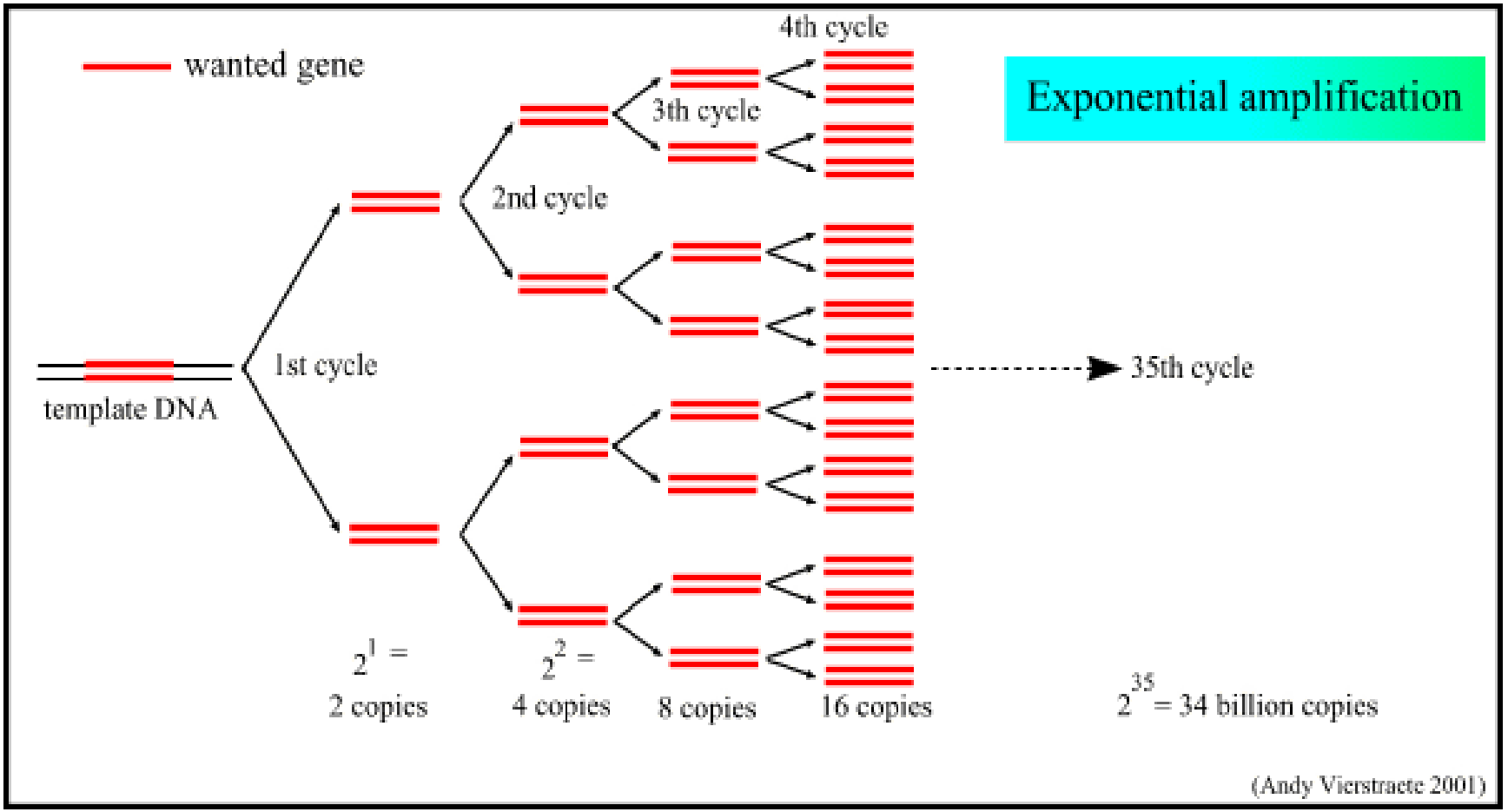
Step 3 : extension

2 minutes 72 °C

only dNTP's

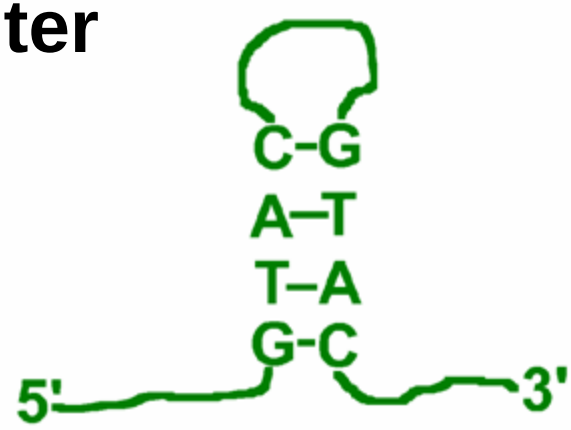
(Andy Vierstraete 1999)

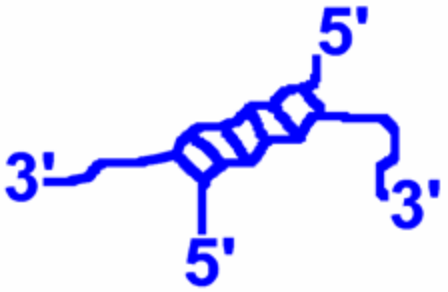
Amplification



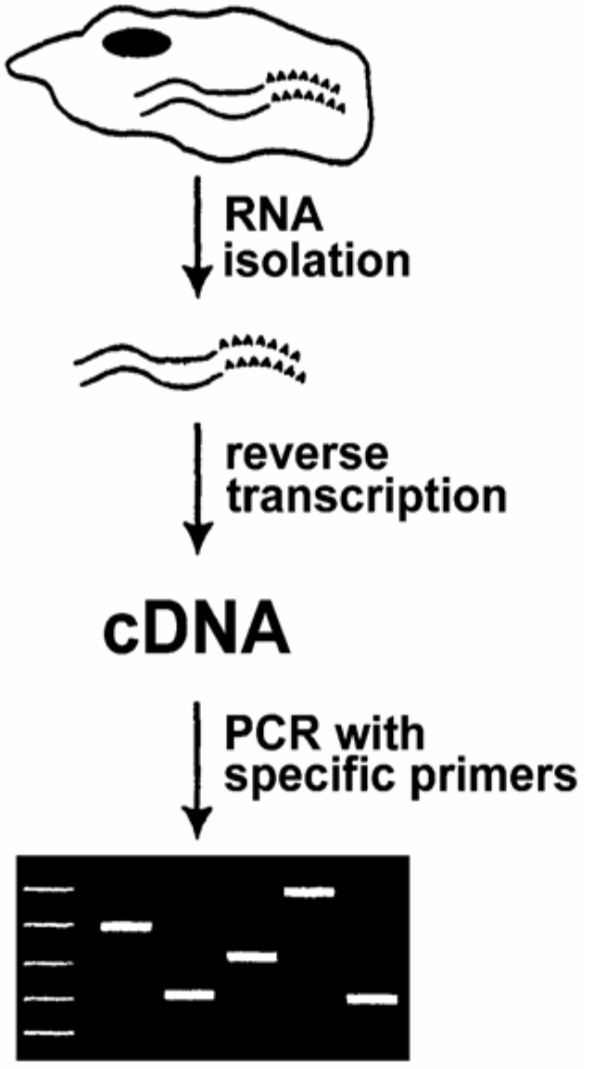
Design of Oligonucleotide Primers

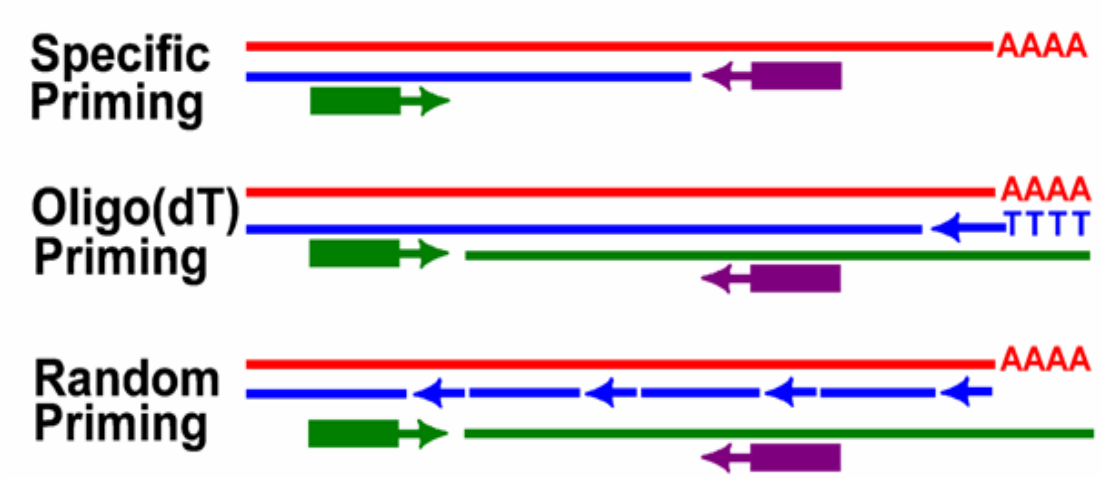
- analyze sequence with computer
  - amplicon length (250-1000 bp)
  - uniqueness (18-28 bases)
  - T_m > 55°
  - 50% GC composition
  - 3'-GC 'caps'
  - no internal complementarity
  - no 'primer dimers'
- HPLC purification (optional)





RT-PCR


RNA
isolation
reverse
transcription
cDNA
PCR with
specific primers


Specific
Priming
AAAA
Oligo(dT)
Priming
AAAA
TTTT
Random
Priming
AAAA

Producing cDNA

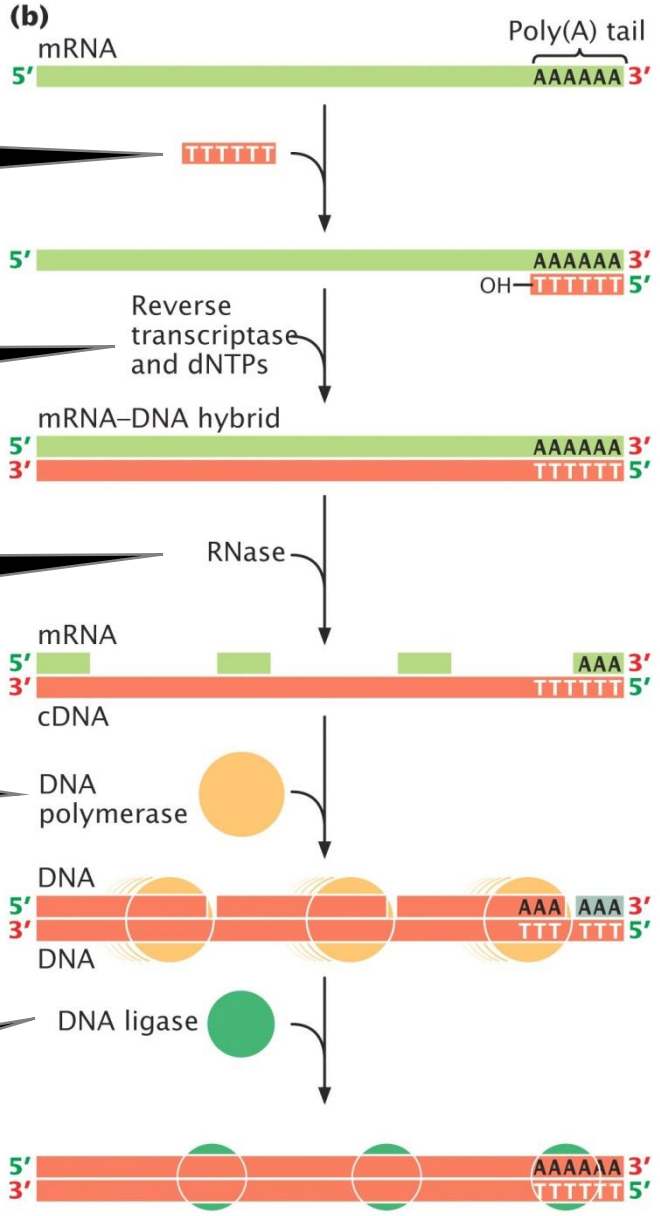
Primer binds to poly-A tail

DNA produced from RNA template

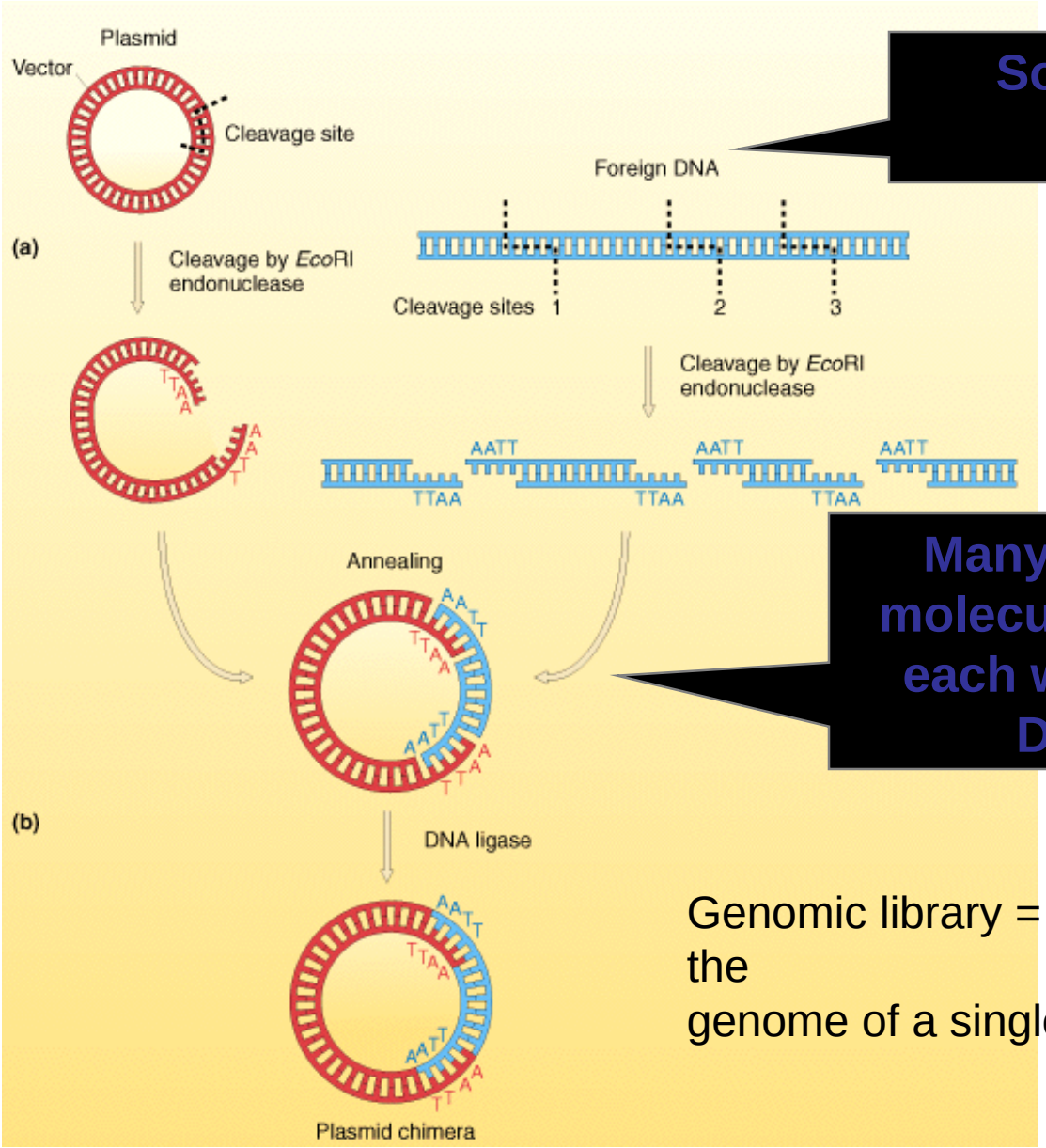
Partial digestion of RNA template

Second DNA strand produced using remaining RNA segments as primers

Gaps in backbone sealed



Producing a Genomic Library



Source of DNA for Library

Many recombinant molecules are formed, each with a different DNA insert

Genomic library = all of the sequences from the
genome of a single organism

In situ hybridization

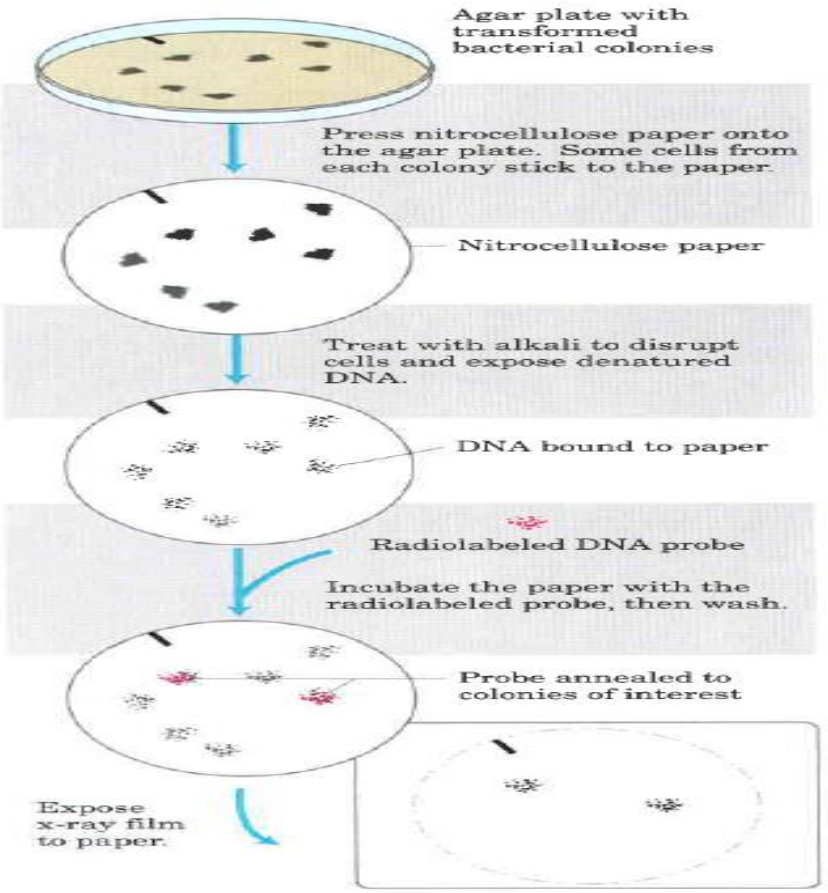


FIGURE 9–8 Use of hybridization to identify a clone with a particular DNA segment. The radioactive DNA probe hybridizes to complementary DNA and is revealed by autoradiography. Once the labeled colonies have been identified, the corresponding colonies on the original agar plate can be used as a source of cloned DNA for further study.