2010

Programme Booklet

Department of Biochemistry

NSS Enriching Knowledge for the Biology Curriculum:

Biotechnology – Recombinant DNA Technology

生物化學系（理學院）
Biochemistry(Science)

香港中文大學
The Chinese University of Hong Kong

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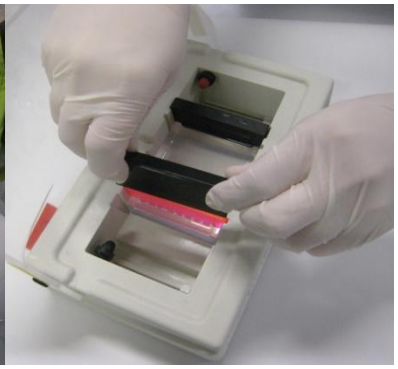

Preface

To all participants:

We would like to extend our warmest welcome to all participants of this training workshop. This workshop is specifically tailored for biology teachers who are interested in biotechnologies.

As an important part of the New Senior Secondary School (NSS) curriculum, the biotechnology module in the science curriculum is not only a new domain of knowledge for students, but also a great challenge for teachers to teach the abstract science concepts. In view of these challenges and the availability of our expertise in the area, we have developed this training workshop that aims to equip participants with common practical skills in molecular biology and to enrich them with the essential knowledge to develop suitable experiments for their students.

This one day training course is composed of both lecture and practical work. The intensive lecture session will introduce the major principles of recombinant DNA technology and their applications in medical and agricultural aspects. The practical work session comprises a series of exercises on common molecular techniques such as the extraction of DNA, polymerase chain reaction (PCR) and DNA finger-printing. Instructors will also share their experience in planning and conducting an experiment of molecular biology for students.

Finally, we wish all participants a fruitful workshop and enjoyable stay in the campus.

The Organizing Committee
April 2010

生物化學系（理學院）
Biochemistry(Science)

Programme Schedule

NSS Enriching Knowledge for the Biology Curriculum: (1) Biotechnology – Recombinant DNA Technology

Date*: 9 Apr 2010 (Friday) / 23 Jun 2010 (Wednesday) / 29 Jun 2010 (Tuesday)
Time: 8:45 - 17:00
Venue: Room 619 - 620, Mong Man Wai Building, The Chinese University of Hong Kong, Shatin, New Territories.

Time	Programme
8:45 - 9:00	Registration and briefing
9:00 - 12:00	Task 1: Extraction of Genomic DNA from buccal cells and amplification of D1S80 loci using polymerase chain reaction (PCR) Lecture 1: Principles of recombinant DNA technology
12:00 - 13:00	Task 2: Heat-shock transformation of DNA vectors into bacterial cells Task 3: Spreading transformed bacterial cells on agar plates
Lunch break	
14:00 - 16:00	Lecture 2: Applications of recombinant DNA technology Task 4: Analysis of the PCR products by agarose-gel electrophoresis
16:00 - 16:30	Demonstration 1: Bacterial colony selection and determination of transformation efficiency Demonstration 2: Detection of recombinant Orange Fluorescent Protein (OFP)
16:30-17:00	Q&A Session

* It is a one-day course. Each participant shall take one course only.

Principles of Recombinant DNA Technology

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The Chinese University of Hong Kong

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Concept Map

- Recombinant DNA Technology
  - DNA Structure
  - The Central Dogma: DNA → RNA → Protein
  - DNA Fingerprinting
    - DNA Isolation
    - Restriction Enzymes
    - VNTR
    - Agarose Electrophoresis
    - DNA Detection by Fluorescence
    - Polymerase Chain Reaction (PCR)
  - Recombinant Protein Production
    - Bacterial Plasmid
    - Bacterial Transformation and Culture
    - Production of Recombinant Protein by Bacteria

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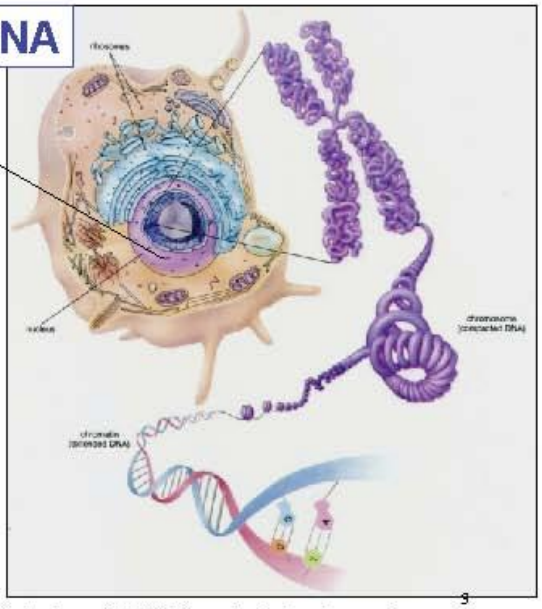
Remarks :

Cell-Chromatin-DNA

nucleus

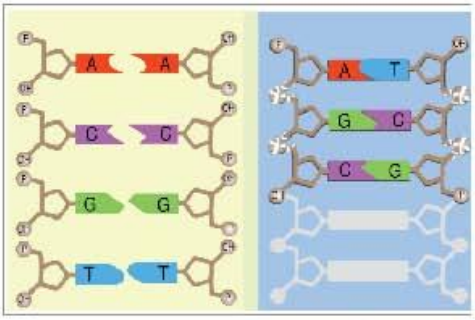


DNA is a very large molecule that must somehow be packed into a small nucleus. These packing problem is solved by **coiling DNA** & **packing it with proteins (e.g. Histone).**

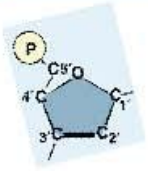
https://www.msu.edu/course/isb/202/ebertmay/2004/drivers/cell_to_dna_sm.jpg

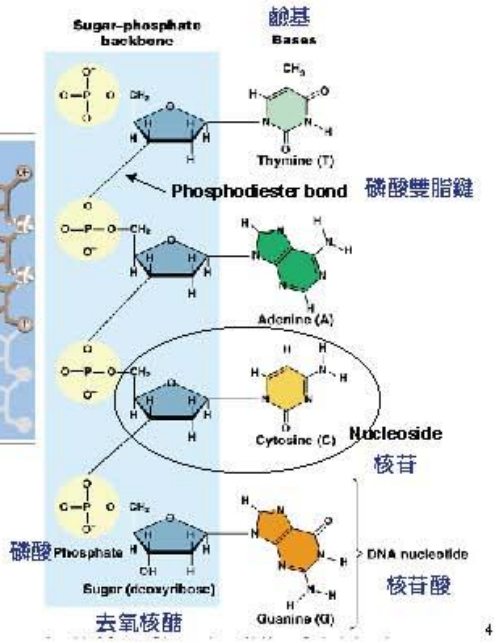
DNA Structure

DNA: Deoxyribonucleic acid



- 3'-, 5'-ends
- Direction
- Charge of DNA
- Base pairing





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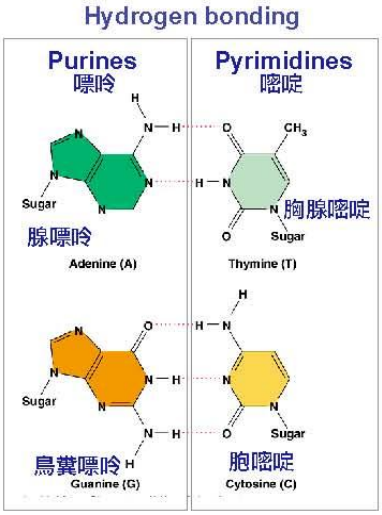
DNA Structure



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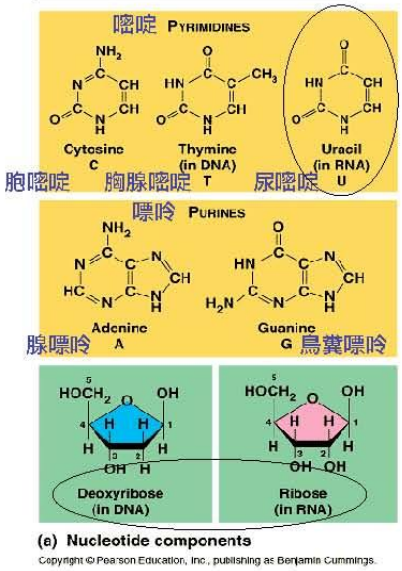
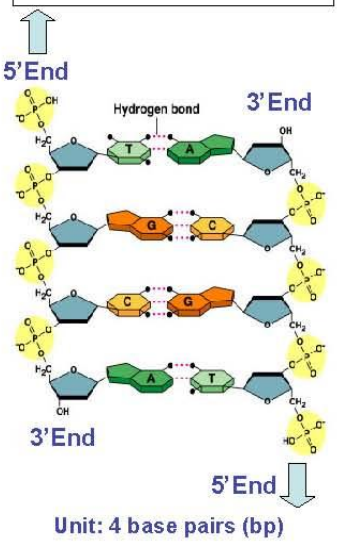
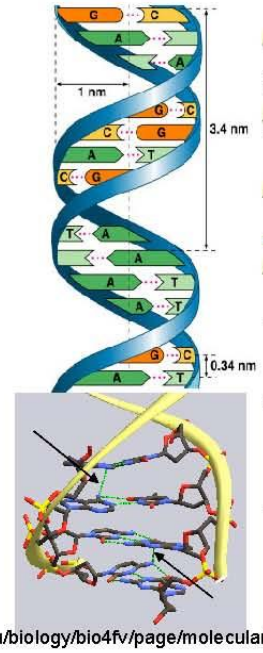
DNA and RNA



(a) Nucleotide components

DNA Structure





Summary:

- DNA is double stranded. Two single strands lie side by side, parallel to each other.
- Held together by H-bonds between adjacent bases.
- Watson & Crick Base Pairs: (A – T) (G – C)
- The double strand is wound into a helix, known as the double helix.
- The two strands of DNA are Anti-Parallel.

http://academic.brooklyn.cuny.edu/biology/bio4fv/page/molecular%20biology/dna-structure.html

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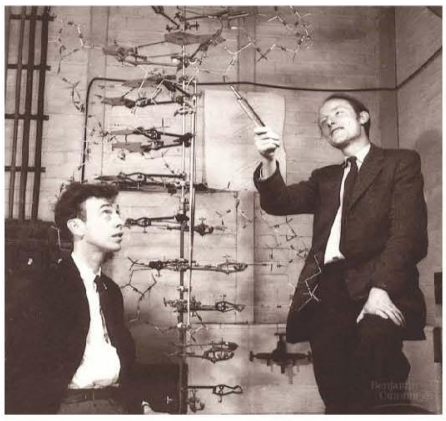
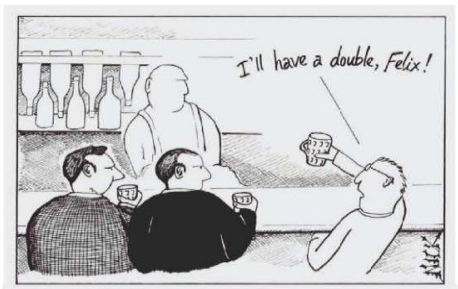
Remarks :

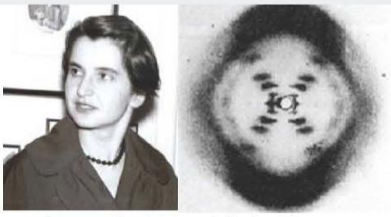
DNA Structure



JD Watson (left) & Francis Crick (Cambridge) with their model of DNA in1953.



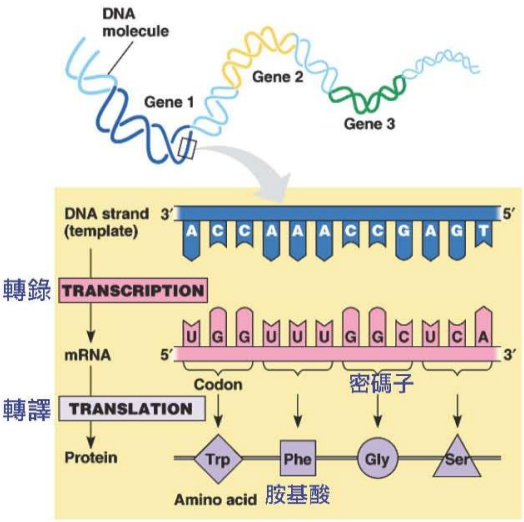
Cambridge, 1953. Shortly before discovering the structure of DNA, Watson and Crick, depressed by their lack of progress, visit the local pub.



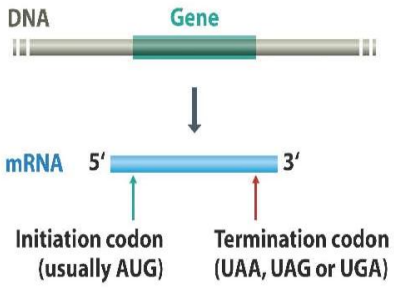
Rosalind Franklin's X-ray diffraction photo of DNA, 1953.

The Central Dogma: DNA→ RNA → Protein

(Flow of Genetic Information) 1957 proposed by Francis Crick

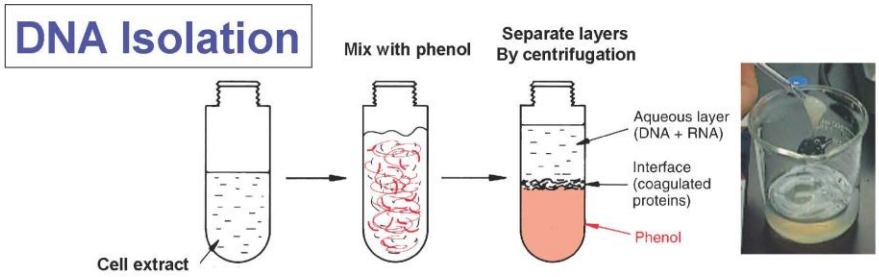


- BRCA1 (breast cancer 1, early onset):
  Mutation → Cancer
  Location: 17Q21
- Rnd2 (~21 kDa): G-Protein signaling (GTPase)
  Location: 17q21
  Centromeric neighbor of BRCA1



Remarks :

DNA Isolation



- **Cell Breakage:** Sonication, grinding, detergent digestion
- **Removal of Protein:** By enzymes (proteinase K)
  By solubility: phenol/chloroform extraction
- **Removal of RNA:** By enzyme (RNase)
- **Concentration of DNA:** Precipitating DNA in the aqueous phase with alcohol
- **Determination of the purity of DNA**
  Pure DNA: OD_{260}/OD_{280} = ~1.8.
  Pure RNA: OD_{260}/OD_{280} = ~2.0.
  Low ratios: with protein or phenol contamination.

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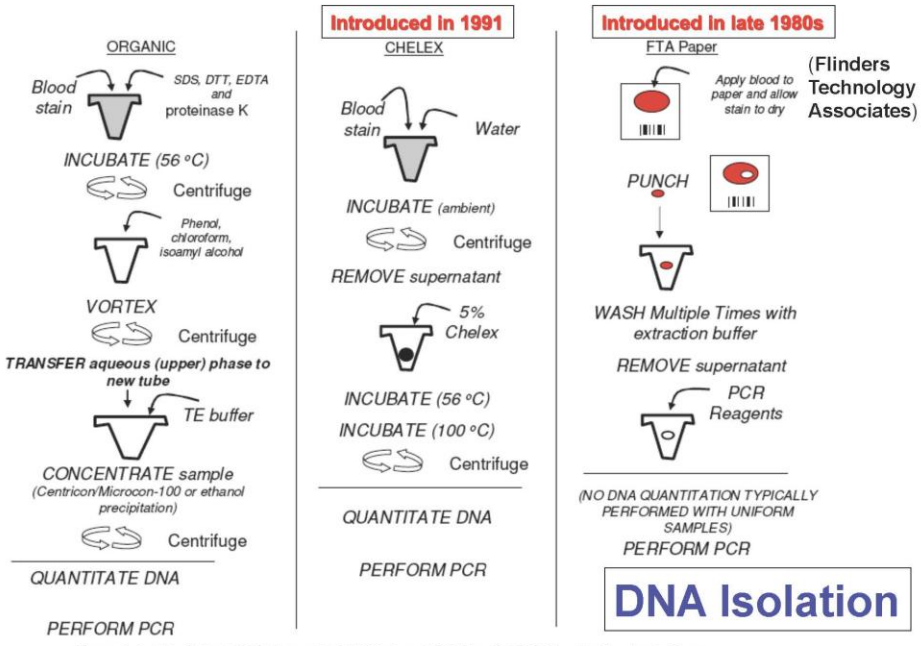

Figure 3.1, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Remarks :

DNA Isolation

Technical Tips:

- Safety: Phenol, Low vapour pressure → Poisoning (anesthetic).
- Avoid hydrodynamic shearing of high mol wt DNA, pipette DNA solution slowly.
- DNA solution storage: pH > 8.5 to avoid deamidation.
- DNase contamination from skin. Use sterilized plastic tubes & solutions.
- Add DNase inhibitor in solution: e.g. EDTA (ion chelator) (~50mM).
- Heavy metals promote the breakage of phosphodiester bonds (EDTA).
- EB: EB caused photo-oxidation of DNA. Store the DNA gel stained with EB in dark.
- DNA storage:
  - Short-term usage The best temp for is 4-6°C without freezing & thawing.
  - Long term storage: -70°C or below.
  - Storage at -20°C is not recommended as the molecular bound water is not frozen. Freezing & thawing at -20°C cause DNA breakage.

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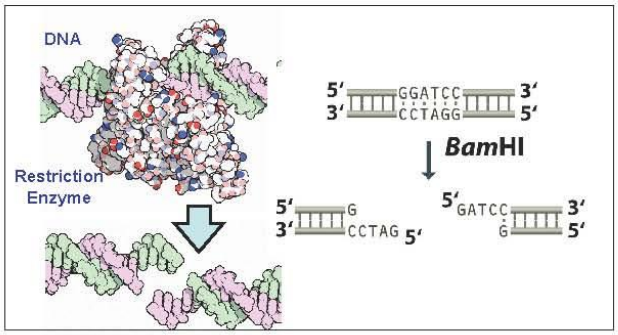
Restriction Enzyme (限制性內切酶)

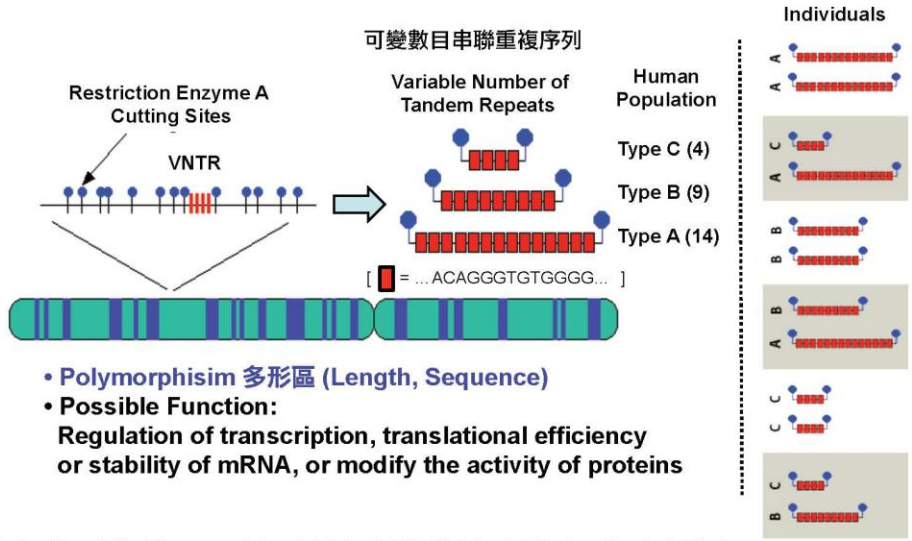


Enzyme	Recognition Sequence
BamH I	GGATCC CCTAGG
Not I	GCGGCCGC CGCCGGCG
Sau3A I	GATC CTAG
Sac I	GAGCTC CTCGAG
Sst I	GAGCTC CTCGAG
Hinf I	GANTC CTNAG
Xho II	PuGATCPy PyCTAGPu

http://hpdb.hbu.cn/molecule/restriction_enzyme/restriction_enzyme.asp

Remarks :

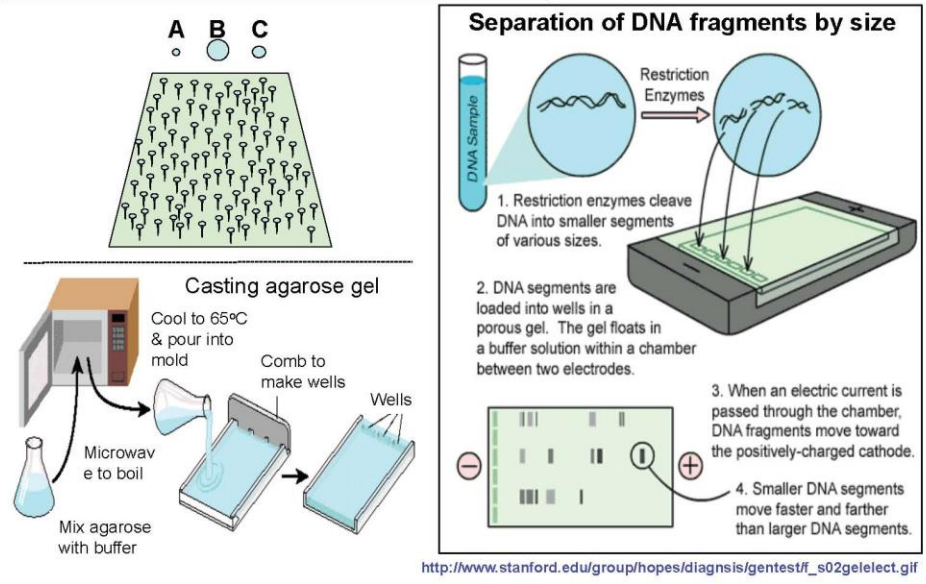
VNTR (variable number of tandem repeats)



- Polymorphisim 多形區 (Length, Sequence)
- Possible Function:
  Regulation of transcription, translational efficiency or stability of mRNA, or modify the activity of proteins

Animation: http://ihome.cuhk.edu.hk/~z045513/virtuallab/animation/vntr.html

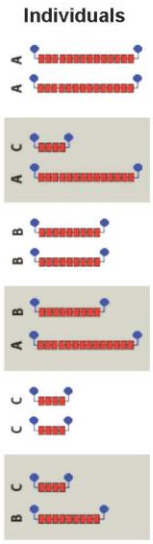
Agarose Electrophoresis

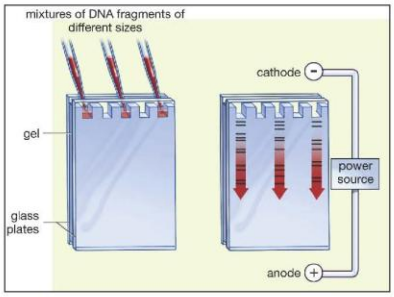


http://www.stanford.edu/group/hopes/diagnsis/gentest/f_s02gelelect.gif

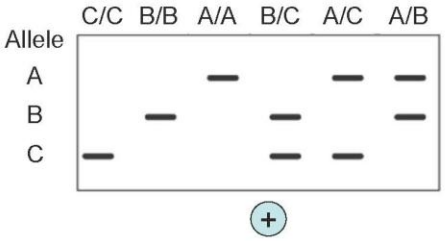
Remarks :

DNA Electrophoresis





- 6 diploid genotypes are present in this population
- These DNA fragments are separated by agarose electrophoresis



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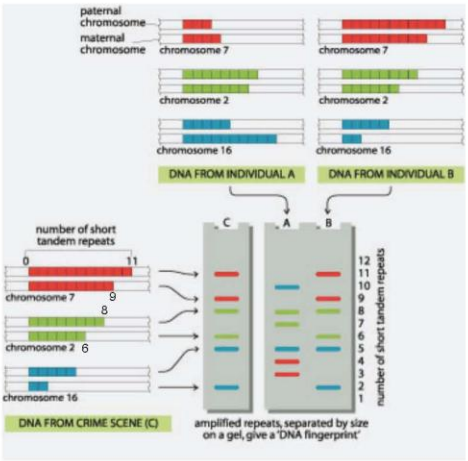
DNA Fingerprinting



http://www.randomhouse.com/knopf/authors/watson/images7.html

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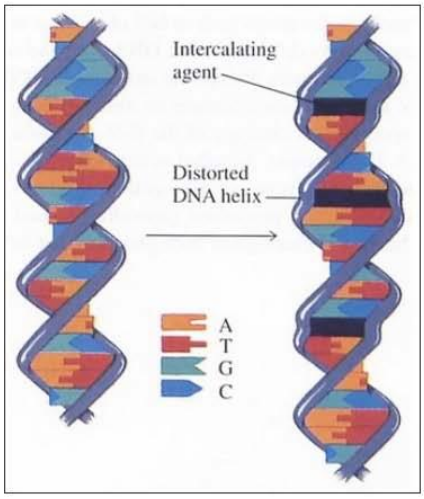
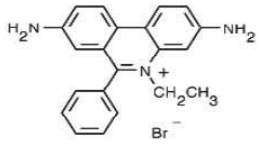
Remarks :

DNA Detection by Fluorescence



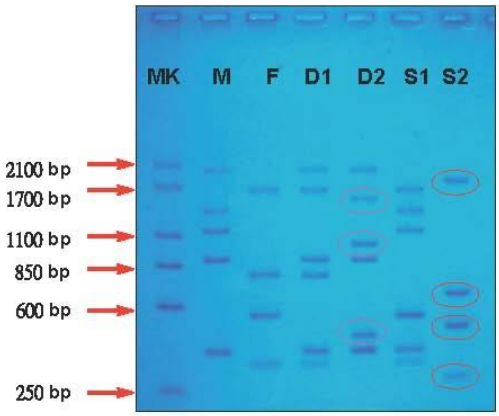


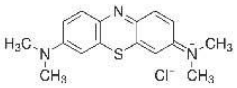
Ethidium Bromide (0.5 µg/ml)
Ex: UV ~260nm, Em ~590nm
May be a carcinogen !!!

http://sandwalk.blogspot.com/2007/07/ethidium-bromide-binds-to-dna.html

LAB

DNA Fingerprinting Results





Methylene Blue Staining

Lane MK: DNA marker
Lane M: Mother
Lane F: Father
Lane D1: Daughter 1
Lane D2: Daughter 2
Lane S1: Son 1
Lane S2: Son 2

(Biologic child / Adopted child / Stepchild)

Department of Biochemistry
The Chinese University of Hong Kong
香港中文大學生物化學系

Remarks :

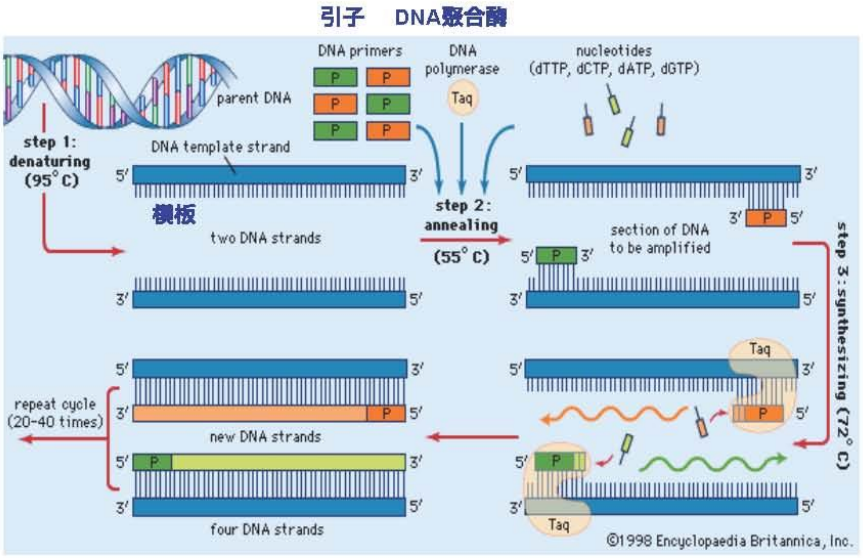
DNA Fingerprinting

Technical Tips:

- Store restriction enzyme at -20°C in a freezer that is not frost free.
- Aliquots with ~30µl/tube.
- Use 10 units or more of RE/µg DNA (>20-fold excess enzyme).
- Agarose electrophoresis buffer:
  1) TAE (Tris-acetate EDTA):
     - low pH buffering capacity, for high voltage electrophoresis.
     - bromophenol blue migration ~1cm/hr at 10V/cm.
     - dye co-migrates with the smallest DNA.
  2) TBE buffer (Tris-borate EDTA):
     - high pH buffering capacity, for DNA <12000 bp.
     - DNA migration in TBE slower than that in TAE → DNA bands are sharper.
     - Pore size of agarose in TBE (borate) < that in TAE.
- DNA loading dye solution: Add urea (~5M) to denature protein → reduce smear.
  glycerol or sucrose (~10%) to increase density → U shape DNA band.
  Other reagent: Ficoll 400 (15-20%).
- DNA pre-stained with EB: mobility reduced ~15%.
- Safety: - To avoid electric shock electrophoresis, disconnect positive lead first.
  - UV light: Wear protective face shield.

Helpful Tips

Polymerase Chain Reaction (PCR) (聚合酶鏈式反應)

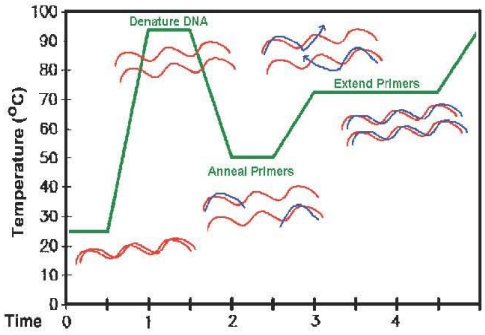


Remarks :

Polymerase Chain Reaction (PCR) (聚合酶鏈式反應)

The polymerase chain reaction (PCR) is a biochemical technique that can isolate and exponentially amplify a DNA fragment of interest via a chain of enzymatic reactions.

PCR是Kary Mullis博士於1983年想出來的方法, PCR將一段極少量的DNA, 倍增成為極多具相同排列的複本。而這項技術使Mullis博士獲得了1993年諾貝爾化學獎。



http://www.copernicusproject.ucr.edu/ssi/HighSchoolBioResources/Genetic%20Engin%20Hum%20Genome/pcr.jpg

http://www.youtube.com/watch?v=_YgXcJ4n-kQ

• A very good animation to show the outcomes after PCR cycles.

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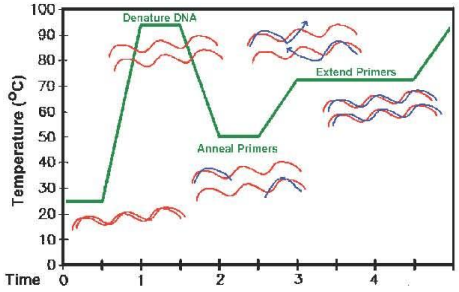
Thermocycler



Kary B. Mullis Winner of the 1993 Nobel Prize in Chemistry

http://oregonstate.edu/dept/ncs/newsarch/2002/Jan02/dna.htm

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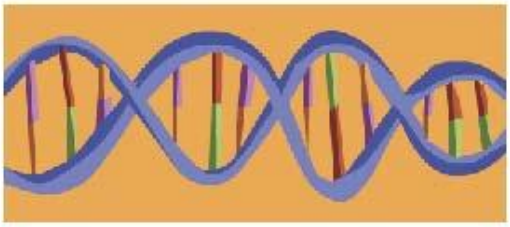
http://www.kscience.co.uk/as/module2/dna_structure/DNA_files/History%20of%20DNA%20research.htm

Remarks :



Q&A:

1) We usually use the DNA from cheeks cells for DNA-fingerprinting test. Will the bacteria or food residues in our mouth affect the paternity test?

2) DNA fingerprinting is a very powerful technique in solving criminal cases and paternity relationship. Name some **undesirable effects/outcomes of this technique.**

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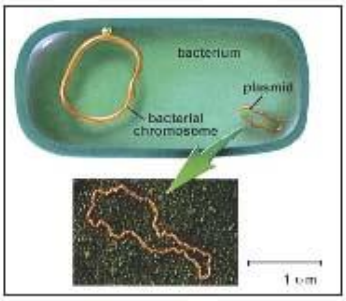
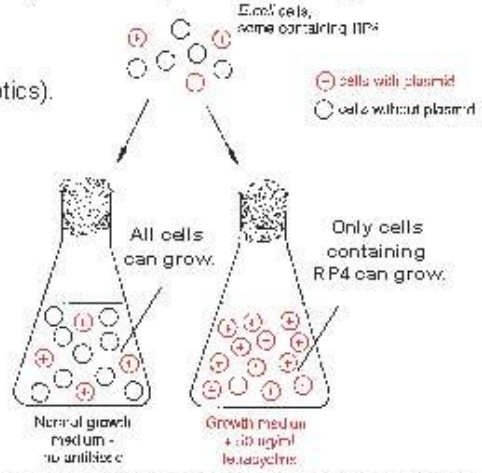
Bacterial Plasmid (質粒)

Plasmid (質粒):
- A circular, extra-chromosomal DNA,
- Can replicate itself.
- Sometimes >1 copy/cell.
- Carries a particular trait

(e.g. produce enzymes to destroy antibiotics).



RP4 Plasmid: With genes to produce enzymes to destroy ampicillin (block cell wall synthesis), tetracycline & kanamycin (both act on protein synthesis).

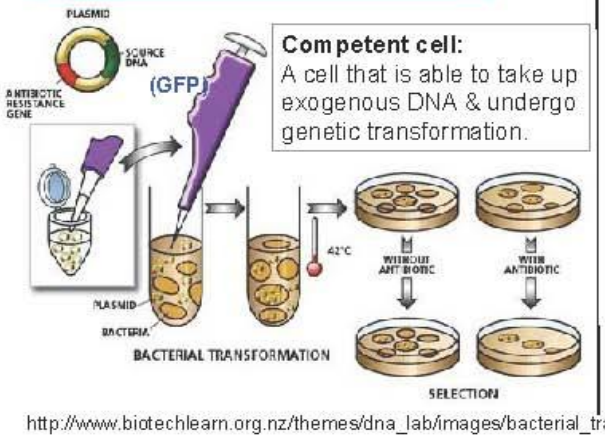


http://www.agen.ul.edu/~chyn/age2062/OnLineBiology/OLBB/www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookDNAfinity_2.html

Remarks :

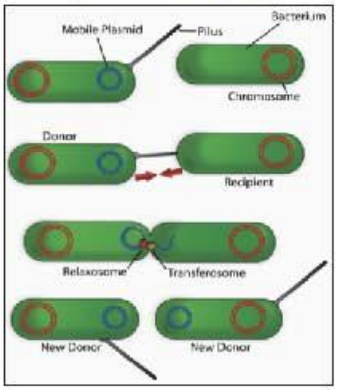
Transformation (細菌轉化)

Heat Shock:
1) Bacteria on ice for 20 min.
2) Heat bacteria at 42°C for 30-45 sec.
3) Culture bacteria at 37°C afterwards.



Competent cell:
A cell that is able to take up exogenous DNA & undergo genetic transformation.

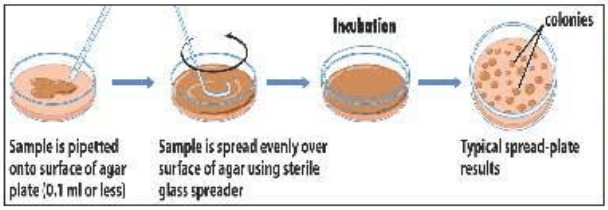
Bacterial Conjugation (細菌接合作用)



http://www.biotechlearn.org.nz/themes/dna_lab/images/bacterial_transformation

Bacterial Culture

Spread plate method (塗佈培養法)



Colony = Single bacterium

Flame your spreader with alcohol – Beware of fire!

- Bacterial colonies with GFP grown in agar plate with antibiotics.
- GFP emits green fluorescence (荧光) under UV excitation.

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Remarks :

Transformation (細菌轉化)

Heat Shock Mechanism:

- Adding the plasmid to the cells on ice makes the plasmid **adhere** to the cell wall.
- The heat shock **opens the pores** and gets the plasmid to enter the cell.
- **Lowers the membrane potential (?)**, facilitates DNA to cross inner membrane of E. coli.
- Placing the cells on ice after heat shock closes the pores.
- Many bacteria use filaments called **type 4 pili** to create pores in their membranes. Grab and **pull DNA** through the pores into the cytoplasm.

Transformation Efficiency:

- A measure of the number of cells within the bacterial culture that are able to take up DNA.
- Results are expressed in number of (**cfu / µg DNA**), (cfu = colony forming units).
- **Example:** How much DNA is plated?
  1 µL of 0.1 ng/µl DNA is added to 100 µL of competent cells. 900 µL of medium is added prior to expression. 10 µL (equivalent to 0.001 ng DNA) is then diluted in 990 µL medium and 100 µL is plated (equivalent to 0.0001 ng DNA).

  If 100 colonies are counted on the plate, the transformation efficiency is:
  100 transformants / 0.0001 ng X 1000 ng/µg = 1 x 10^9 transformants/µg.

27

Transformation (細菌轉化)

Troubleshooting

Possible Cause	Solutions
Impurities in the DNA	Remove phenol, proteins, detergents, and ethanol from the DNA solution.
Excess DNA or volume	Add 1 to 10 ng of DNA in no more than a 5-µl volume per 100 µl of bacterial cells.
Improper heat-shock procedure	Follow exactly the protocols as written
Slow or no growth of cells	Incubate the transformed colonies longer.
Overgrowth (little or no selection)	Use correct antibiotic and concentration.
Too many satellite colonies (tiny colonies that surround the big antibiotic-resistant colony) as the antibiotic-resistant cells destroy the antibiotics in their immediate vicinity on the plate.	Don't use the old plate (old meaning that the antibiotic is partially degraded). Don't plate cells at high density.
Freeze/Thawing of bacterial cells	Cells that are refrozen will loss their activity.

Reference: Trond Erik Vee Aune & Finn Lillelund Aachmann (2010) Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Appl Microbiol Biotechnol, 85:1301–13. (A good introduction about history and methods of bacterial transformation)

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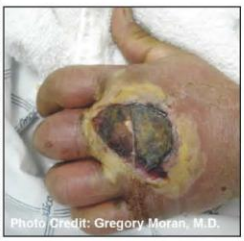
Remarks :

Follow-up Action

- Kill bacteria by autoclave before disposal.
- Autoclave: Steam heat under pressure, 121°C, 15 psi, for at least 15 min.



A patient's hand showing MRSA infection.

近年肆虐中港台韓

抗藥惡菌源自日本

【本報訊】抗藥性金黃葡萄球菌（MRSA）在全球肆虐，不論是醫院或社區感染個案也不斷增加。一項最新的國際研究發現，近年在本港、內地、台灣、日本及韓國等流行的抗藥性金黃葡萄球菌品種，源頭是來自日本，惡菌可於短時間在鄰近地區流行，研究更發現抗藥惡菌的變種速度驚人，未來治療會越來越困難。

經基因分析鑑定

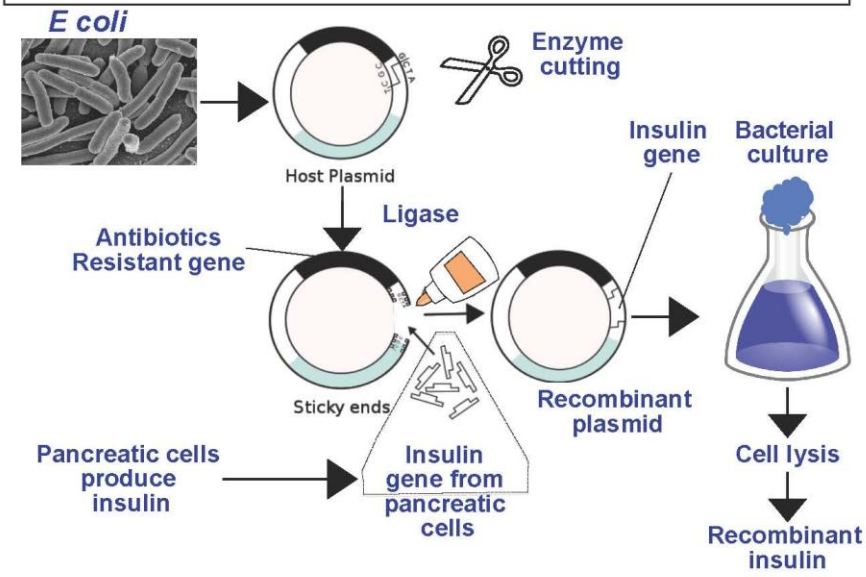
核國際研究組織共分析了135個從22個國家收集的抗藥性金黃葡萄球菌，基因分析能鑑定在不同區域抗藥惡菌的衍生源頭。組織成員之一韓國成均館大學醫學院教授高官洙表示，分析顯示現時在東亞地區肆虐的抗藥性金黃葡萄球菌，其實是源自日本的ST5類菌株，惡菌透過頻繁的旅客交往接觸而大規模傳播，估計目前在韓國發現的抗藥惡菌感染個案，約一半是屬於日本型的ST5惡菌，或由此型惡菌變種而來，有關研究結果刊於最新一期美國國家科學研究院學報（PNAS）。

現時各國衛生部門沒有聯手對付抗藥惡菌傳播的策略，高官洙強調，今次研究證實抗藥性金黃葡萄球菌是一種可跨境散播的高危惡菌，並非單純是個別國家的衛生問題，故各國應該盡快確立控制跨境傳播的政策，或可以減慢病例擴散的速度。

抗藥性金黃葡萄球菌分醫院感染或社區感染，後者近年爆發速度驚人，患者皮膚會長出膿瘡，惡菌更會產生毒素，一旦入血或引發致命性併發症，常用的抗生素不能殺死此惡菌。

Methicillin-resistant Staphylococcus aureus (MRSA)

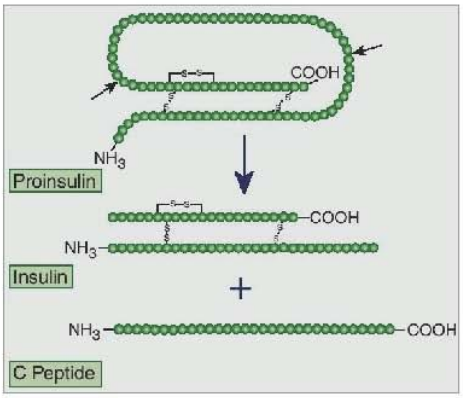
Use Bacteria to Produce Recombinant Proteins



Remarks :

Use Bacteria to Produce Recombinant Insulin

- Insulin is synthesized in pancreatic islet cells.
- It is made as a single polypeptide chain: Preproinsulin.
- Preproinsulin is proteolytically processed to form insulin.
- In mature insulin, the A (21 aa) & B (30 aa) chains are linked by –S-S- bridges.



Synthetic Insulin Chain A & Chain B sequences cloned separately into expression vector

↓

Bacterial Transformation & recombinant protein expression

↓

Mix purified insulin chains A & B

↓

Refold under oxidising conditions to promote disulphide bond formation

↓

Humuli (developed by Genentech in 1978)

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http://medical-dictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name=insulin.jpg

Use Bacteria to Produce Recombinant Insulins

- A fermenter used to grow recombinant bacteria.
- The final steps are to collect the bacteria, break open the cells, and purify the recombinant insulin.
- Recombinant insulin
  (From *E. coli:* trade name = Humulin®).
  (From Yeast: trade name = Novolin®) (1987).

Case study: Harvard Business School: Race to Develop Human Insulin.1991,9,191-121.

Remarks :

Applications of Genetic Engineering

Professor P.C. Shaw (邵鵬柱)
Department of Biochemistry
The Chinese University of Hong Kong

Genetic Engineering

- To use in vitro techniques to produce new combination of heritable material
- the alteration of genetic code by artificial means
- therefore different from traditional selective breeding

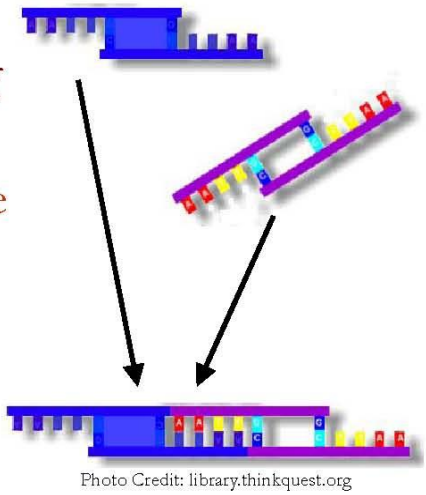


Photo Credit: library.thinkquest.org

Remarks :

Genetic Engineering - Theory

- Genetic code is universal – that is genes can be transferred from one organism to another
- A gene codes for the same protein whether it is in a human cell or any other cells
- Genetic Engineering can therefore cross species barrier

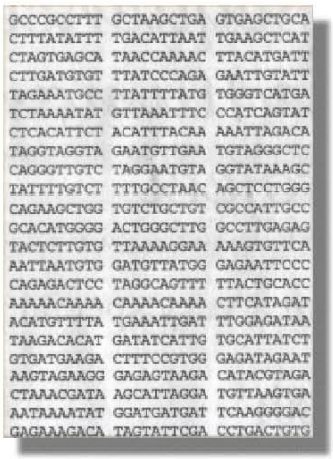
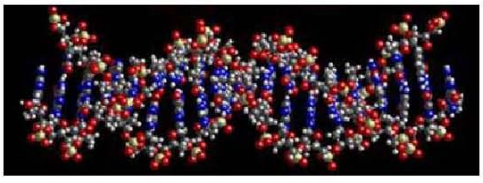


Photo Credit: academy.d20.co.edu

Genetic Engineering - History

- 1944 – O. Avery showed that DNA can alter the heredity of bacteria
- 1953 – James Watson and Francis Crick discovered that DNA is in the shape of a double-helix
- 1961 – Discovery of mRNA
- 1966 – Genetic Code is cracked
- 1970 – Discovery of restriction enzyme
- 1972 – Developed methods for generating recombinant DNA



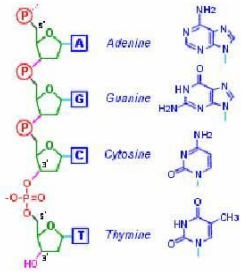


Photo Credit: www.eugenicsarchive.org, www.geneticengineering.org, www.astrobio.net

Remarks :

Genetic Engineering - History

- 1976 – Application of DNA technology to diagnose thalassemia
- 1977 – Rapid sequencing of DNA, development of protein engineering
- 1980 – Production of insulin by recombinant DNA procedures
- 1984 – Identification of gene for development
- 1985 – Polymerase chain reaction, a method for amplifying a minute amount of DNA

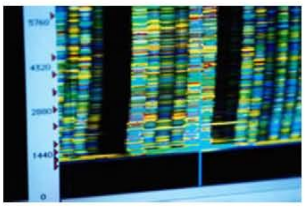


Photo Credit: www.biology.iupui.edu, www.utoronto.ca

Genetic Engineering - History

- 1988 – the first US patent on a genetic engineered higher animal – a mouse that develops cancer
- 1990 - Clinical trial of gene therapy on the deficiency of adenosine deaminase
- 1990 – the first genetically engineered gene product (recombinant rennin for making cheese) approved by FDA

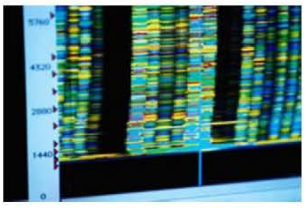


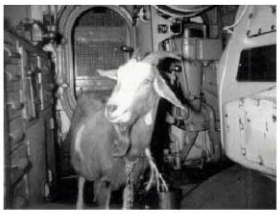
Photo Credit: www.biology.iupui.edu, www.utoronto.ca

Remarks :

Genetic Engineering - History

- 1992 – first sold of a genetically engineered crop plant – a tomato more resistant to rotting
- 1990s – sequencing of human genome, finished in 2003
- 2009 – FDA approved a blood thinning drug called ATryn produced from a genetically engineered goat

http://www.thenibble.com/reviews/nutri/matter/images/GoldenRice-230_000.jpg



http://www.treehugger.com/files/2009/02/first-drug--genetically-engineered-animals-approved-fda.php

How to generate recombinant DNA?

- The DNA is cleaved by restriction enzyme to generate compatible ends
- The two DNA fragments are joined together by DNA ligase

Watson J.D. et al. Recombinant DNA. 2nd ed. W.H. Freeman and Company

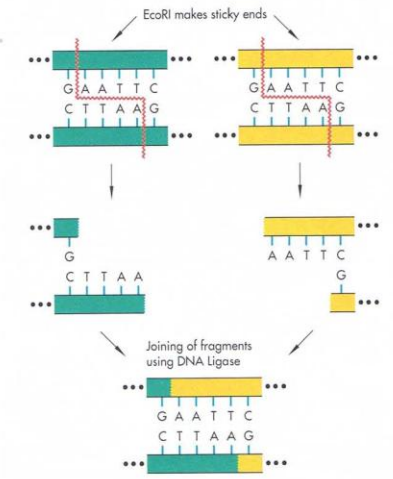


FIGURE 5-7
The *Eco*RI restriction enzyme makes staggered, symmetrical cuts in DNA away from the center of its recognition site, leaving cohesive, or "sticky," ends. A sticky end produced by *Eco*RI digestion can anneal to any other sticky end produced by *Eco*RI cleavage.

Remarks :

How to generate recombinant DNA?

- The foreign DNA is inserted to a plasmid vector, which acts as a vehicle for carrying the DNA to the cell.
- After entering the cell, the host becomes antibiotic resistant, because the presence of the plasmid.
- The host is propagated and selected by culturing in an antibiotic containing medium

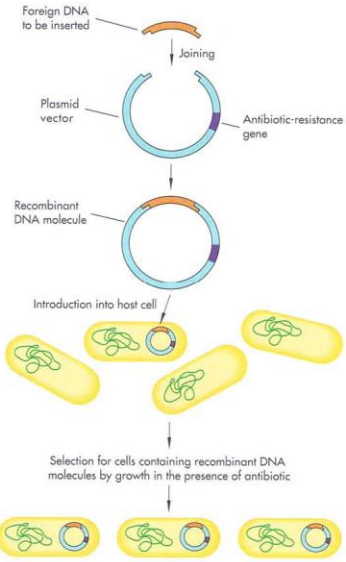


FIGURE 5-11
The cloning of DNA in a plasmid.

Example of Genetic Engineering – Production of interferon

- Interferon is an anti-viral agent made by human cells during viral attack
- mRNA of interferon is converted to DNA

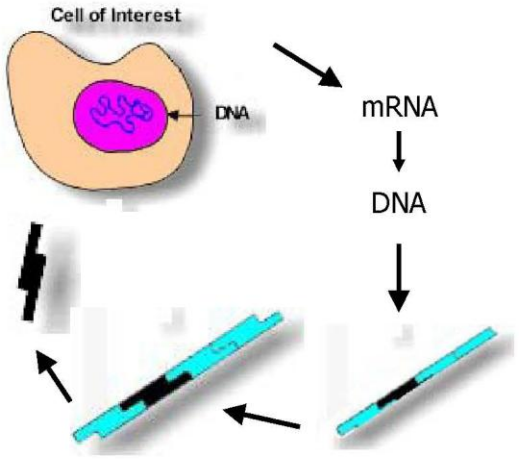


Photo Credit: library.thinkquest.org

Remarks :

Example of Genetic Engineering – Production of Growth Hormone

- Plasmid DNA is extracted from bacterial host
- They are cut open using a restriction enzyme
- Restriction enzyme cleaves which cut the DNA at specific base sequence

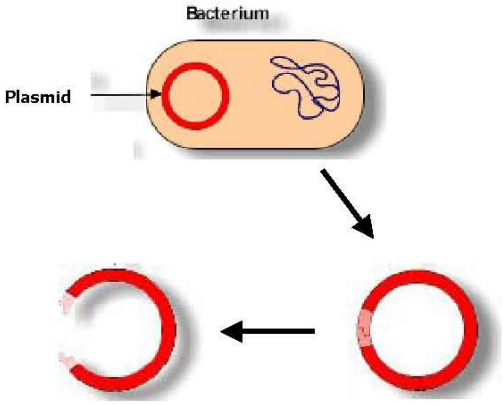


Photo Credit: library.thinkquest.org

Example of Genetic Engineering – Production of Growth Hormone

- The interferon DNA and the plasmid are mixed and they are joined together by DNA ligase
- The newly formed plasmid is known as a recombinant plasmid

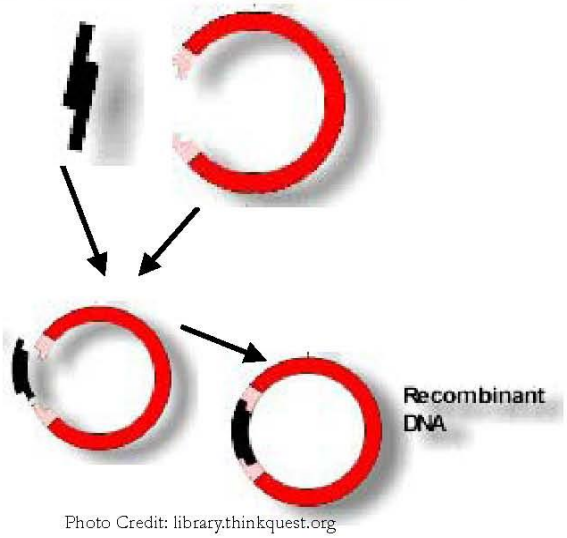


Photo Credit: library.thinkquest.org

Remarks :

Example of Genetic Engineering – Production of Growth Hormone

- The recombinant plasmids are mixed with host cells, which would absorb them
- The genetically modified E. coli would then be cultured in a fermenter and the interferon is extracted and purified
- 1978---$50,000/impure dose, 1980's---$1/dose via genetically engineered bacteria

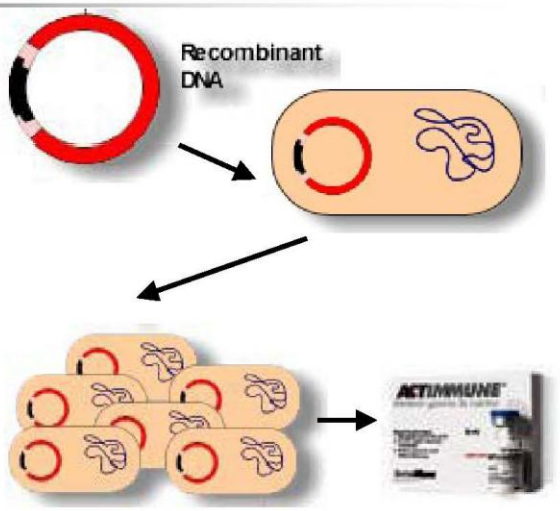


Photo Credit: library.thinkquest.org

Example of Genetic Engineering – Human Gene Replacement Therapy

- Gene therapy is the treatment of genetic disease by altering the genotype
- One technique is to insert the 'healthy' gene into the affected cells if the disease is caused by the 'defective' gene
- The best cells to use are stem cells, which can continue to divide and proliferate to different cell types
- An example is the curing the Rhys Evans who is suffering from severe combined immune deficiency (SCID), caused by lack of adenine deaminase (ADA) deficiency

Photo Credit: www.ananova.com

Remarks :

Example of Genetic Engineering – Human Gene Replacement Therapy

- Retroviruses with the 'functioning' gene are mixed with the stem cells.
- The viral genome and the concerned gene is inserted into the chromosome
- Stem cells containing the working gene divide to T-cells
- The therapy was performed in December, 2001, now the child was allowed to mix with other children and played outdoor

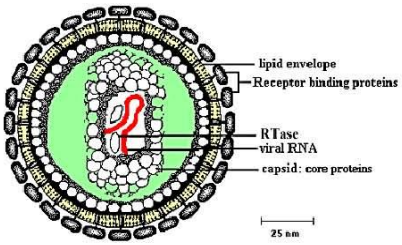


Diagram of a Retrovirus

Photo Credit: www.accessexcellence.org

Human Gene Replacement Therapy for inherited blindness

- In 2008, UK researchers successfully introduced the RPE65 gene to the retinal cells of patients with Leber's congenital amaurosis (LCA), caused by an abnormality RPE65. The condition appears at birth or in the first few months of life and causes progressive loss of vision.
- A patient had significant increase of night vision.
  - http://www.ucl.ac.uk/media/library/Genetherapyblind

Remarks :

Example of Genetic Engineering – Human Gene Replacement Therapy

- Single gene disorders provide the easiest options for gene therapy, Examples include:
  - neurological disorders, e.g., Parkinson disease, Huntington disease
  - muscular dystrophies
  - immunological disorders, e.g., severe combined immunodeficiency syndrome (SCIDS)
  - blood abnormalities, e.g., thalassemias, hemophilia
  - certain cancers
- Multiple gene disorders, e.g. heart disease, diabetes and high blood pressure are more difficult

Uncertainties of Human Gene Replacement Therapy

- Problems of inserting genes to the genome – stability, site of insertion, multiple insertion, gene expression after insertion
- If gene is not inserted, have to repeat the therapy from time to time
- Immune responses to the vector – causes toxicity, inflammation and deaths
- Deaths in some clinical trials
  - In 2007, a woman died in the treatment of rheumatoid arthritis
  - In 2003, three children got leukemia in therapy for SCID
  - In 1999, a man died during a trial for ornithine transcarbamylase disorder

Remarks :

Example of Genetic Engineering – Present situation of GM crops

- In mid 90s, the first genetically modified tomato (FlavrSavr), which was modified to ripen without softening, was marketed.
- In 2005, 222 million arces (900,000km^2) were used for growing GM crops.
- Major countries for growing GM crops in 2003: United States (63%), Argentina (21%), Canada (6%), Brazil (4%), China (4%), and South Africa (1%).
- In the US, by 2006 89% of the planted area of soybeans, 83% of cotton, and 61% corn were GM varieties.

http://en.wikipedia.org/wiki/Genetically_modified_food

Example of Genetic Engineering – Present situation of GM crops

- herbicide- and insecticide-resistant soybeans, corn, cotton, etc.
- sweet potato resistant to a virus
- crops with increased iron and vitamins
- plants able to survive weather extremes, e.g. tomato against frost

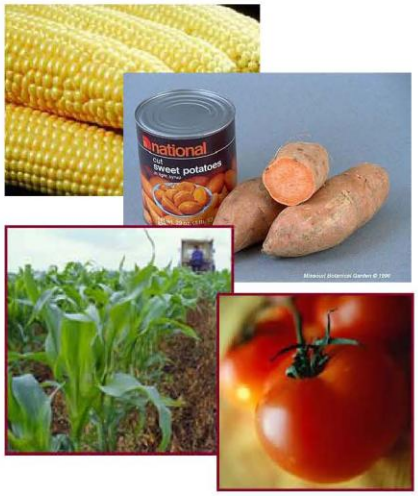


Photo Credit: www.millenniumassessment.org, free-stock-photos.com, www.mobot.org

Remarks :

Example of Genetic Engineering – Environmental Protection

- Dying of jeans:
  - Prior to dying jeans, the fabric is bleached by hydrogen peroxide
  - a lot of water is used to wash out the bleach before applying dye
  - catalase, an enzyme produced by genetically manipulated bacteria can neutralize the bleach
  - saving water, energy and time.

Photo Credit: www.google.com

Example of Genetic Engineering – Environmental Protection

- Management of Wastes:
  - used to decompose garbage and to break down petroleum products
  - For example, an oil-eating "nonnatural manmade microorganism" is used for cleaning up oil spills.
  - In 1980 Dr Ananda Chakrabarty of G.E. patented first genetic engineered life - oil eating bacteria

Photo Credit: www.cornellcollege.edu

Remarks :

Example of Genetic Engineering – increases convenience

- Biological Washing Powder:
  - 90 % of the enzymes in washing powder are produced by genetic engineering
  - Lipase for breaking down fat
  - Protease for breaking down proteins

Photo Credit: www.schoolscience.co.uk

Example of Genetic Engineering –Transgenic Animals

- In New Zealand, Lincoln University created the first transgenic animal (sheep with a mouse keratin gene) in the early 1990's aimed at increasing wool growth.

Photo Credit: genetech.csiro.au

Remarks :

Example of Genetic Engineering –Transgenic Animals

- King Salmon underwent GE experiments in New Zealand between 1995 and 1999 on salmon to increase growth rate
- The fish can reach market size in 18 months, rather than 36 months
- Scientists also believed the salmon could grow to 550 pounds, compared to the wild salmon weighing 100 pounds

Photo Credit: www.localfishermennews.com

Human Genome Project

- Begun in 1990, the Human Genome Project aimed at:
  - ***determine*** the sequences of the 3 x 10^9 base pairs that make up human DNA,
  - ***improve*** tools for data analysis and storage,
  - ***transfer*** related technologies to the private sector,
  - ***address*** the ethical, legal, and social issues (ELSI) that may arise from the project.

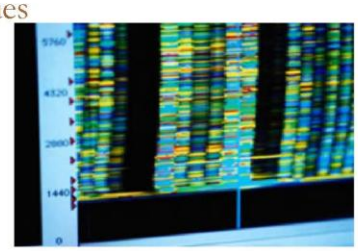


Photo Credit: www.utoronto.ca

Remarks :

Human Genome Project

- ***Sequencing*** completed in 2003,
- ***Found*** that human being has about 30,000 genes
- ***Uses*** of human genome –
  - Study human evolution and races
  - Identify individual
  - Detect diseases
  - Generate useful products
  - Gene therapy

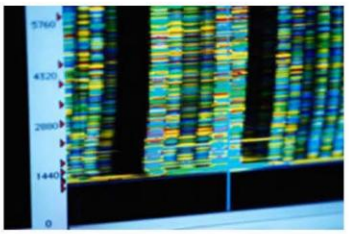
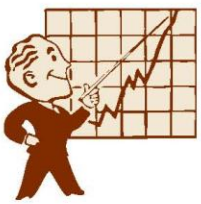


Photo Credit: www.utoronto.ca

Advantages of genetic engineering

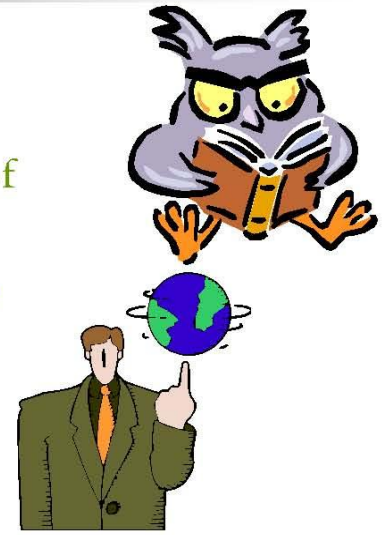
- Improve health and diagnostic methods
- Increase nutrients, yields, and stress tolerance
- Improve resistance to disease, pests, and herbicides
- "Friendly" bioherbicides and bioinsecticides
- Conservation of soil, water, and energy
- Improve product quality and quantity
- Reduce the cost of products



Remarks :

Concerns

- Genetic engineering breaches natural species boundaries
- Violates the natural integrity of species and animal rights
- Increasing dependence on the industrialized countries



Concerns

- Gene therapy and recombinant drugs: will only the rich be able to afford?
- Animal rights: Some of the salmon in New Zealand under development had lumps on their heads and other deformities

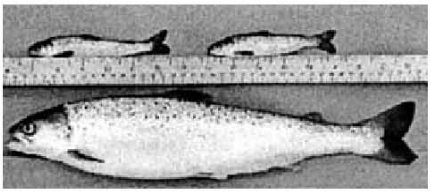


Photo Credit: www.fao.org

Remarks :

Concerns

- Transgenic animals and plants may contaminate the environment
- They may out-compete the natural species
- Resistant plants may induce super-bugs
- The effects of long-term consumption are not known

Photo Credit: news.bbc.co.uk, www.stuff.co.nz

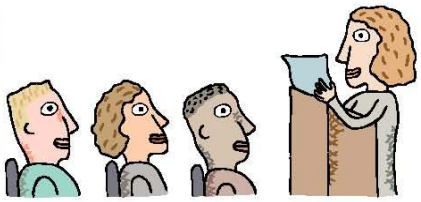
Concerns in the use of human genome

- Fairness in the use of genetic information
- Clinical issues
- Privacy and confidentiality
- Reproductive issues
- Commercialization
- Psychological impact
- Conceptual and philosophical implications

Remarks :

Points to be considered

- Can benefits outweigh drawbacks?
- Are drawbacks controllable?
- Guidelines on R and D
- Increase public awareness
- Input from different sectors



End

Remarks :

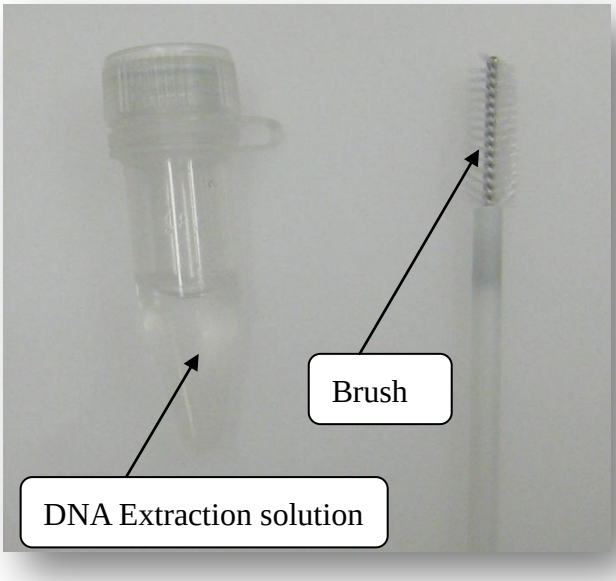
Task 1 – Part A
Extraction of Genomic DNA from buccal cells

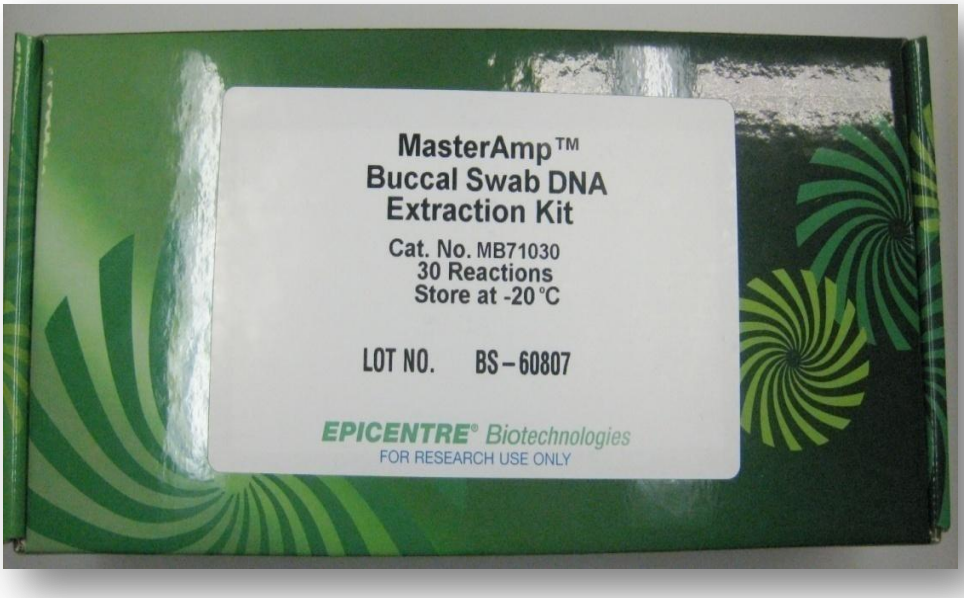
Introduction

In this exercise, you will learn to obtain your own DNA sample from the buccal cells using the MasterAMP™ buccal swab DNA extraction kit. The sterile buccal swab brushes are provided in paper pouches for collection of tissue. The DNA extraction requires only heat treatment to lyse the epithelial cells. Following heat treatment and centrifugation to pellet debris, your DNA sample will be used as a template for PCR in the next exercise.

Solutions / tools

DNA extraction solution	2 ml
50 mM Tris-HCl (pH 10.5)	
1 mM EDTA	
MasterAMP PCR enhancer	
Buccal brushes	2 pieces

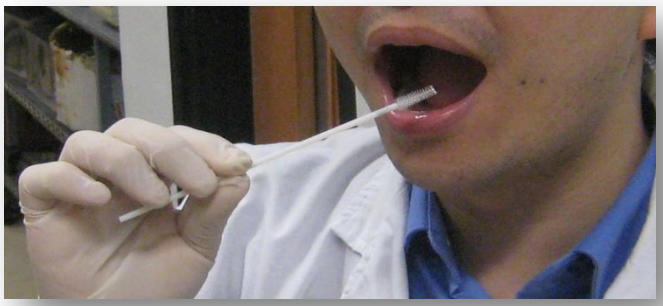




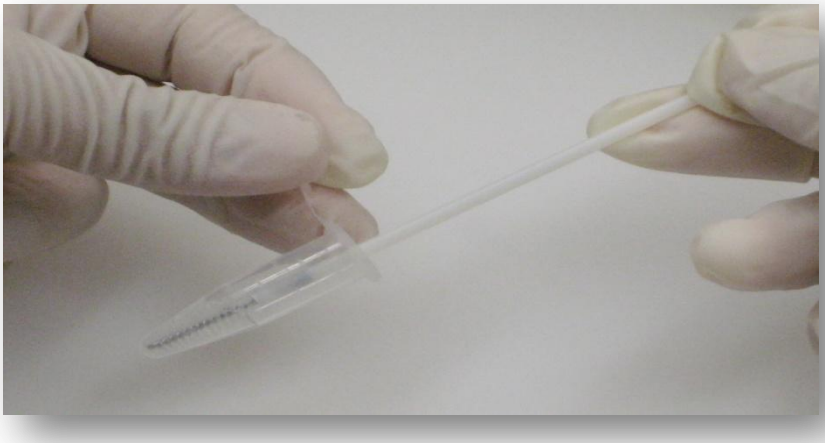
Procedures

You may obtain your own DNA samples for this exercise.

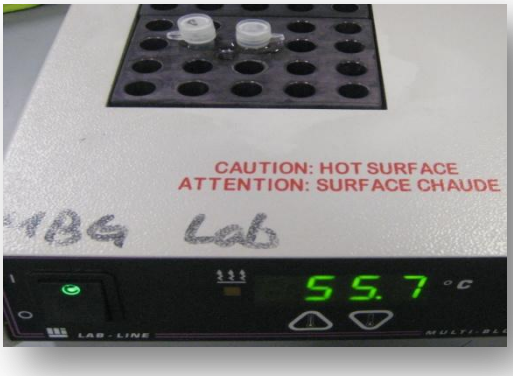
1) Rinse mouth twice with water.
2) Roll the Buccal Brush firmly on the inside of the cheek (about 30 times on each side).
3) *If you are getting samples from your relatives and do not extract the DNA immediately, air dry the brushes for 10–15 minutes at room temperature and then store them at 4℃ refrigerator. Pack them in a sealed plastic bag and bring them to the lab the following day.*



4) Place the Buccal Brush into DNA Extraction Solution and rotate the brush a minimum of 20 times.

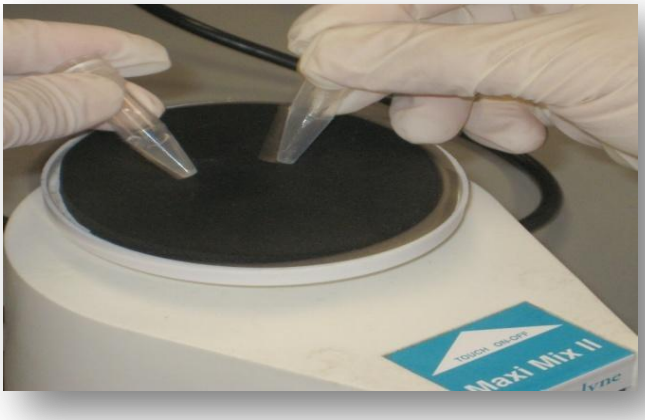


5) Press the brush against the side of the tube and rotate the brush while removing it from the tube to ensure most of the liquid remains in the tube.



6) Screw the cap on the tube tightly and vortex mix for 10 seconds. Incubate the tube at 60°C for 30 minutes.

7) Vortex mix for 15 seconds.



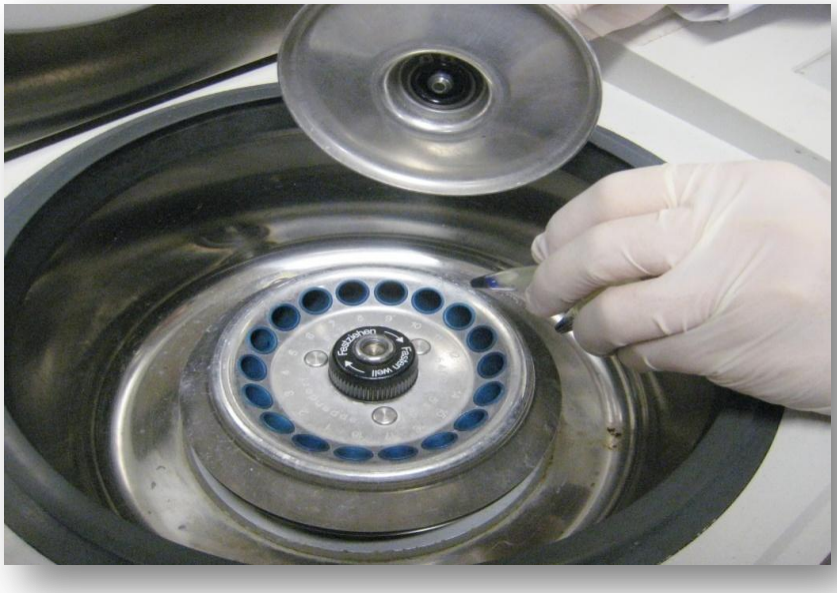
8) Transfer the tube to 98°C and incubate for 8 minutes.

9) Vortex mix for 15 seconds.

10) Return the tube to 98°C and incubate for an additional 8 minutes.

11) Vortex mix for 15 seconds. Chill the tube on ice for 5 minutes to reduce the temperature.

12) Pellet cellular debris by centrifugation at 4°C for 5 minutes.



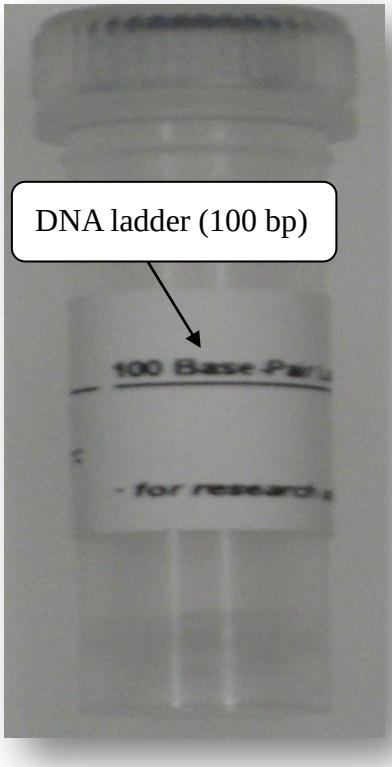
13) Carefully transfer 50 µl of the DNA-containing supernatant to a clean tube **without including any of the beads**.

Task 1 – Part B
Amplification of D1S80 Loci from buccal cell DNA using polymerase chain reaction (PCR)

In this exercise, you will conduct a polymerase chain reaction (PCR) to amplify a segment of noncoding region of chromosome 1 called D1S80, which has a repeat unit of 16 base pairs. At this locus, most individuals have alleles containing between 14 and 40 repeats, which are inherited in Mendelian fashion from the maternal and paternal copies of chromosome.

Solutions / chemicals (preparation list)

Taq polymerase	3 µl
10× buffer	20 µl
Primer mix	10 µl
dNTPs (2.5m M each)	10 µl
DNA sample	10 µl
dd H_2O	200 µl



Procedures

1) In a reaction of 50µl, add

   36.5 µl dd H_2O

   5 µl 10X buffer

   1 µl dNTPs (2.5 mM each)

   2 µl Primer mix (10 µM)

   0.5 µl Taq DNA Polymerase

   5ul DNA sample

Question: How to choose a suitable ladder?

2) In this experiment, you can prepare a master mix for 3 reactions

   109.5 µl dd H_2O

   15 µl 10X buffer

   3 µl dNTPs (2.5 mM each)

   6 µl Primer mix (10 µM)

   1.5 µl Taq DNA Polymerase

3) Add 5µl of the below templates into corresponding PCR tubes:

- 5 µl H_2O (blank control, negative control)
- 5 µl DNA of Student A
- 5 µl DNA of Student B

4) The reaction tubes are placed into a thermal cycling machine programmed to run 38cycles with the following parameters.

- 1 cycle: 94℃ for 1 minute
- 38 cycles: 94℃ for 15 seconds (denaturation)
  55℃ for 15 seconds (annealing)
  72℃ for 45 seconds (elongation)
- 1 cycle: 72℃ for 10 minutes
- 1 cycle: 4℃ for indefinite period

Remarks :

Task 2
Heat-shock transformation of DNA vectors into bacterial cells

In this experiment, you will put a plasmid containing the Green Fluorescent Protein (GFP) or Orange Fluorescent Protein (OFP) into the bacteria (*E. coli*) by a process called transformation. You will have your target DNA fragment, together with the plasmid, amplified by *E. coli* in order to obtain sufficient amount of DNA for subsequent use and/or analysis.

Transformation is the process to acquire naked DNA by an organism like bacteria; competence is the state of the organism to be able to take up the naked DNA by transformation. In certain bacterial strains, there is a natural mechanism in which bacterial cells acquire "competence" – the ability to take up DNA – as they grow. It should be noted that certain bacteria in nature are capable of undergoing transformation, yet many other bacteria are not naturally competent; in other words, they are engineered to become competent by the exposure to a variety of artificial treatments such as high concentrations of divalent cations. The mechanism of this artificial competence is still not very clear: but it has been suggested that divalent cations might play some roles: (1) The divalent cations can shield the negative charges of DNA (in the phosphate groups) such that the DNA molecules to be transformed can come in close association with the cell surface; (2) The combination of low temperature and divalent cations might facilitate the crystallization of certain regions of the plasma membrane to make channels for DNA uptake more accessible.

E. coli cells are not naturally competent; they are made competent by culturing log-phase cells in calcium chloride solution. These competent cells can be used immediately or frozen by liquid nitrogen and then stored at –70 ^{o}C freezer for later use.

Transformation includes several steps:

1) *Adsorption of plasmid DNA onto E.coli cell surface.* Plasmid is mixed with *E.coli* competent cells and being adsorbed onto cell surface under low temperature (on ice). Do not disturb the cell in this process;

2) *Heat Shock.* The cells are exposed to a short period of elevated temperature (42^{o}C) to trigger DNA uptake. It is immediately followed by exposure at a low temperature (one ice) to close DNA uptake channels.

3) *Recovery of cell.* LB medium is added to the cell to repair the damage of the cells during the heat shock process. The growth rate of cells and the expression of antibiotic gene in the cells during the recovery period are important factors for the subsequent antibiotics selection step on LBA plate.

(I) Solutions / Chemicals

Plasmid containing GFP/OFP 10 µl

Competent cells (DH5α) 1 tube (100 µl per tube)

LB medium (autoclaved) 2 ml

Per liter:

Tryptone	10g
Yeast extract	5g
NaCl	10g

LBK agar plates (containing 50µg/ml kanamycine) 2 plates

LBA agar plates (containing 10µg/ml ampicilin) 2 plates

(LBK agar plates for GFP; LBA agar plates for OFP)

(II) Procedures for Green Fluorescent Protein (GFP)

1) Gently thaw the competent cell (DH5α) from -80°C on ice. For each transformation, 100 µl of competent cell is used.

2) Prepare 2 big snap-cap tubes and add 100 µl competent cell into each tube with the presence of open flame.

3) Add 10 µl of plasmid (pEGFP-c1) into each tube and incubate the mixture on ice for 10 minutes.

4) Incubate the competent cell-plasmid mixture in 42°C water bath for 90 seconds. Incubate the mixture immediately on ice for at least 2 minutes.

5) Apply 900 µl of LB medium to the tube with the presence of open flame, then incubate the tube at 37°C for 1 hour with shaking at 250 rpm.

(III) Procedures for Orange Fluorescent Protein (OFP)

6) Prepare for the competent cell (BL21 DE3). For each transformation, 100 µl of competent cell is used.

7) Prepare 2 big snap-cap tubes and add 100 µl competent cell into each tube with the presence of open flame.

8) Add 10 ng of plasmid into each tube and incubate the mixture on ice for 10 minutes.

9) Incubate the competent cell-plasmid mixture in 42°C water bath for 40 – 60 seconds. Incubate the mixture immediately on ice for at least 2 minutes.

10) Apply 900 µl of LB medium to the tube with the presence of open flame, then incubate the tube at 37°C for 1 hour with shaking at 250 rpm.

Remarks :

Task 3
Spreading transformed bacterial cells on agar plates for the estimation of transformation efficiency

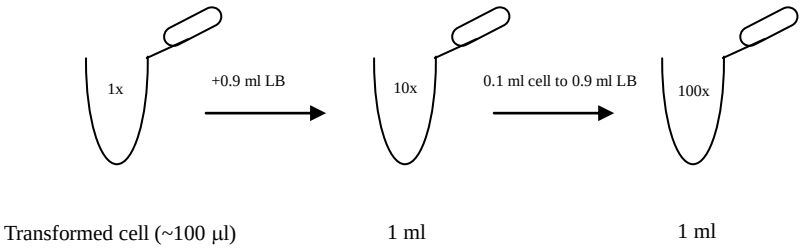
(A) Plating out the transformants

11) During the incubation time, prepare two LBA plates (LB agar supplemented with kanamycins). Label one plate as 10× dilution and the other as 100× dilution as well as other essential information such as date, group name, strain of bacteria, contents of the plates, etc.

12) Clean up the bench with 100% ethanol. Use open flame to sterilize the spreader. Make sure all the ethanol has been already burnt away. Then place the spreader near the flame until use.

13) Plate 100 µl of the transformed bacteria onto the LBK or LBA plate labeled 10×. Cool down the spreader and spread the cells thoroughly and evenly by rotating the plates until the bacterial medium has been absorbed into the agar.

14) Transfer 100 µl of the transformed bacteria into 900 µl of LB in an autoclaved 1.5 ml microcentrifuge tube. Plate and spread 100 µl of the mixture onto another LBK or LBA plate labeled 100×.



15) Leave the plates on bench for 5 minutes to let the agar absorb the liquid. Incubate the plates upside-down in a 37°C air-bath overnight.

(B) Estimating transformation efficiency

16) On the next day, count and record the number of florescent colonies on each plate.

17) Calculate the transformation efficiency from the plate with colony number statistically significant and countable.

Some Important Information:

(A) Preparation of liquid media for bacterial growth

LB broth, per L:

10g tryptone
5g yeast extract
10g NaCl
1ml 1M NaOH

Add water to 1L and autoclave.

(B) Preparation of solid media for bacterial growth

LB agar plates:

10g tryptone
5g yeast extract
10g NaCl
1ml 1M NaOH
15g agar

Add water to a final volume of 1L, dissolve, and autoclave.

To pour plates, allow agar to cool to 50°C. Add antibiotics (or other additives) if needed, and mix. Pour liquid agar (about 30 to 40ml) into 100-mm sterile disposable petri dishes and allow plates to cool, with consequent hardening of the agar. It is generally advisable to pour plates at least 1 day in advance to allow time for the plates to dry at room temperature before use. If more rapid drying is needed, incubate the solidified plates with the lids slightly ajar in a 37 °C incubator for about 1 hr. After drying, store plates at 4 °C until needed.

(C) Preparation of antibiotics (if required for selection of recombinant vectors with an antibiotic resistance gene)

Ampicillin, 50mg/ml in water, sterile filtered (1000x stock)
Tetracycline, 12mg/ml in 50% (v/v) ethanol (1000x stock)
Chloramphenicol, 50mg/ml in ethanol (1000x stock)
Kanamycin, 25mg/ml in water, sterile filtered (500x stock)

Store antibiotic stock solutions at -20 °C until needed; they can be aliquoted for ease of use. Add 1ml of 1000x stock or 2ml of 500x stock per L to broth for liquid culture, or agar solution (after cooling to 50 °C) before pouring plates.

(D) Growth of bacteria in liquid culture

1. Overnight culture:
   The overnight culture is a small-scale liquid culture growth used to initiate a larger-scale growth. Using sterile technique, 2ml liquid medium is transferred to a sterile 15-ml tube. The liquid medium is inoculated with an isolated colony from an agar plate using a sterile inoculating loop. The tube is capped and incubated at 37 ^{o}C with shaking, or placed on a roller drum rotating slowly in a 37 ^{o}C incubator. The culture grows until saturation (1 to 2 x 10^{9} cells/ml), typically over an 8-hr to overnight period.

2. Preparative culture:
   Preparative cultures are inoculated with cells from an overnight culture. The overnight culture is diluted at least 100-fold during the inoculation to assure a uniform population of exponentially growing cells in the preparative growth. The preparative culture is grown with vigorous orbital shaking (>200 rpm). The volume of the flask should be at least four times the volume of the liquid media used for preparative growth to allow proper aeration.

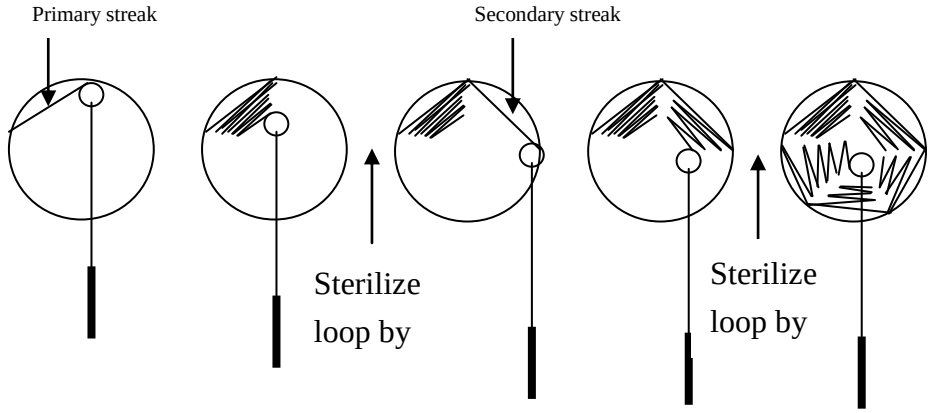
3. To measure bacterial density during growth
   For many procedures, the growth of the cells needs to be monitored. The cell number per ml can be easily estimated by measuring the scattered light at 600nm using a visible spectrophotometer. To measure bacterial density, blank a visible spectrophotometer at 600nm with a cuvette containing the bacteria growth medium (eg, LB broth). Add 1ml of the growing bacteria to another cuvette and measure the optical density (OD). An OD reading of over 0.6 is beyond log phase. An OD reading between 0.2 and 0.6 represents cells in late log-phase growth. The OD reading is linear with cell number: 1 OD/ml = 8 x 10^{8} cells/ml.

(E) Growth of bacteria on agar plates

Single colonies of bacteria can be obtained by growing bacteria on agar plates. The technique employed include streaking, spreading and pouring plate.

1. Streaking plate

a) Sterilize a platinum transfer loop by flaming until it glows light red. Allow it to cool in air. (The loop may be cooled more rapidly by dipping it into sterile water or medium or by stabbing it into sterile agar medium.)
b) Dip the cooled loop into the bacterial suspension. Streak the bacteria adhering to the loop onto a segment of a plate containing agar medium.
c) Resterilize the loop by flaming and cool it by stabbing into a region of the agar medium that is free of bacterial cells.
d) Pass the loop once across one end of the primary streak and spread the bacteria that adhere to the loop into a fresh region of the agar medium.
e) Sterilize and cool the loop again and streak from one of the secondary streak.
f) Repeat step c twice more, serially.
g) Replace the lid on the plate. Label the bottom of the plate and incubate it in an inverted position at 37℃ for 16-24 hours. Well-separated colonies should be visible in the area of the final streak.



2. Spreading Technique
   a. Transfer bacteria from an agar slant or liquid culture to a tube containing 1ml of liquid medium. Vortex thoroughly to disperse any clumps of bacteria.
   b. Dilute the cells by using a sterile micropipette to transfer 10µl from the first tube to a fresh tube containing 1ml of medium.
   c. Repeat step b serially two or three times.
   d. Spot a small aliquot (10-50µl) of each dilution in the centre of a plate containing hardened agar medium. Spread the cells over the entire surface of the medium by moving a sterile, bent glass rod back and forth gently over the agar surface and, at the same time, rotating the plate by hand or using a rotating wheel. The glass spreader can be sterilized by dipping it into a beaker containing 95% ethanol and then holding it in the flame of a bunsen burner to ignite the ethanol. The spreader should then be cooled first in air and then by touching the surface of a plate of sterile agar medium.

(F) Preparation of Competent Cell

1) Streak *E.coli* strain DH5α on fresh LB plate. Grow overnight at 37^{o}C.
2) Inoculate a single colony into 5 ml LB. Grow overnight, shaking at 37^{o}C.
3) Inoculate 50 ul overnight cell culture into 5ml LB (1% inoculation), shaking at 37^{o}C until OD600 = 0.5. (This will take about 3 hours.)
4) The cell culture is aliquot into 1.5ml centrifuge tube. Each group will be given 3 tubes of cell culture. Centrifuge the cell at 6000rpm, 2 min.
5) Decant medium and resuspend cells in 1ml ice-cold 50mM $CaCl_2$.
6) On ice, 15 min.
7) Centrifuge the cells at 6000rpm, 2 min.
8) Decant medium and resuspend cell pellet in 0.2ml ice-cold 50mM $CaCl_2$.
9) Cells are now ready for transformation.

(G) Short-term storage of bacterial strains

Colonies of most strains of bacteria can be maintained for periods of a few weeks on the surface of agar media if the plates are tightly wrapped in parafilm and stored inverted at 4℃.

(H) Long-term storage of bacterial strains

Bacteria can be stored for many years in media containing 15% glycerol at low temperature without significant loss of viability.

1. Inoculate a culture flask containing 5-10ml of liquid medium with a single bacterial colony and grow the culture overnight.
2. Transfer 0.85ml of the overnight culture to a sterile vial containing 0.15ml of sterile glycerol. Place the cap on the vial and mix the contents thoroughly by vortexing.
3. The glycerinated cultures can then be stored at -20℃ for a few years without loss of viability. Alternatively, the glycerinated suspension can be stored at -70℃. In this case, viable bacteria can be recovered many years after the initial freezing. Viable bacteria can be recovered by simply scratching the surface of the frozen stock with a sterile platinum loop or wire. The frozen suspension can then be returned to the freezer. Several vials of each strain should be frozen.

Remarks :

Task 4
Analysis of PCR products using agarose gel electrophoresis

In this exercise, the PCR products obtained from the previous experiment will be resolved and analyzed by agarose gel electrophoresis. You need to study the pattern of the bands, which correspond to different alleles and determine their relationships.

Procedures

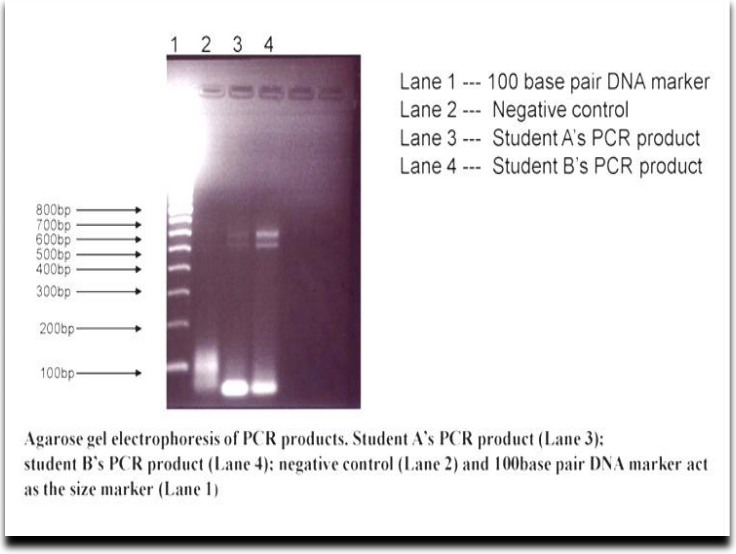
1) Prepare a 2% agarose gel (0.6g agarose powder in 30ml 1X TBE buffer) with 2µl 10 mg/ml ethidium bromide solution added.

2) Label a set of new microfuge tubes. When the PCR reaction is finished, add 4 µl of 6X agarose gel loading buffer and 20 µl PCR product to each tube and then mix well.

3) Load the following samples onto the 2% agarose gel, and electrophoresis at 100V until the loading dye runs to the edge of the gel.

100 bp-ladder-DNA size marker (1 ug)	10ul
Negative control PCR product	24ul
Student A's DNA PCR product	24ul
Student B's DNA PCR product	24ul

4) Slide the gel off the gel bed and observe the DNA with an UV transilluminator. Ultraviolet radiation can damage eyes and skin, so always wear a plastic face mask. Photograph the gel.

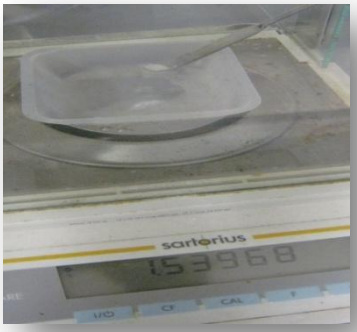


Agarose gel electrophoresis of PCR products. Student A's PCR product (Lane 3); student B's PCR product (Lane 4); negative control (Lane 2) and 100base pair DNA marker act as the size marker (Lane 1)

Precautions: **Ethidium bromide** is a suspected **carcinogen**. Wear gloves to handle the gel with eithidium bromide and discard the gel in a special container.

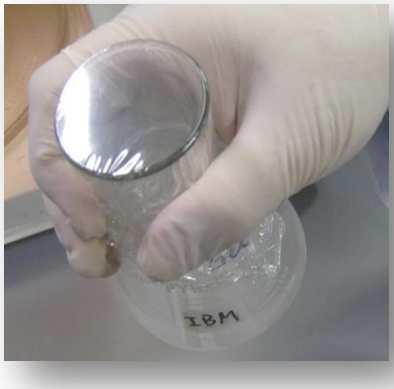
A) Preparation of Agarose Gel (2%)

1) Prepare the gel mould for an agarose gel as shown by the demonstrator. Three groups will share and prepare one gel.



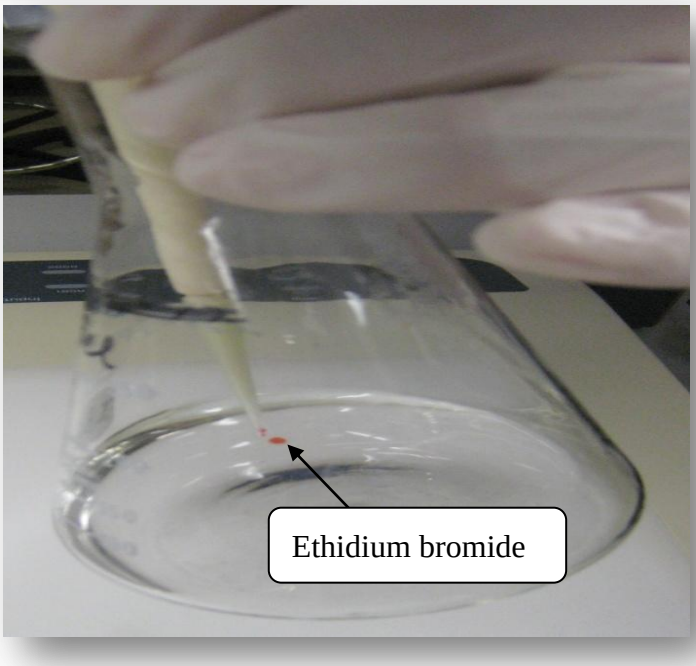
2) Add 0.6 g agarose powder and 30 ml 1X TBE buffer into a 250-ml conical flask.

3) Use a microwave oven to melt the agarose.

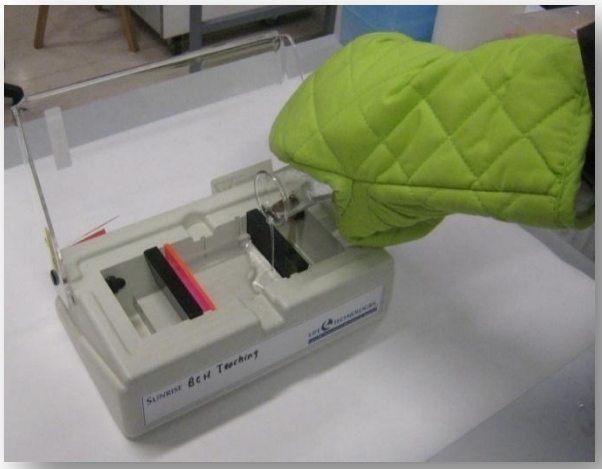


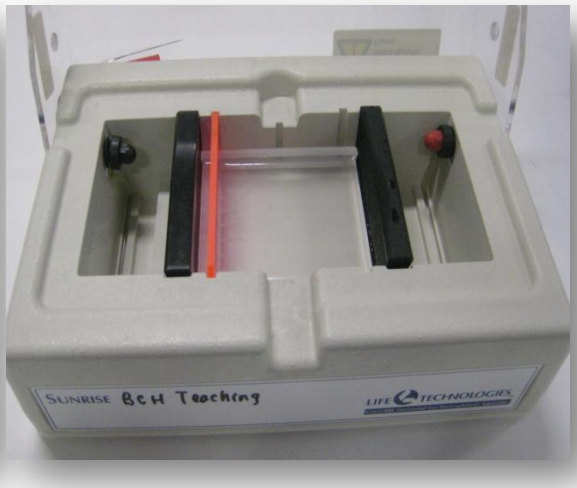
4) Allow the agarose solution to cool to hand-hot, then add 2µl 10 mg/ml eithidium bromide solution.

5) Pour the agarose solution into the gel mould and place it aside to set at least 20 minutes.



Question: Why is the EB so carcinogenic?



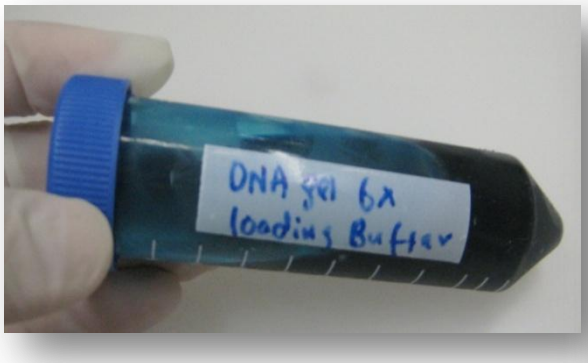


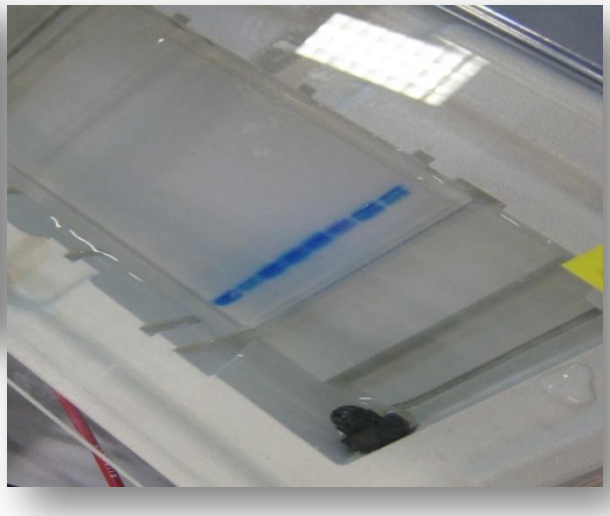
(B) Agarose Gel Electrophoresis

1) Mix the PCR products with 4 µl of 6X agarose gel loading buffer. Spin down the solution.

2) Put the gel bed into the gel tank and add TBE buffer until the gel is just immersed. Load samples and the 100 bp DNA marker onto the 2% agarose gel, and electrophoresis at 100V until the loading dye runs to the edge of the gel.

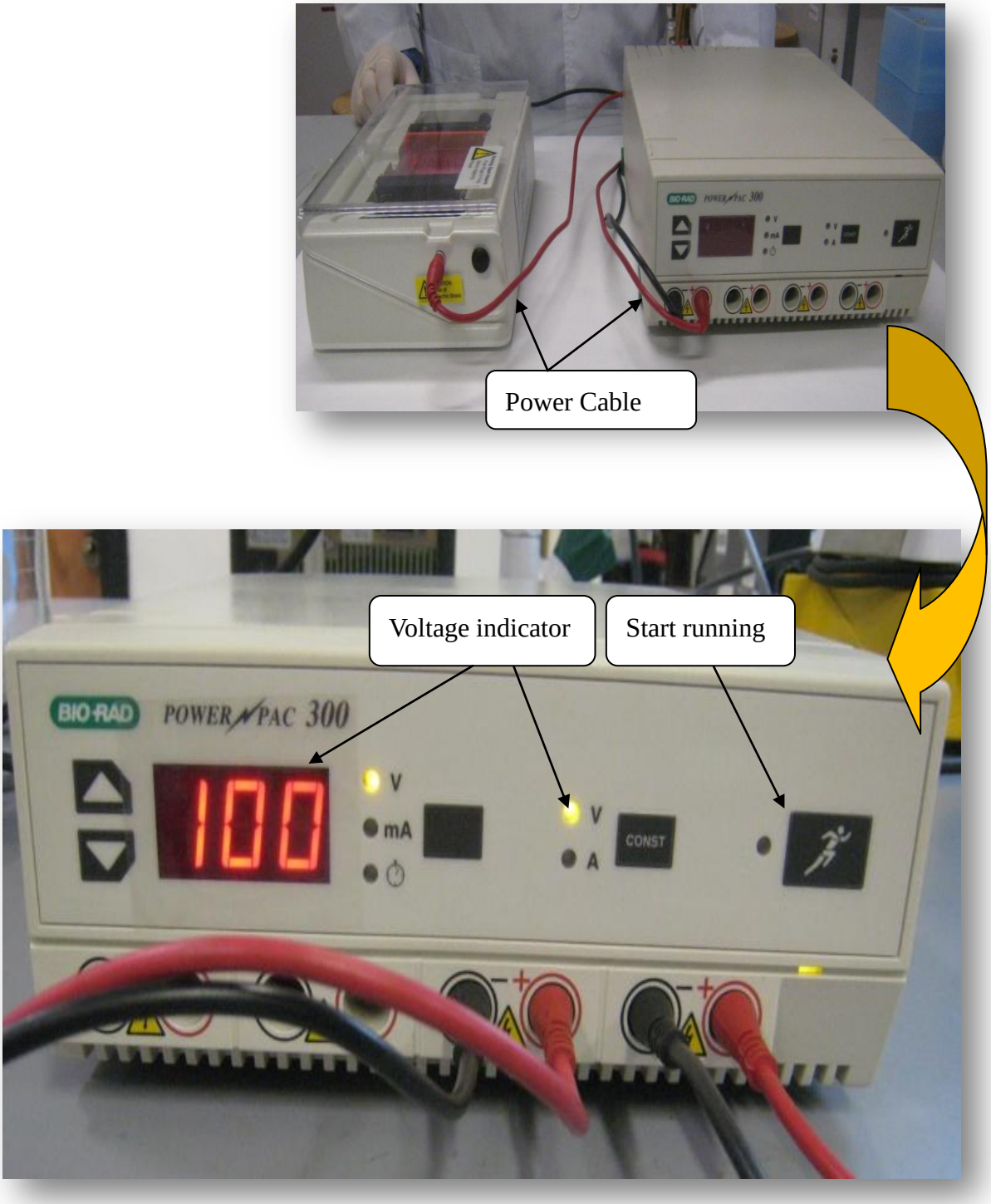
3) Slide the gel off the gel bed and observe the DNA with an UV transilluminator. Ultraviolet radiation can damage eyes and skin, so always wear a plastic face mask. Photograph the gel.





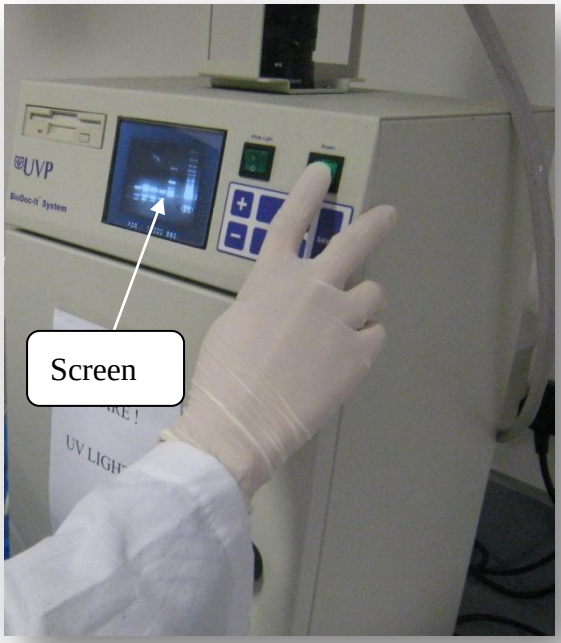
Question: What is the function of loading buffer?

Question: Could you think of any way to speed up the DNA migration? What factors will you consider?



Note: Make sure the cables have been correctly connected. Red cable has to be connected to the "+" socket. Black cable has to be connected to the "-" socket. If the connection is incorrect, the direction of DNA migration will be reversed leading to loss of DNA samples.


UVP
BioDoc-It System
LIVE
CAPTURE
SAVE
Agarose gel


UVP
BioDoc-It System
Screen

Remarks :

Practical skills of molecular biology

Practical skills – 1

- Micropipetting skills
  - *Different types of pipettes (single channel / multi-channels)*

    Many companies are manufacturing various types of pipettes, which suit different purposes. For example, there are single channel pipettes for manipulating one sample at one time; multi-channel pipettes are designed to handle 8 to 12 samples at one time. Auto-pipettes are designed for handling relatively larger amounts of volumes (10 – 50 mL). (Fig. 1)

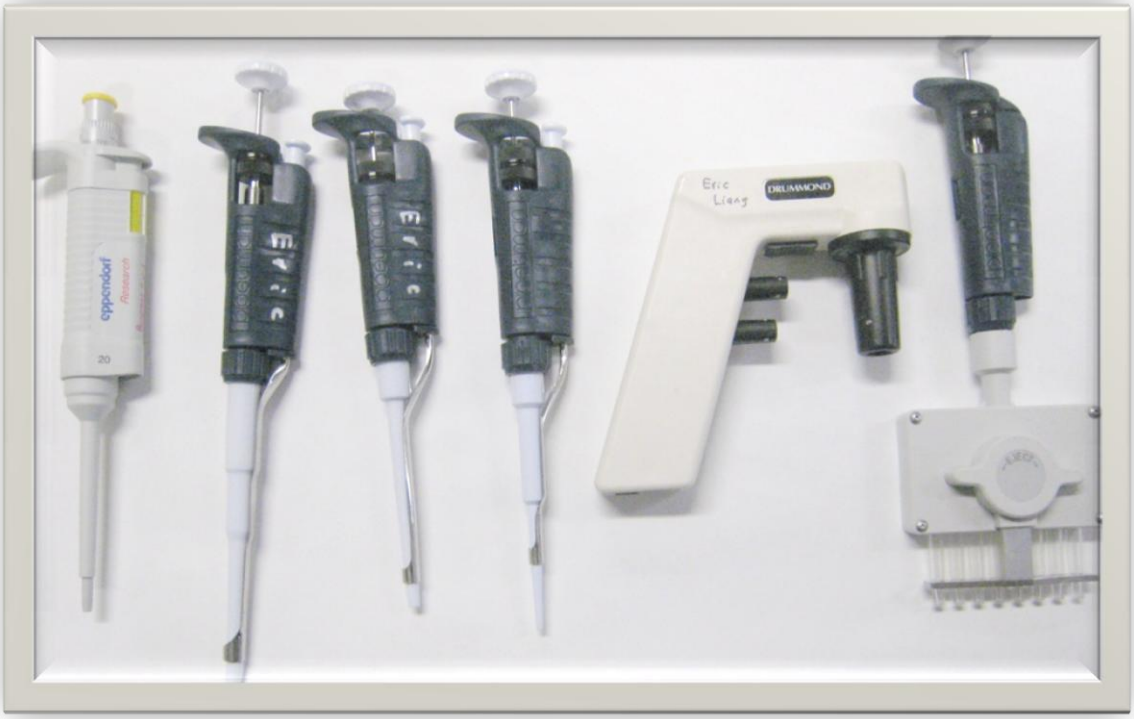


Fig. 1. Different types of micropipettes. (The first four items from the left hand side are single-channel pipettes, followed by an auto-pipette and a multi-channel pipettes)

  - *Different sizes of pipettes*

    Since in the experiments of molecular biology, regents with different volumes will be manipulated, pipettes designed for transferring different tiny volumes of reagents are designed to meet this purpose. Common volume include 0 – 2 µl, 0 – 10 µl, 2 – 20 µl, 20 – 200 µl, 200 – 1,000 µl, etc. (Fig. 2)

Fig. 2. Different sizes of pipettes. (Starting from the left hand side, the maximum pipetting volumes are 1000 µl, 200 µl and 20 µl, respectively)

- *Different sizes of pipette tubes*

  As there are different sizes of pipettes, there are different sizes of pipette tubes for the corresponding pipettes. In general, the smallest pipette tube (0 – 10 µl) is white in colour; yellow pipette tubes are suitable for pipetting volume from 2 – 200 µl; blue pipette tubes are suitable for pipetting volumes from 100 – 1,000 µl. (Fig. 3)

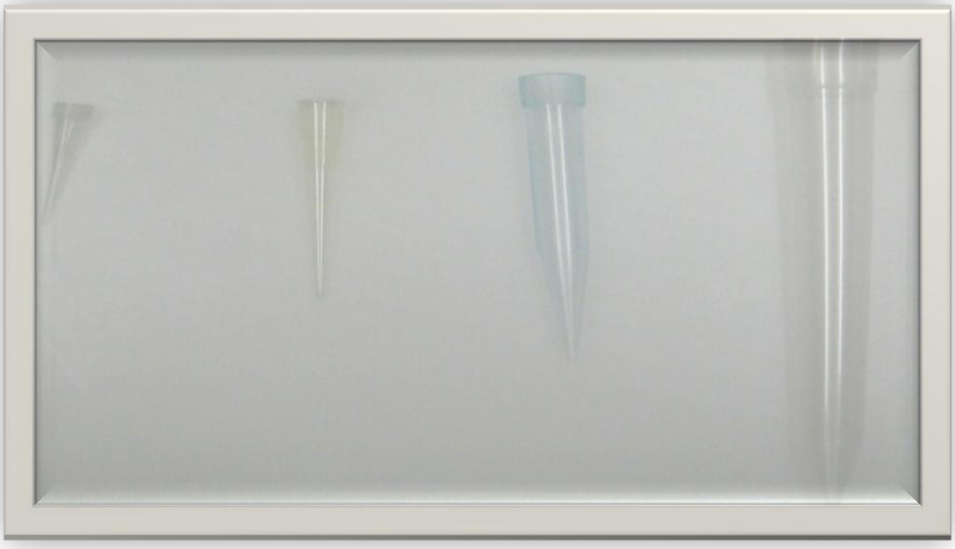


Fig. 3. Different sizes of pipette tubes. (Starting from the left, the volumes of the pipette tips are 20 µl, 200 µl, 1000 µl, 5000 µl, respectively)

- *Use of filtered tubes*

  Some pipette tubes are equipped with a filter to prevent contamination. These tubes are designed for experiments handling delicate samples, such as DNAs and RNAs. It should be noted that with the filter present, the volume of reagents to be transferred should not exceed the maximum level and thus touching the filter inside the tube. (Fig. 4)

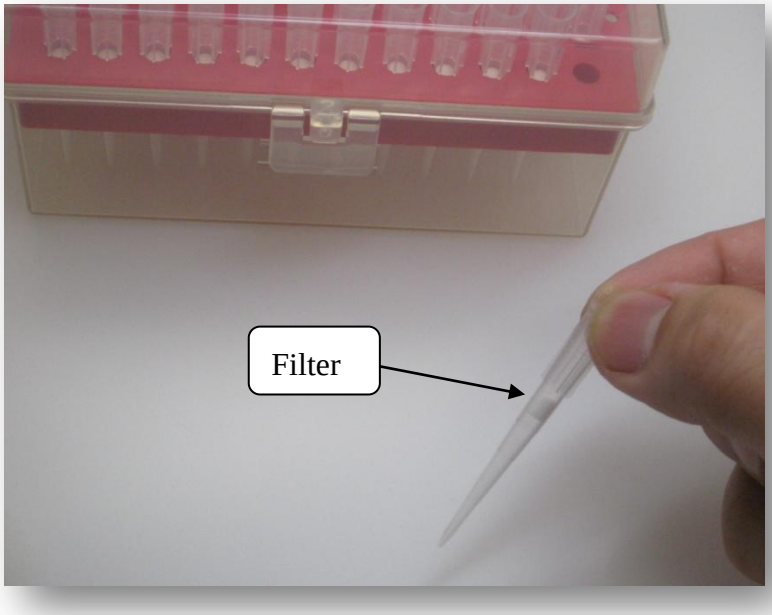


Fig. 4. Filtered tips. A filter is fit inside a pipette tube to prevent potential contaminants of the pipette from reaching the reagents to be transferred; in the mean time, the interior of the pipette tube is prevented from contamination even though the pipette-tube box has been opened.

- *Important note to use the pipettes*

  Since a small drop of reagent tends to reside on the tip of a pipette tube, there is a special design of a pipette to eliminate this problem. To press a pipette with your thumb, you can feel 'two steps' with difference 'resistance'. To draw a reagent into the tube, only the 'first step' is used; while to expel the reagent, you can see the reagent moving out as you are pressing the 'first step' of the pipette. Finally, you will have to press the second step to remove the drop of residual reagent at the tip.

Before pressing

Pressing to the first step: for drawing and expeling

Pressing to the second step: for expeling only

Fig 5. When pressing a pipette with your thumb, you can feel there are 'two steps' with different resistance: the first step is light; while the second step is heavier. Use the first step only to draw a reagent; to expel a reagent from a pipette tube, press the first step to expel most of the reagent volume and the press the second step to remove the residual reagent at the tip of the pipette tube.

- *Consideration of pipetting small volumes*
  In the experiments of molecular biology, small volumes of reagents (~ 1 µl) are often handled. Care should be taken when small volumes are to be transferred with the aid of a pipette. For example, air bubble or small amount of residue reagent on the outer wall of the tubes can significantly affect the accuracy of the volume to be transferred.

- *Prevention of bubble build-up*

  During the pipetting process, care should be taken to release the thumb slowly and gently to prevent the build-up of bubbles that will affect the accuracy of pipette volume of the reagent.

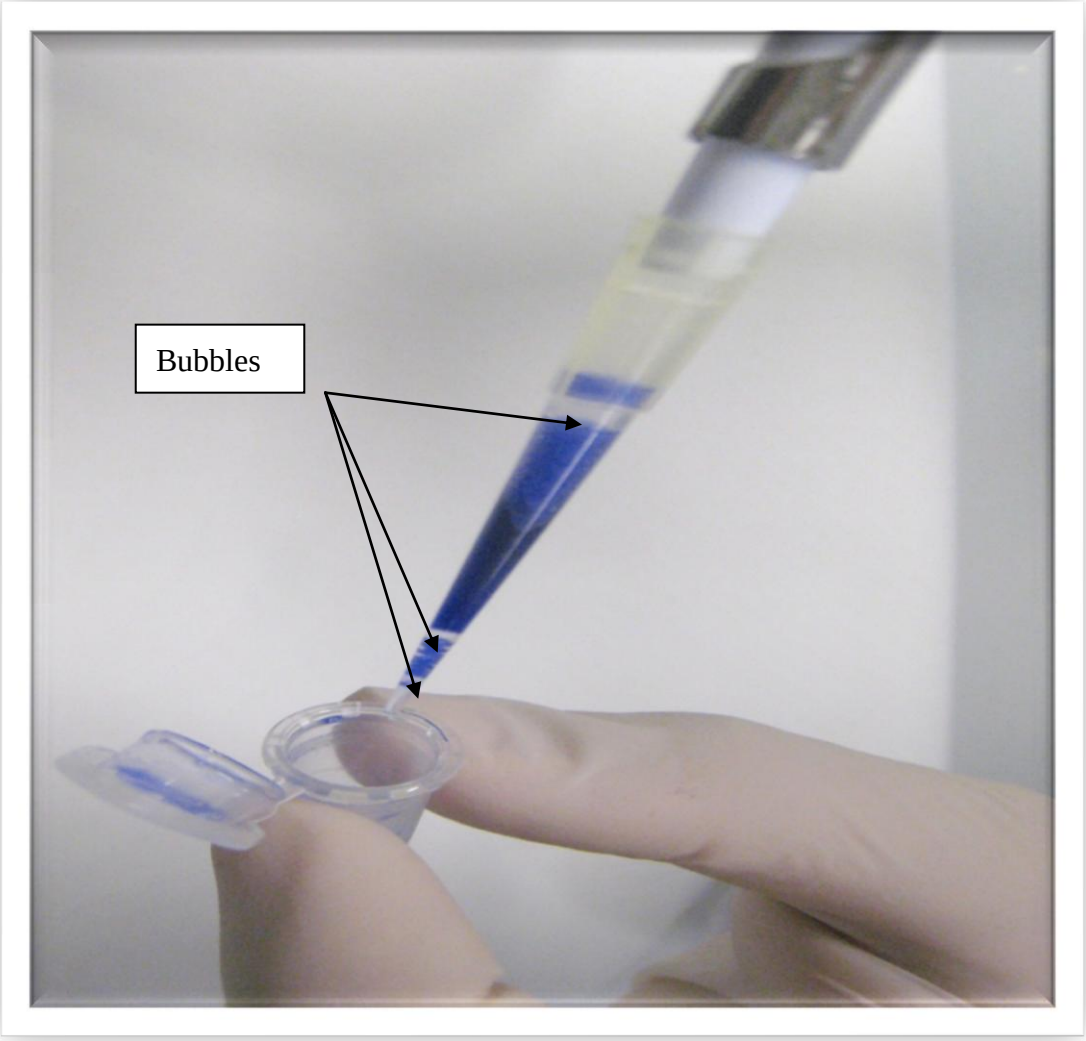


Fig. 6. Prevention of bubble buildup during pipetting. If air bubbles are generated and get mixed with the reagent inside the pipette tube, the space occupied by the bubbles instead of reagents will affect the accuracy of the pipette volume.

- *Consideration of solution temperature*

  Since the volume of reagents vary with temperature, for example, the volume is higher at higher temperature and vice versa, the reagents to be transferred should be at room temperature as possible to make sure a standardized volume to be transferred.

- *Calibration of pipettes*
  Since the accuracy of pipettes may decline over prolonged use, the pipettes should be calibrated on a regular basis to ensure accurate pipetting.

- Essential skills of working with DNA samples, primers and enzymes
  Storage
  - *Effects of freeze-thaw cycle on the samples*
    The term 'freeze' indicates keeping the reagent below freezing point; while the term 'thaw' indicates melting or warming of the reagents, which are previously kept below freezing point. Repeated freeze-thaw cycle is known to decrease the chemical stability of the active compounds in a reagent. It is recommended that in order to preserve the activity of the reagent, the number of freeze-thaw cycle should be minimized.

  - *Storing samples in small aliquots*
    To avoid massive contamination of reagents, it is recommended that all the stock reagents are divided into small aliquots for long term storage. Furthermore, if the reagents are stored below freezing point, small aliquot reduce the overall number of freeze-thaw cycle of the reagent.

  - *Use of screw-cap microcentrifuge tubes to reduce evaporation*
    Since the evaporation of solvent affects the concentration of the active ingredient(s) of a reagent during long term storage, it is recommended that the containers of the reagents should be air-tight; thus, in stead of using microcentrifuge tubes, screw-cap microcentrifuge tubes are recommended for storing reagents long term.

  - *Storing samples in a refrigerator (-20℃ or lower)*
    If a reagent has to be stored for long term, it is recommended that it should kept at a temperature as low as possible. As long as a freezer is available, the reagents should be kept at -20℃or below.

  - *Don't not use frost-free refrigerator*
    Since a frost-free refrigerator may have fluctuating temperature, which may affect the quality of the stored reagents, during the de-froze process, it is recommended to store the reagents at a frost-free refrigerator.

- *Proper labeling (use of oil-base markers)*

  For any reagents to be stored term, they should be labeled properly. Essential information, such as the name of the reagent, storage time, user, volume, etc should be labeled on the container with a permanent marker. (Fig. 7 & 8)

Fig. 7. An oil based marker. A permanent oil-based marker is recommended for label. The information written can remain clear after prolonged storage and even the containers get wet.

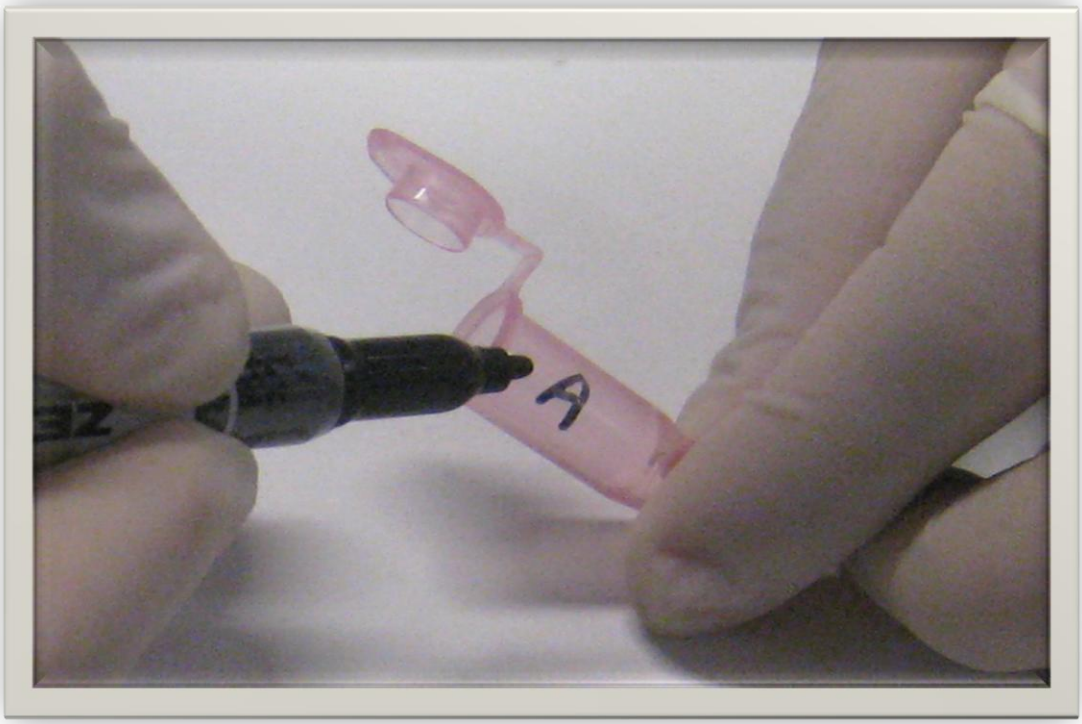


Fig. 8. Proper labeling. Label each micro-centrifuge tube on rough surface with an oil-based marker, when necessary, write on a tape and then stick the tape on the containers.

Precaution of contamination

- *Use of gloves*

  Since our skin contains a lot of contaminants such as enzymes, bacteria, etc, we should wear gloves during the experiments to avoid contamination of the samples.

- *Prevention of contamination from various sources (e.g. coughing, enzymes, bacteria) by improving personal hygiene*

  Good practice of personal hygiene can help reducing the incidence of contamination. For example, avoid speaking and coughing during an experiment; wash the hands and tidy up the hair before experiment may help to reduce contamination.

Fig. 9. An autoclave machine.

- *Use of autoclaved consumables (e.g. tubes, buffers)*

  The apparatus and equipment to be used in an experiment can be sterilized by autoclave process. The use of sterilized apparatus and equipment can reduce contamination. (9 - 11)

Fig. 10. Two pipette tips boxes. The pipette tips need to be autoclaved before use.

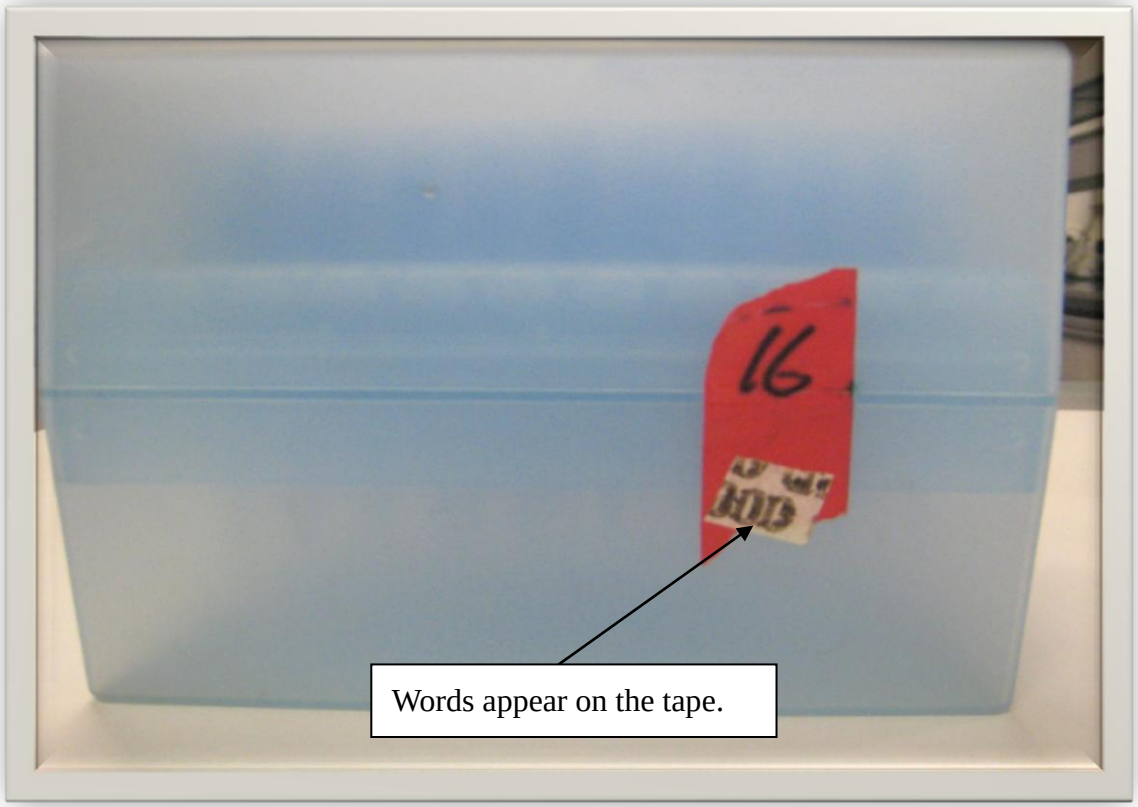


Fig. 11. Autoclave indicator. A short segment of autoclave tape is usually put on the box. After the autoclave process, an indicator (e.g. word) on the tape will appear.

- *Use of fumehood and biosafety carbinet*

Fig. 12. Safety cabinet (left); Fumehood (right)

For certain experiments requiring high cleanliness standard, they should be performed in a carbinet which provide a sterilized working environment. (Fig 12)

- *Use of log-book*

  It is a general practice to record the activities in the laboratory; each person should keep a record on their daily activities in the laboratory. Important information like the date, the place, the chemicals handled should be clearly presented on the log-book such that the writer as well as other readers are able to understand the information after years.

- Use of centrifuges
  - *Use of mini-centrifuge, bench-top centrifuge (Beckman)*

    Many different types of centrifuge machine are designed to suit different purposes, for example, there are different machine for centrifuging different volumes of samples. Care should be taken to ensure the right machine is used for an experiment. (Fig. 13)

Fig. 13. A bench-top centrifuge. Make sure proper balance is set up; all the caps and covered are securely fastened before the operation of the centrifuge (Maximum speed is 13,000 r.p.m.).

- *Place test tube in centrifuge holder*
  Since the tubes will undergo high-speed rotation, it is recommended that the centrifuge tubes should be securely fit in a centrifuge holder during centrifugation process to avoid any flying tubes resulted from the centripetal force.

- *Balancing skills*
  During centrifugation, a balance with another test tube filled to the same level in the opposite holder is essential to ensure smooth rational movement of the rotor. Imbalanced rotor will lead to vigorous vibration, which will otherwise damage the centrifuge. (Fig. 14)

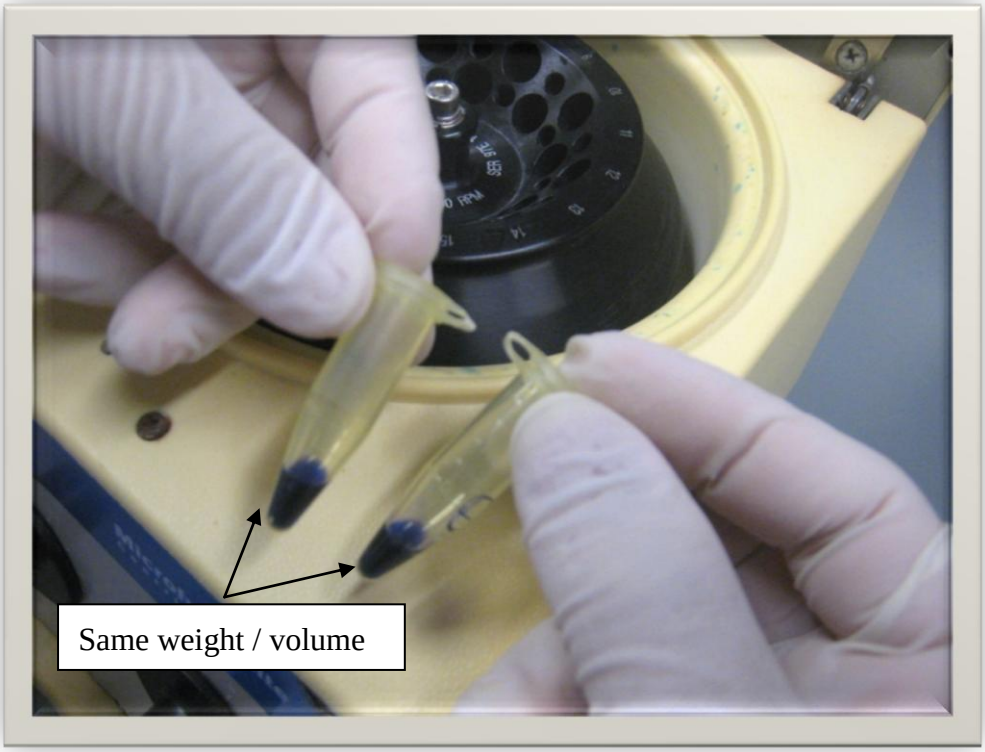


Fig. 14. Balancing the centrifuge tubes. A balancing tube contains the same weight or same volume of liquid.

- *Covering the cap and cover*
  Before centrifugation, it is essential to close the cover and turn the knob. Centrifugation might take some time, it should be noted that that the centrifuge is turned off with the switch and the spinning is completely stopped in order to open the cover and remove the samples. It ensures effective separation.

- *Choosing suitable tubes for different speeds*
  During high-speed centrifugation, the centrifuge tubes might receive heavy centripetal force. It is recommended suitable tubes are used for centrifugation with different speeds, such that the tubes can withstand the force to avoid splashing and even damage of the tubes.

- Lab safety measures
  - Access to the laboratory is limited to authorized personnel only during operations and to others at times and under such conditions as designated by written rules or as established by the laboratory supervisor.

- All chemicals must be stored properly, according to compatibility. Any chemicals, which pose a special hazard or risk, should be limited to the minimum quantities needed for the research project, and materials not in actual use should be stored under appropriate safety conditions.
- Updated animal and chemical data sheets must be kept in the laboratory.
- An MSDS file will be maintained for all chemicals purchased in the laboratory for which they are available. These files should be accessible to the workers in the laboratory.
- Organic and flammable solvents must be kept in the standard safety cabinets.
- All chemical wastes should be disposed of through proper channels.
- Any spills or accidents in the laboratory should be cleaned up or reported to the person-in charge immediately.
- No eating, drinking or smoking is allowed in the laboratory.
- Appropriate information, such as the telephone numbers of the person-in-charge, or any alternates should be posted on the outside of the laboratory door or the adjacent wall.
- All work with hazardous kinds or quantities of materials should be performed in a fume hood, or in specially designed and totally enclosed systems.
- Working with explosives, toxic chemicals, and hazardous materials should be limited to the minimum quantities needed.
- Systems representing other physical hazards, such as high voltage, radiation, intense laser light beams, high pressure, etc. should be interlocked so as to prevent inadvertent injuries. The interlocks should be designed to be fail-safe such that no one failure of a component would render the safety interlock system inoperative.
- It shall be mandatory to wear any personal safety equipment required for conducting operations safely in the laboratory, such as protective eye wear, face masks, and hand protection.
- The facility should include a separation of workspaces and desk areas as well as a second exit, equivalent to the arrangement shown in the standard laboratory module.
- Wear lab coat, googles, gloves.
- No eating, hairs
- Refer to the MSDS data sheet.

  For certain potentially hazardous materials, their MSDS data sheets should be obtained for guiding the handling of these materials.

Practical skills – 2

- *Reconstitution of chemicals from commercial testing kits*
  - Dissolving powdered chemicals according to the manufacturer's instructions

    In some reagent kit, the components are stored in dry powder form for long term storage. Users of the kit are required to reconstitute the powder into solution form with the solvent suggested by the kit manufacture. (Fig. 15)

Fig. 15. DNA polymerase kit. Some reagents are manufactured and stored in powder form. These reagents need to be reconstituted according the instruction provided by the manufacturer.

  - Store samples and reagents in proper temperatures (ranging from 4℃ to -80 ℃).

    In a reagent kit, different components may need to be stored at different temperature, for example, some components have to be stored at 4℃to avoid freezing; while others may require freezing at -20℃ for storage long term.

  - Some reagents need to be freshly prepared

    Certain components of a reagent kit are highly unstable once they are reconstituted. For these components, they should be freshly prepared before each experiment.

- Spectrophotometric determination of concentration and purity of nucleic acids.
  In certain kits involving the handling of nucleic acids, the concentration of the nucleic acids (DNA and/or RNA) can be determined with a spectrometer according to the manufacturer's instructions.

Practical skills – 3

Visualizing the DNA to be resolved by agarose gel electrophoresis

- *Pre-stain: EB & CYBR-safe*
  The gel for electrophoresis can be pre-stained with DNA stains, such as ethidium bromide (EB) and CYBR-safe. The procedure is to apply these stain when the gel is being casted; as a result, the DNA samples will be stained as soon as they enter the gel.

- *Post-stain: Methylene Blue*
  After being resolved by the gel, the DNA samples can be post-stained by methylene blue. The procedure is to immense the gel containing the DNA samples in a buffer containing the stain. Then the DNA samples in the gel will be stained and visualized.

- *Casting of agarose gel and acrylamide gel (refer to the appendix at the end of this booklet)*
  - Real practice with agarose gel
  - Demonstration for acrylamide gel

Remarks :

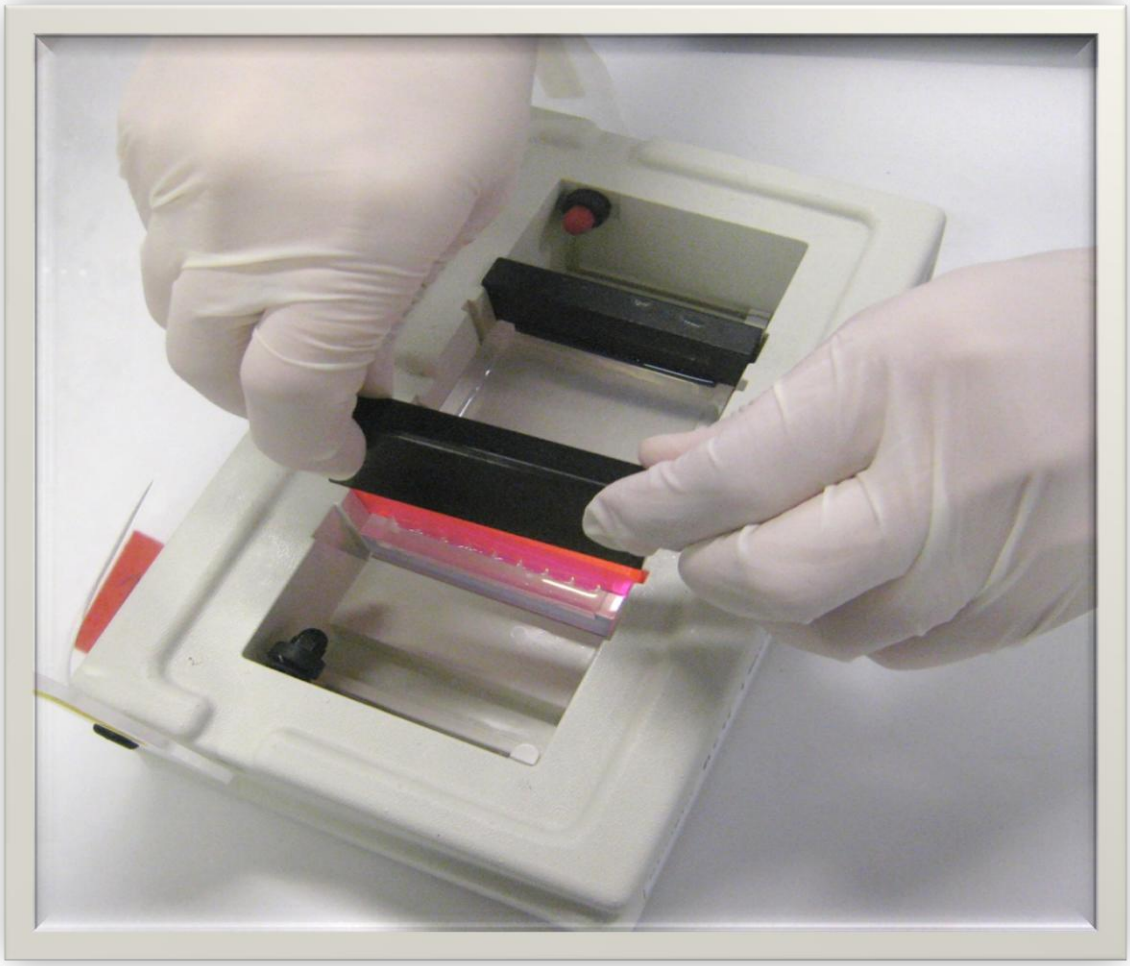


Fig. 16. Casting gel. A comb (Orange) has to be inserted into the melt agarose before it is cooled down to form a gel. The comb will be removed to leave on the gel eight wells that are used to hold DNA samples.

- *Skills of sample loading*
  - Functions of sample loading dye

    There are two major functions of sample loading dye. The first function is to increase the density of the samples, such that the samples can sink into the wells during application; the second function is to visualize the progress of electrophoresis. With the aid of dye front, the progress of electrophoresis can be traced by simple visual recognition. (Fig 17)

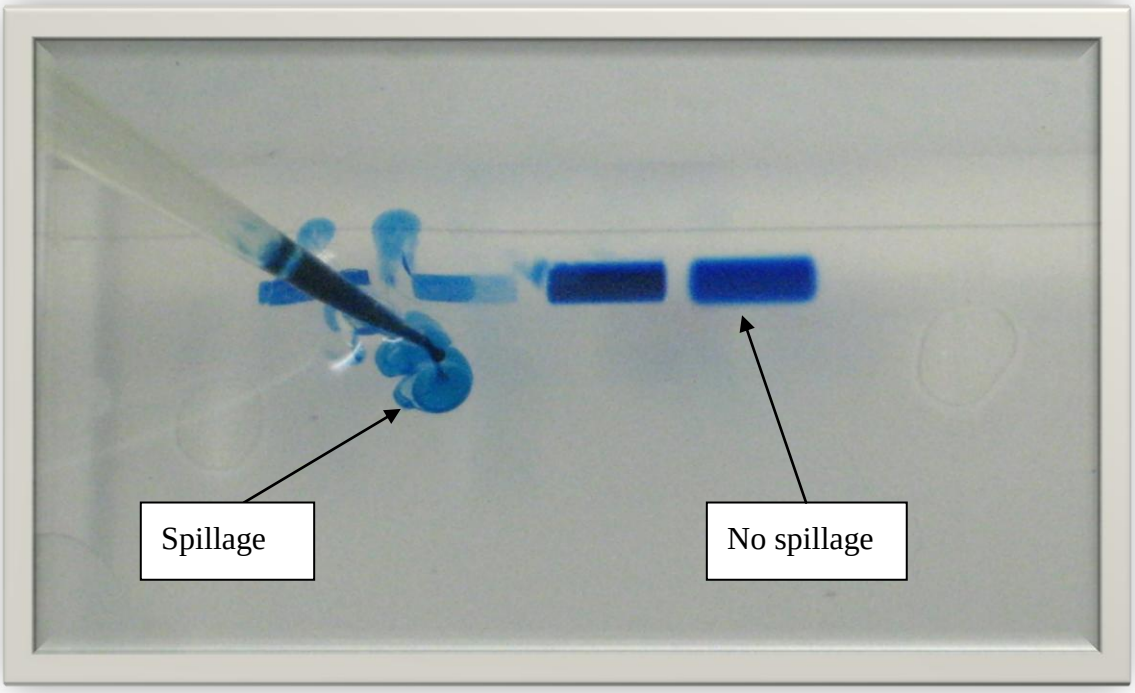


Fig. 17. Sample loading. In case of sample spillage, some blue liquid will be left around the well.

Practical skills – 4

- *Software for densitometry (Bio-Rad)*
  Densitometry is a technique to qualify the intensity of the bands for the analysis of DNA gel electrophoresis. Briefly, the computer software is able to measure the band intensity and present the intensity in numerical values for direct comparison. Trial version of the software can be downloaded from Bio-Rad website (www.bio-rad.com)

Remarks :

- *Setting PCR machine*
  - Set up thermal cycles. (Fig. 18)

Fig. 18. Two sets of PCR machines. The keypad allows users to set the time, temperature and the number of cycles of the PCR reaction.

- *Waste handling and disposal*

Solvent wastes

- Each lab is requested to set up three glass bottles for daily storage of solvent wastes (some 2.5-L bottles are recommended). These bottles should be clearly labeled for halogenated, non-halogenated and organic acid wastes, respectively.
- Once these bottles have been set up at a secure place in the lab, every disposal of solvent wastes to these bottles should be recorded on the corresponding standard log sheets.
- When these bottles are filled, the coordinator(s) of the laboratory may arrange transferring the wastes to the 25-L standard waste cans which are removed by specialized companies.

- *Sharps*
  - Each laboratory is requested to set up a special waste can for sharps and needles (top left photo). Please reduce the physical volume of this waste as much as you can. For example, do not put a long broken column in a small waste basket. Break it into small pieces before disposal.

- *Biohazardous Wastes*
  - If the chemical waste is believed to be biohazardous (such as agar plates containing bacterial colonies), it should be handled with extra care and stored in an appropriate container with clear labeling (top right photo). A complete log sheet for disposal is required. All biological waste should be sterilized by autoclave (bottom photos)

- All the apparatus used in any experiment is assumed to be contaminated. The apparatus should be sterilized by bleach for at least one hour or by autoclave. After experiment, the bench should be disinfected by disinfectant (such as bleach solution).
- In case of the spillage of potentially hazardous microorganisms, the spillage has to be handled by qualified teachers or technicians with full protective equipment on. The spills should be covered by a cloth containing disinfectant such as bleach solution for at least 15 minutes. Then, the cloth is used to remove the spill which will be kept in a sealed container for subsequent disposal. After the clearance, the area should be further cleansed up by disinfectant. In any case that the spills have make contact of the skin of the personnel, medical consultation will be necessary.
- Further information is available on: http://cd1.edb.hkedcity.net/cd/science/laboratory/safety/SHB_2002e.pdf/

- *Radioactive Wastes*
  - Standard procedures for disposal of radioactive wastes should be closely monitored. In the meantime, please minimize the use of high activity radioisotopes and reduce the physical size of the waste.

- *Precaution of human samples*
  - Keeping privacy for personal biological data
  - Seek agreement of donor

Remarks :

Appendix 1
Common reagent kits for molecular biology works

Cat No.	Item	Unit Price	Supplier	Telephone no.	Fax. No.
MB100SP	MASTERAMP BUCCAL SWAB BRUSH (SOFT PACK)	HKD 540.90	GENE COMPANY LTD.	2896 6283	2515 9371
MB71030	EPICENTRE MASTERAMP BUCCAL SWAB DNA EXTRACTION KIT (WITH SOFT PACK BRUSHES), 30 PURIF	HKD 822.60	GENE COMPANY LTD.	2896 6283	2515 9371
R0136S	NEB: BAMHI, 10000 UNITS	HKD 424.00	EASTWIN INTERNATIONAL TRADING LTD.	2887 1786	2887 7369
28-4065-60	DNTP SET -A,C,G,T, 500UL -25MM	HKD 1,881.90	GE MEDICAL SYSTEMS HONG KONG LTD.	2100 6314	2100 6338
M0267L	NEB TAQ DNA POLYMERASE WITH THERMOPOL BUFFER, 2,000 UNITS 5,000 UNITS/ML	HKD 2,320.00	VIKING MEDICARES LIMITED	2649 9988	2635 0379
75891-5LT	TBE BUFFER-5X	HKD 725.00	GE MEDICAL SYSTEMS HONG KONG LTD.	2100 6314	2100 6338
SD0061	FERMENTAS PUC19DNA 50UG/PK	HKD 440.00	GOLD PACIFIC ENTERPRISES	2543 8199	2543 8255
N/A	Primer synthesis	HKD ~200 per primer	INVITROGEN HONG KONG LTD.	2407 8450	2408 2280
15510-027	AGAROSE	HKD 2,898.90	INVITROGEN HONG KONG LTD.	2407 8450	2408 2280
F167300	GILSON: PIPETMAN STARTER KIT	HKD 3,024.00	WORLD WAYS CO.(H.K.) LTD.	2543 0760	2815 1767
27-4007-01	100 BASEPAIR LADDER	HKD 1,012.00	GE MEDICAL SYSTEMS HONG KONG LTD.	2100 6314	2100 6338

Appendix 2
List of vendors

Company:	**EASTWIN INTERNATIONAL TRADING LTD.**
Address:	Unit 1808, 18/F, Kodak House II, 39 Healthy Street East, North Point, Hong Kong.
Tel:	**2887 1786**
Fax:	2887 7369
URL:	www.eastwinhk.com

Company:	**GE MEDICAL SYSTEMS HONG KONG LTD.**
Address:	L12, Office Tower, Langham Place 8 Argyle Street, Mongkok, Kowloon, HONG KONG.
Tel:	**2100 6314**
Fax:	2100 6338
URL:	www.gehealthcare.com/worldwide.html

Company:	**GENE COMPANY LTD.**
Address:	Unit A, 8/F, Shell Industrial Building, 12 Lee Chung Street, Chai Wan, HONG KONG.
Tel:	**2896 6283**
Fax:	2515 9371
Email:	info@genehk.com
URL:	www.genehk.com

Company:	**INVITROGEN HONG KONG LTD**
Address:	7/F ADP pentagon Centre, 98 Texaco Road
	Tsuen Wan, HONG KONG.
Tel:	**2407 8450 (ACCOUNTS: MS. PANG)**
Fax:	2408 2280
Email:	hkorder@invitrogen.com.hk
URL:	www.invitrogen.com

Company:	**VIKING MEDICARES LIMITED**
Address:	Rm 1116, 11/F., Metropole Square, 2 On Yiu Street,
	Shatin, P.O. Box 50, Hong Kong.
Tel:	**2649 9988**
Fax:	2635 0379
Email:	viking@twcbiosearch.com

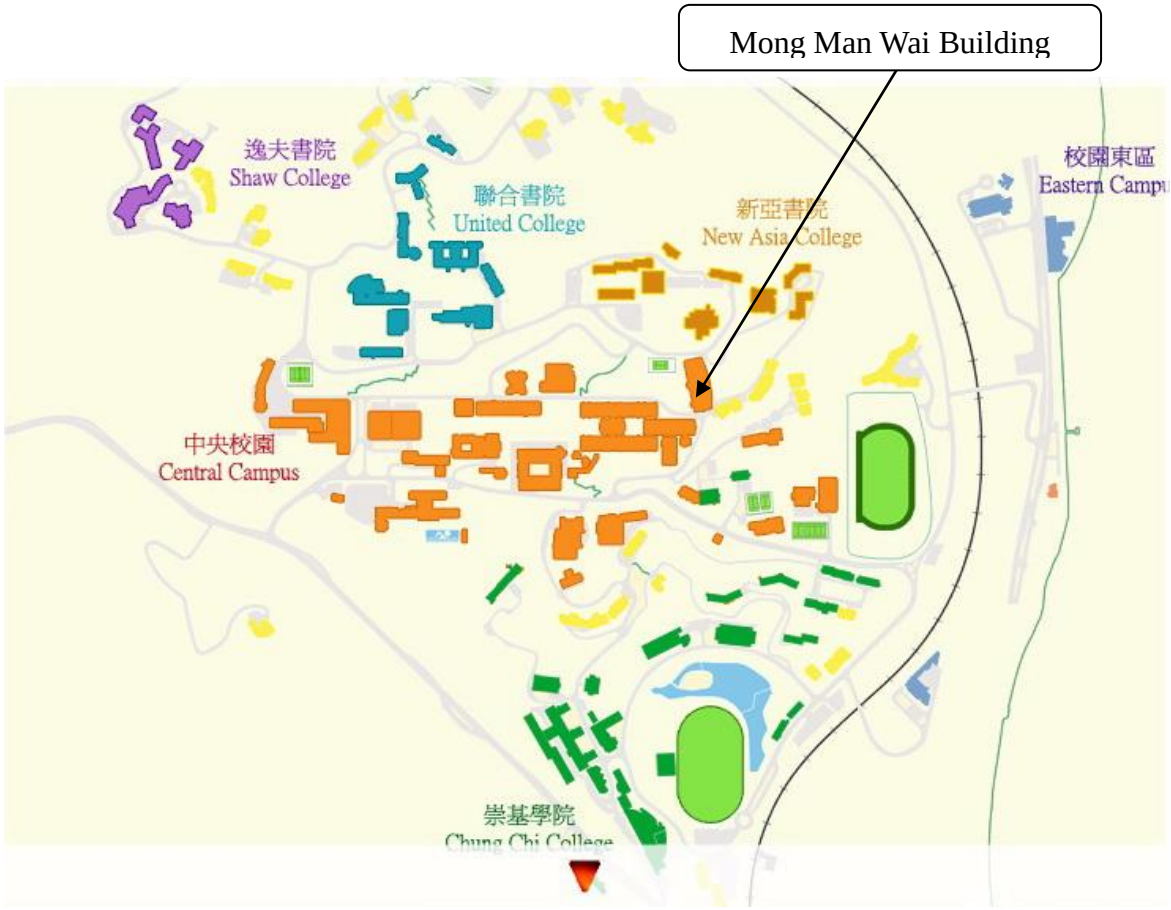
Company:	**GOLD PACIFIC ENTERPRISES**
Address:	16B Casey Building, 38 Lok Ku Road
	Sheung Wan, HONG KONG.
Tel:	**2543 8199**
Fax:	2543 8255
Email:	gpehk2@netvigator.com

Company:	**WORLD WAYS CO.(H.K.) LTD.**
Address:	2/F, Winfull Commercial Building
	172-176 Wing Lok Street
	HONG KONG
Tel:	**2543 0760**
Fax:	2815 1767
Email:	wwhk@hkstar.com
URL:	www.gilson.com

Company:	**GRANDTECH SCIENTIFIC CHINA LTD.**
Address:	Unit 18, 20/F, Grandtech Centre, 8 On Ping Street, Shatin, HONG KONG.
Tel:	**3102 1978**
Fax:	3102 1976
Email:	info@grandtechsci.com

Appendix 3
Remarks on Q and A sessions

Appendix 5
The Map of CUHK



Ref: http://www.cuhk.edu.hk/v6/en/campus_map/campus_map.html

TRANSPORT

MTR (Recommended)

- University Station of the MTR East Rail is located just outside the main campus.

Bus / Mini-bus

- Disembark at Chek Nai Ping station to enter through main entrance
- Disembark at Chung Chi College station to go to Chung Chi College
- Disembark at University Railway Station Bus Terminus to reach University Station of the MTR East Rail

Driving

- University Entrance (enter from Tai Po Road)
- Chung Chi College Entrance (enter from Tai Po Road)
- East Gate Entrance (enter from Tolo Highway)

Appendix *Quick reference of agarose gel electrophoresis*

1. Prepare for the apparatus and materials used in gel eletrophoreis

2. Weight enough agarose powder for gel casting (1 – 2% w/v)

3. Mix agarose powder with TAE buffer before heating

4. Wrap the conical flask with clinging film and make a pore to prevent excessive water evaporation and pressure build-up during heating

5. Heat and melt the agarose with a microwave oven until the agrose powder is completely melt

6. Prepare and set up an eletrophoresis unit

7. Pre-stain the melt agarose gel with ethidium bromide (~0.5 µg/ml)

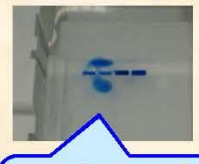
8. Pour the melt agarose onto the gel casting set-up

9. Remove the gel casting unit after the gel is solidified

10. Observe if the wells of the gel are properly set

11. Pour gel running buffer into the eletrophoresis unit fit with agarose gel

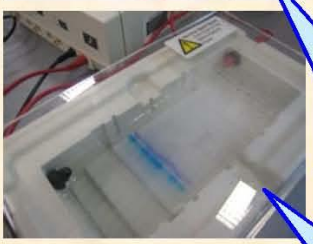
12. Make sure the running buffer cover the gel and the wells



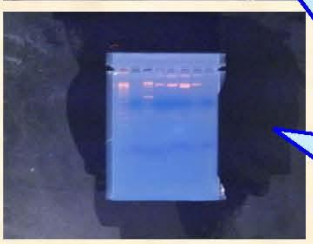
Caution: avoid overloading or splashing the wells

13. Mix the DNA samples with loading dye and apply the samples (25 µl) and DNA markers (10 µl) to the wells carefully

14. Connect the eletrophoresis unit to a power unit and apply a constant voltage (80 - 100 V)



15. Observe the movement of dye front periodically



16. Visualize the DNA bands with UV light

Members of the Organizing Committee

Prof. Shaw Pang Chui, PhD
Chairman, Department of Biochemistry, CUHK.

Prof. Kong Siu Kai, PhD
Professor, Department of Biochemistry, CUHK.

Dr. Patrick Ngai Hung Kui, PhD
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Dr. Lo Fai Hang, PhD
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END

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