Indexed.

Workshop on
"Polymerase chain Reaction (PCR) and its Applications"

5 – 9 October 1999

Department of Chemistry,
University of Colombo

Organized by
National Science Foundation (NSF)
&
Department of Chemistry, University of Colombo

Workshop on *"Polymerase Chain Reaction & its Applications"*

5-9 October 1999

Organized by National Science Foundation & University of Colombo

PROGRAMME

Day 1: ***Tuesday, October 05, 1999***

Venue: Chemistry Lecture Theatre, Department of Chemistry, University of Colombo.

Morning Session

9.30	Inauguration
10.40	Tea and Registration of participants
11.15	Lecture on "DNA structure and physical properties"
12.15	Lunch Venue: Seminar Room 1, Department of Chemistry).

Afternoon Session

Laboratory Course:

Venue: Biotechnology Laboratory (fourth floor), Department of Chemistry.
Instructor: Dr. Anil Jayasekera, Department of Botany, University of Colombo

1.00	Extraction and purification of plant DNA
2.45	Tea Venue: Seminar Room 1, Department of Chemistry
3.00	Extraction and purification of plant DNA
4.45	Discussion

Day 2: Wednesday, October 06, 1999

Laboratory Course:

Venue: Biotechnology Laboratory, Department of Chemistry

Instructors: Dr. Maya B. Gunasekera, Mr. Neil Fernandopulle, Department of Chemistry, University of Colombo
Dr. Anil Jayasekera, Department of Botany, University of Colombo

Morning Session

8.30	Lecture on "Polymerase chain reaction" Venue: Chemistry Lecture Theatre
9.30	Tea Seminar Room 1, Department of Chemistry
9.45	Extraction of DNA from blood and blood stains
11.00	Preparation of reagents for a PCR assay Application: PCR amplification of human short tandem repeat (STR) loci General PCR protocol and multiplex PCR
12.00	Lunch Seminar Room 1, Department of Chemistry

Afternoon Session

1.00	Setting-up of PCR assay
2.00	Preparation of agarose gel
2.45	Tea Seminar Room 1, Department of Chemistry
3.00	Quanitification of DNA by spectrophotometry
4.00	Agarose gel electrophoresis of plant DNA extracts and PCR products
4.45	Discussion

Day 3: ***Thursday, October 07, 1999***

Laboratory Course

Venue: Biotechnology Laboratory, Department of Chemistry, University of Colombo

Instructors: Dr. Maya B. Gunasekera, Ms. Himesha Vandebona, Department of Chemistry, university of Colombo
Dr. Sunil Premawanse, Department of Zoology, University of Colombo

Morning Session

8.30	Lecture on "Optimization strategies for PCR Venue: Chemistry Lecture Theatre
9.00	Optimization of a PCR protocol Application: PCR amplification of NADH dehydrogenase 5 gene of animal mitochondrial DNA (mtDNA) for phylogenetic analyses
10:00	Tea Seminar Room 1, Department of Chemistry, University of Colombo
10.15	Visualization of PCR products of STR analysis and plant DNA Extraction and Purification of PCR products from agarose gels
12.15	Lunch Seminar Room 1, Department of Chemistry

Afternoon Session

1.00	Agarose gel electrophoresis of mtDNA PCR products Preparation of a nonradioactive (DIG) DNA probe using PCR
3.00	Tea Seminar Room 1, Department of Chemistry
3.15	Preparation of gel
4.15	Lecture on "Analysis of STR loci for human identification" Venue: Chemistry Lecture Theatre, Department of Chemistry
4.45	Agarose gel electrophoresis of PCR products

Day 4: Friday, October 08, 1999

Laboratory course
Venue: Biotechnology Laboratory, Department of Chemistry
Microbiology Laboratory, Department of Botany
Instructors: Professor Athula Perera, Department of Agriculture, University of Peradeniya
Mr. J.M.D.T. Everad, Coconut Research Institute.
Mr. Lasantha Ubhayasena, Sugercane Research Institute.

Morning Session

8.30	Lecture on “Preparation of Taq DNA Polymerase in the Laboratory” Venue: Chemistry Lecture Theatre
9.30	Tea Seminar Room 1, Department of Chemistry
9.45	Setting a RAPD-PCR using coconut, papaya, tea and sugercane DNA
12.30	Lunch Seminar room 1, Department of Chemistry

Afternoon Session

1.30	Lecture on “Molecular Biology Applications in Plant Breeding” Venue: Chemistry Lecture Theatre
2.45	Tea Seminar Room 1, Department of Chemistry
3.00	Lecture on “Molecular Markers –RFLP and RAPD Venue: Chemistry Lecture Theatre
4.00	Preparation of agarose and acrylamide gels

Day 5: ***Saturday, october 09, 1999***

Laboratory Course

Venue:	Biotechnology Laboratory, Department of Chemistry
Instructors:	Professor Athula Perera, Faculty of Agriculture, University of Peradeniya Mr. J.M.D.T. Everad, Coconut Research Institute Mr. Lasantha Ubhayasena, Sugercane Research Institute

Morning session

8.30	Gel electrophoresis of PCR products
9.45	Tea Seminar Room 1, Department of Chemistry
10.00	DNA sequence data analysis using computer software
11.00	Lecture on "Analysis of Molecular Data" Venue: Chemistry Lecture Theatre
12.00	Lunch Venue: Seminar Room 1, Department of Chemistry

Afternoon Session

1.00	Scoring the gel
2.00	Data analysis using RAPD distance
4.00	Discussion
4.30	Award of Certificates

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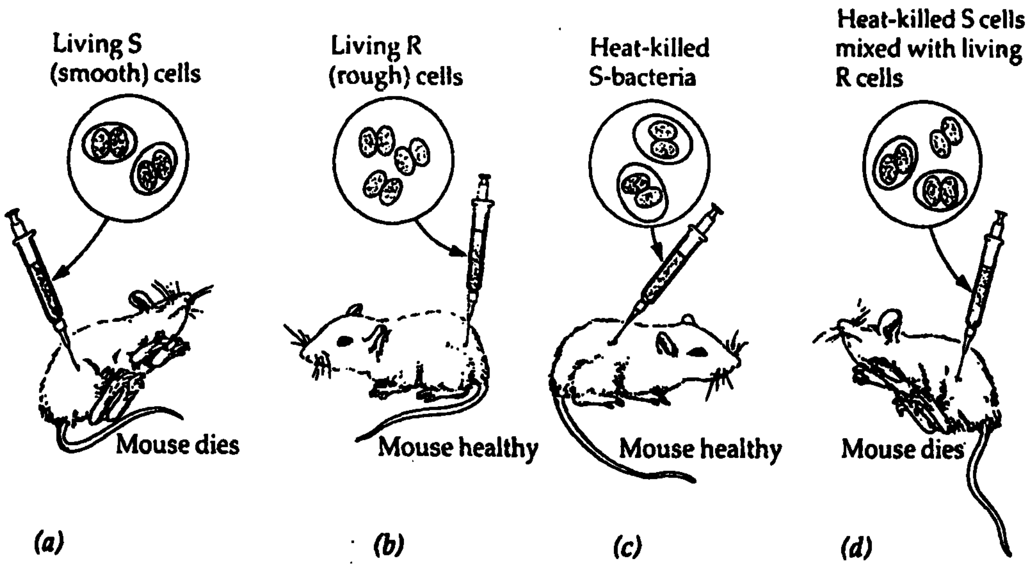
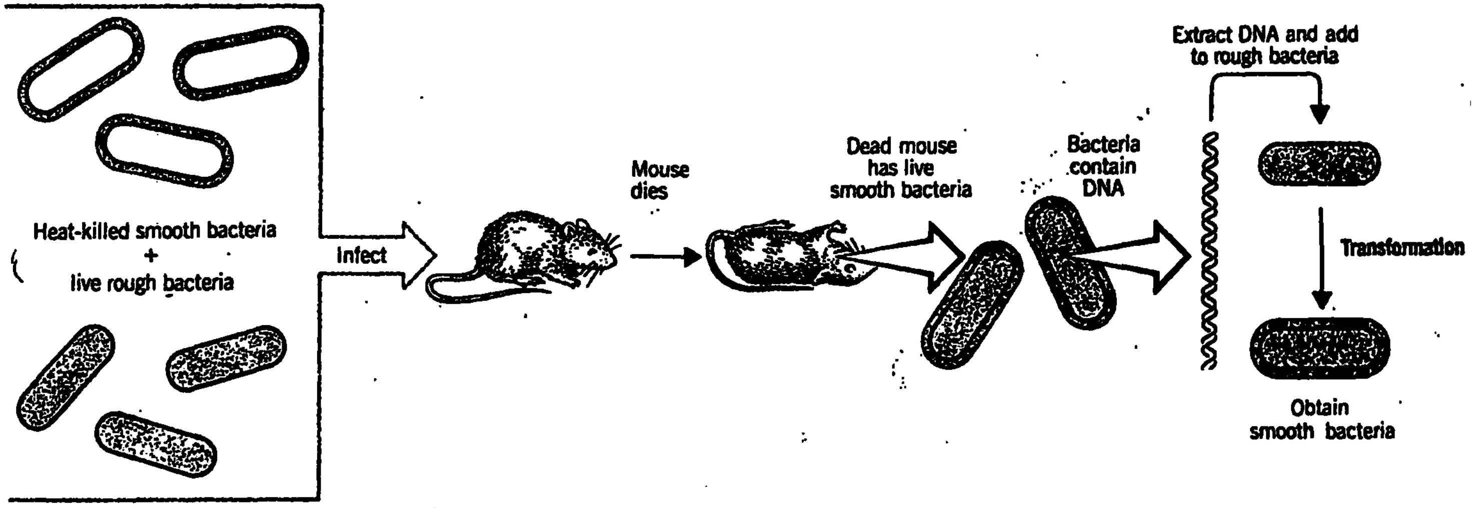
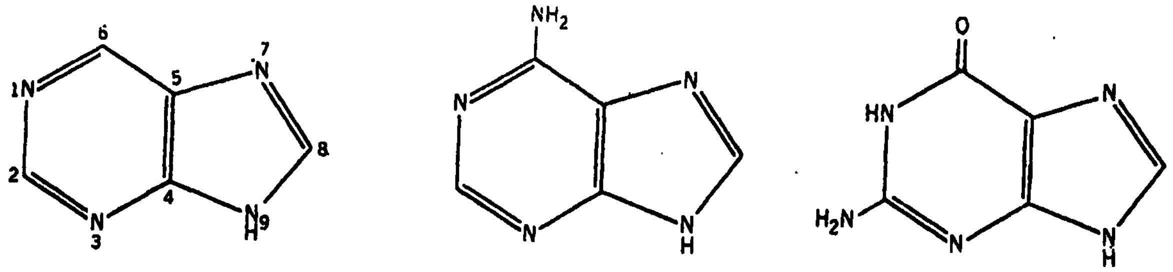


gure 15.1
ansformation of bacteria. Griffith scovered that *(a)* the S strain of the um *Streptococcus pneumoniae,* was protected by a capsule, was thogenic, *(b)* the R strain, a mutant lacking the coat, was nonpathogenic, *(c)* heat-killed S bacteria were harmless, and *(d)* a mixture of heat-killed S cells and live R bacteria caused pneumonia and death. Live S bacteria could be retrieved from the dead mice injecte with the mixture. Griffith concludec that some chemical from the dead S cells had genetically transformed so of the R bacteria into S bacteria.



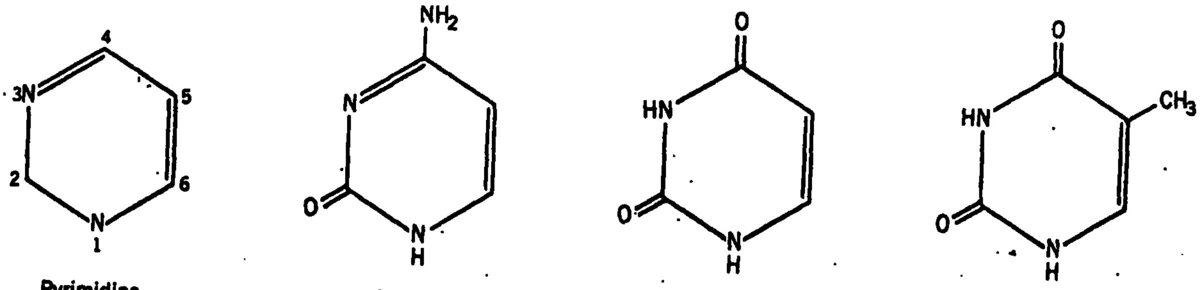
gure 2.1
e transforming principle is DNA.
ither heat-killed smooth bacteria nor live rough (mutant) bacteria can kill mice. But the mixture kills ce; and live smooth bacteria can be recovered from them. The transformation of inactive rough cteria into virulent smooth bacteria can be accomplished *in vitro* by the addition of DNA extracted m smooth bacteria.



Purine

Adenine

Guanine



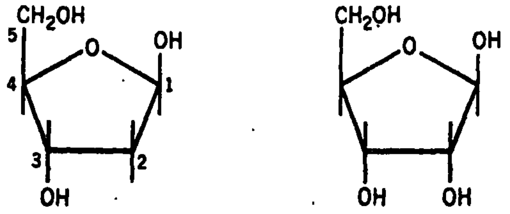
Pyrimidine

Cytosine

Uracil

Thymine

Figure 2.4
Purines and pyrimidines provide the nitrogenous bases in nucleic acids.
The "purine" and "pyrimidine" rings show the general structures of each type of base; the numbers identify the positions on the ring.



2-deoxyribose

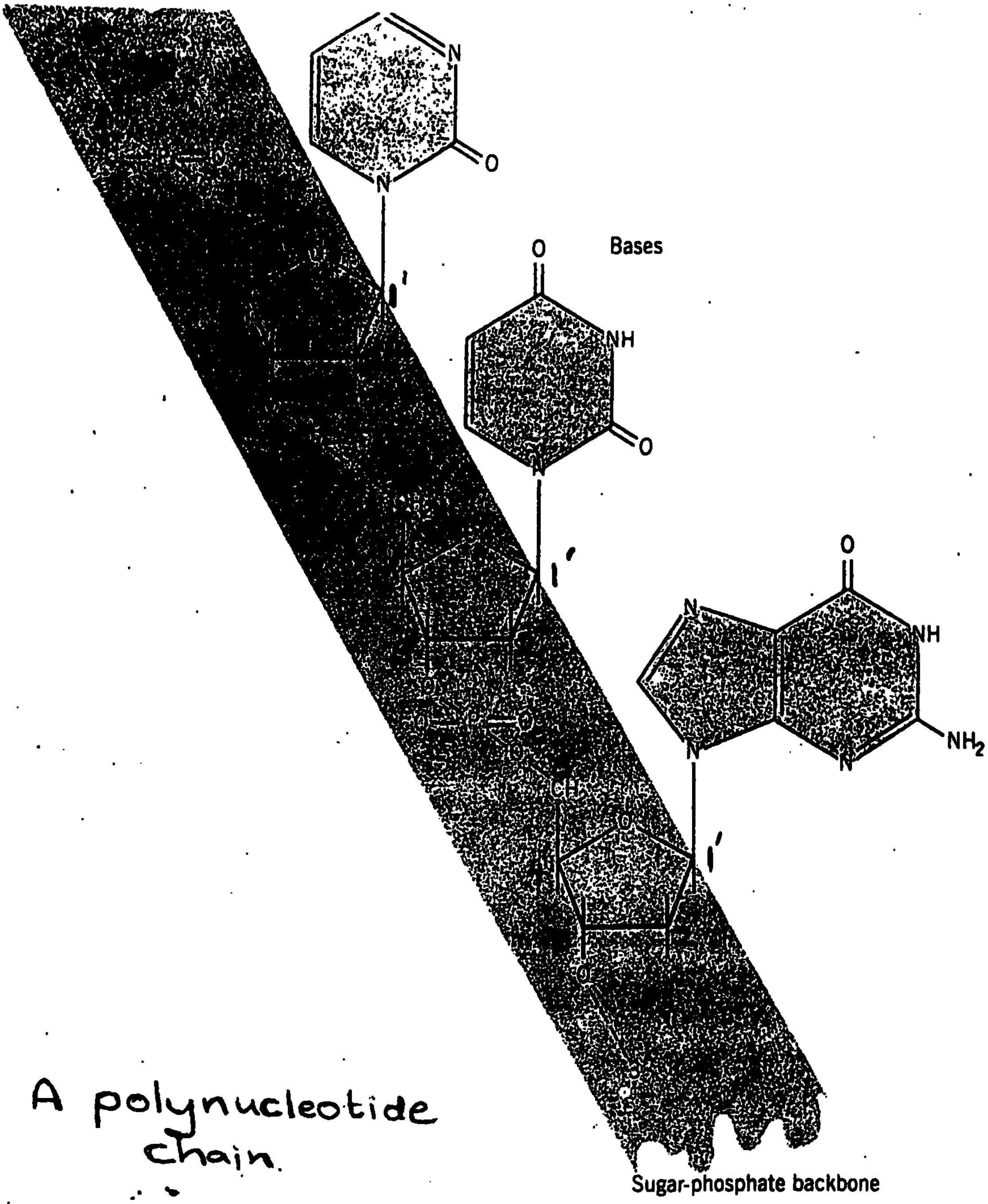
Ribose

Figure 2.5
2-Deoxyribose is the sugar in DNA and ribose is the sugar in RNA.
The carbon atoms are numbered as indicated for deoxyribose.

Bases, nucleosides, and nucleotides have related names.

Base	Nucleoside	Nucleotide	Abbreviation	
			RNA	DNA
Adenine	adenosine	adenylic acid	AMP	dAMP
Guanine	guanosine	guanylic acid	GMP	dGMP
Cytosine	cytidine	cytidylic acid	CMP	dCMP
Thymine	thymidine	thymidylic acid		dTMP
Uracil	uridine	uridylic acid	UMP	

Abbreviations of the form NMP stand for nucleoside monophosphate; "d" is used to indicate the deoxy form and its absence implies the presence of a 2′-OH group.



A polynucleotide chain.

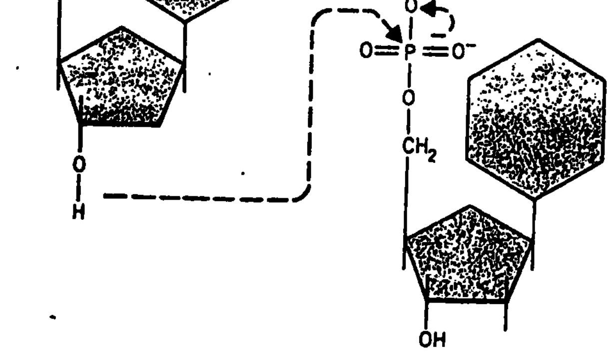
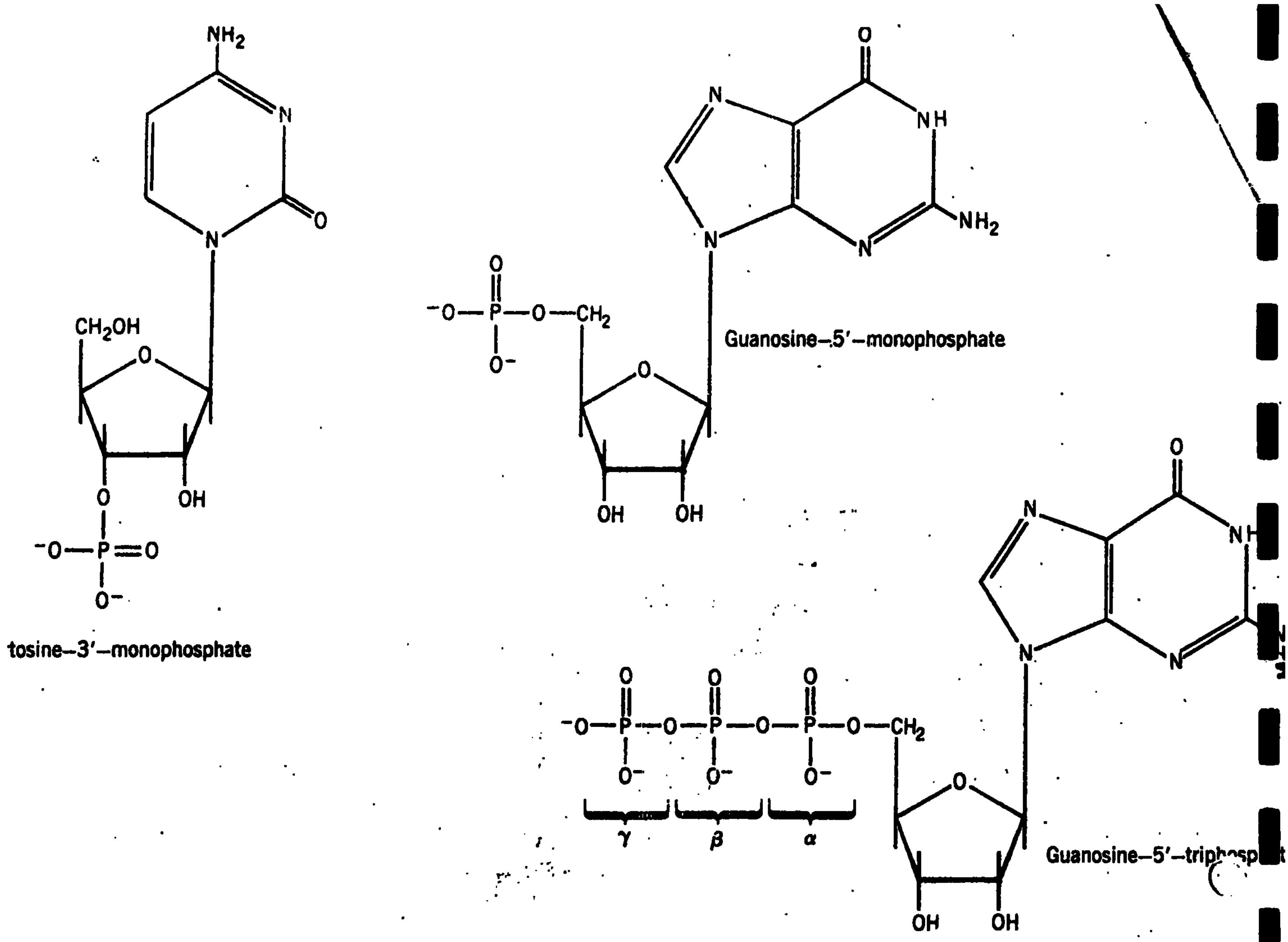
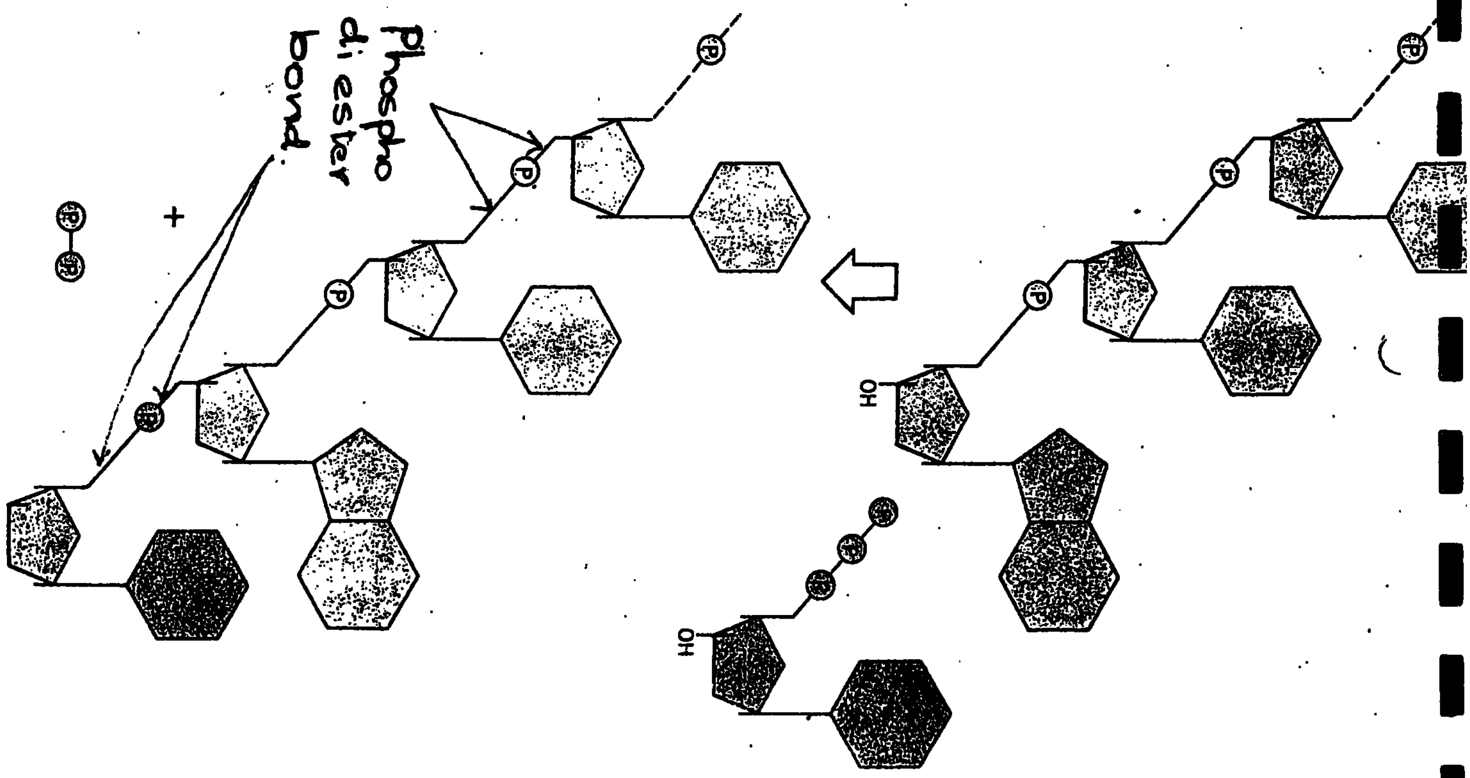


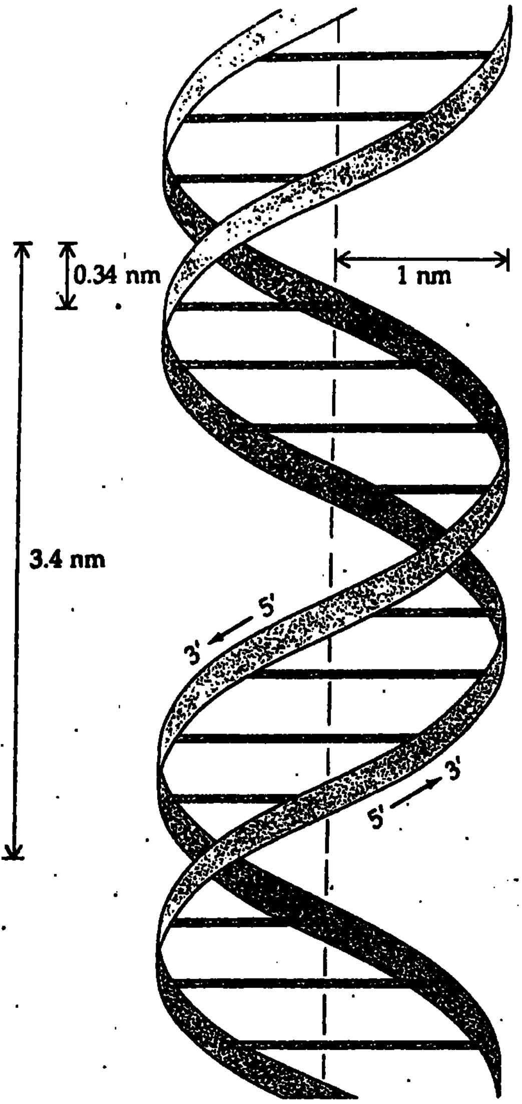
Figure 9.3
Phosphodiester bond formation involves a hydrophilic attack by the 3′—OH group of the last nucleotide of the chain on the 5′ triphosphate of the incoming nucleotide, with release of pyrophosphate.

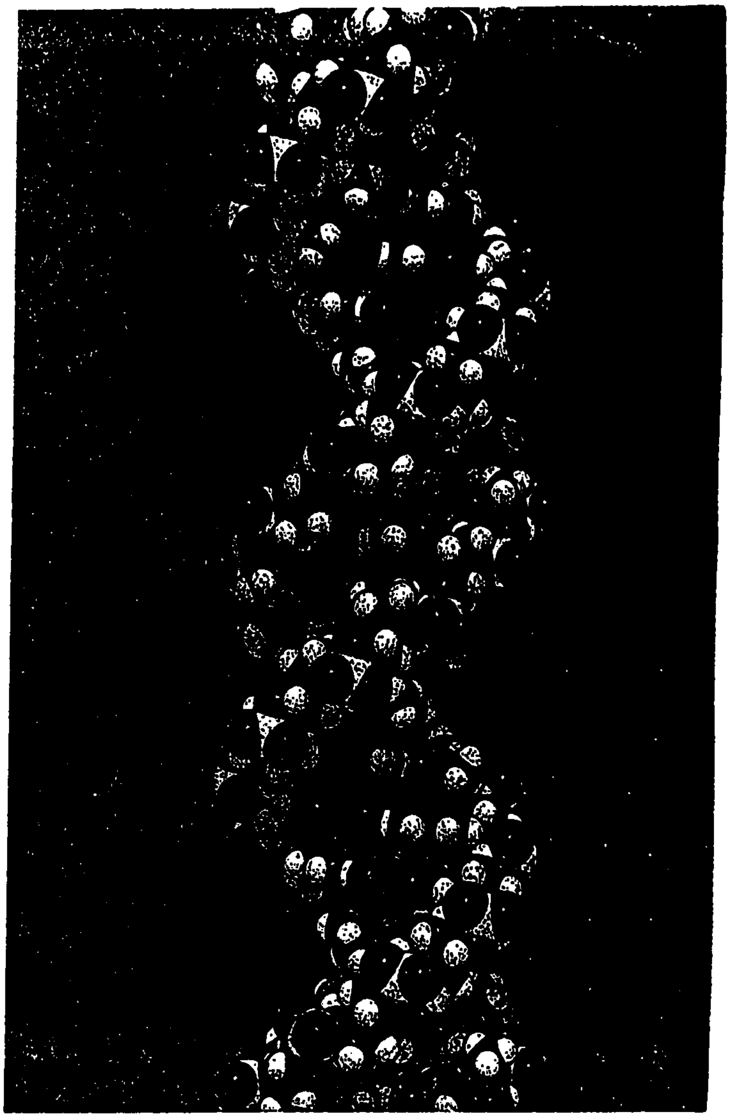


gure 2.7
ıcleotides may carry phosphate in the 5′ or 3′ position.



C4





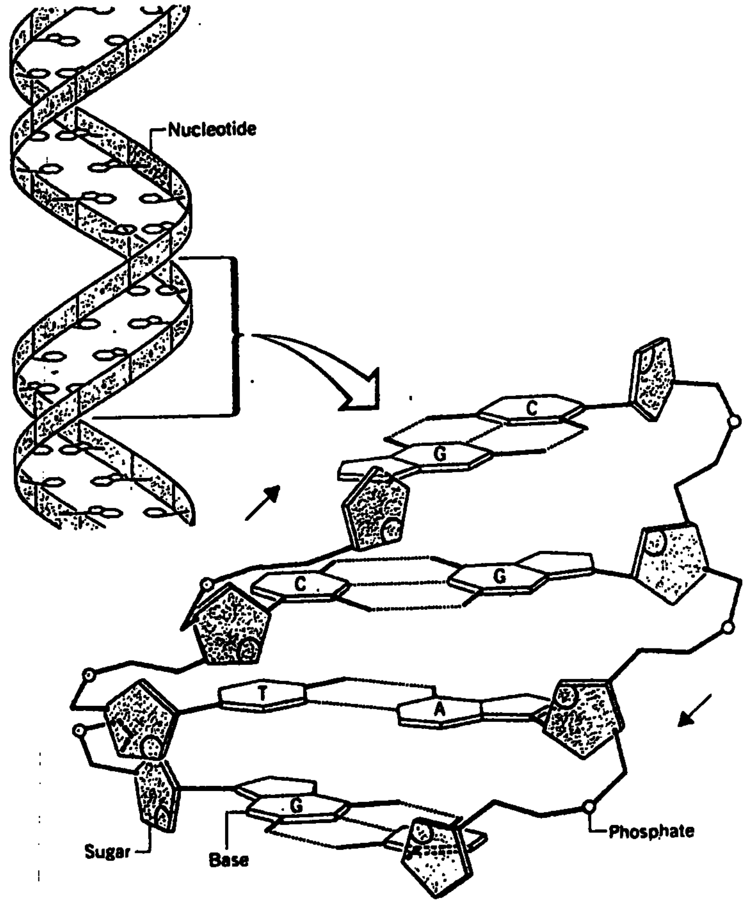


Figure 2.11
Flat base pairs lie perpendicular to the sugar-phosphate backbone.

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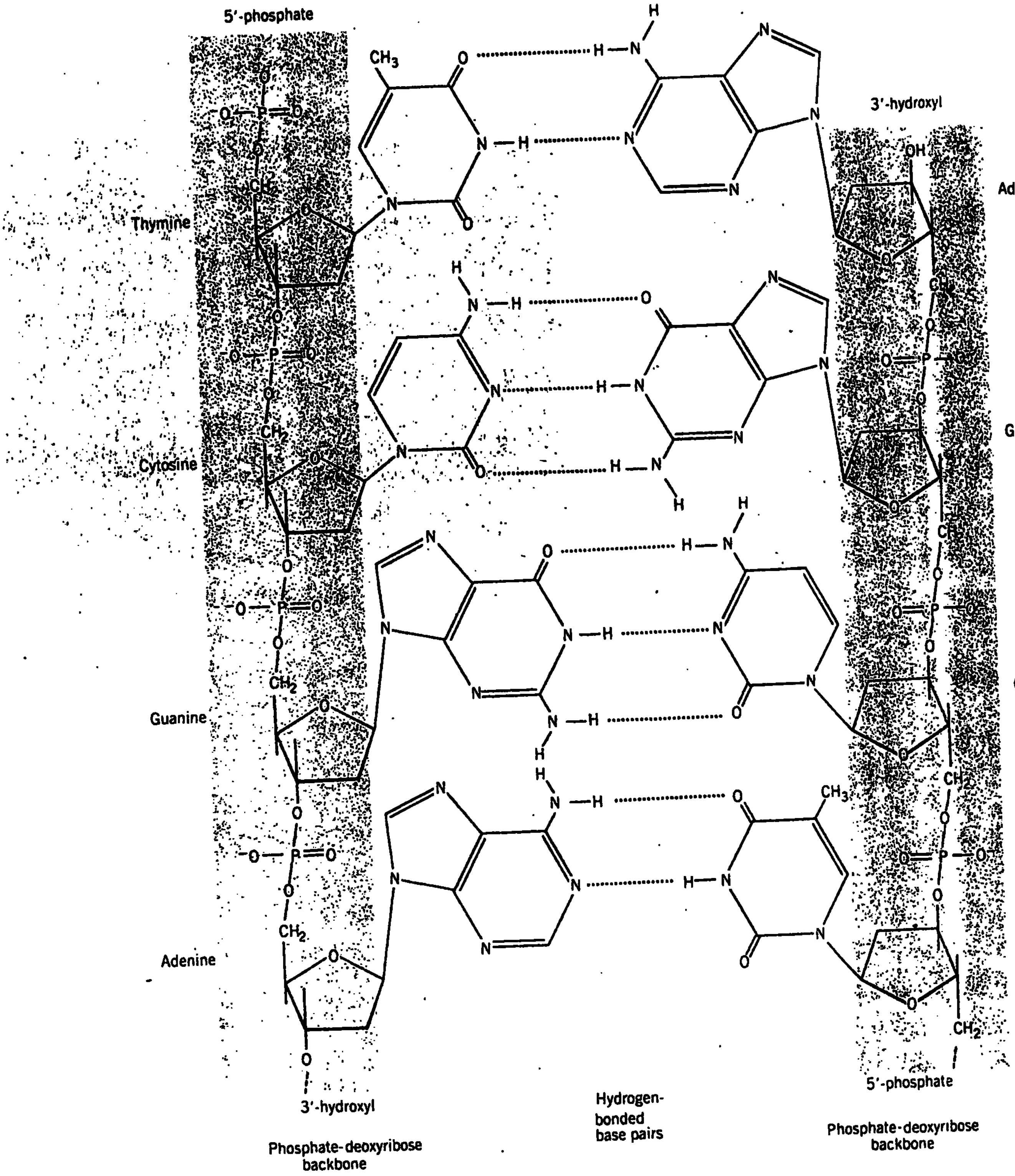


Figure 2.10
The double helix maintains a constant width because purines always face pyrimidines in the complementary A-T and G-C base pairs.
Reading down the page, the strand on the left runs 5'–3' and the strand on the right runs 3'–5'. The figure is diagrammatic and does not show the winding of the two strands about one another.

PCR IN BIOLOGY

(1) PCR Amplification of mitochondrial-cytochrome oxidase *b* gene from genomic DNA samples of a mammalian species.

Analysis of mitochondrial DNA (mtDNA) for sequence variation is a powerful tool used in molecular phylogenetic studies. As opposed to nuclear DNA, mtDNA has several advantages in phylogenetic analysis:

1) mtDNA has a high mutation rate and therefore, nucleotide changes accumulate quickly even between species that diverged quite recently.
2) mtDNA is maternally inherited (haploid) and does not recombine and therefore it is much easier to analyse than nuclear DNA. Furthermore, individuals sharing the same mtDNA sequences must have had a common female ancestor.

The genetic relatedness between species, subspecies and sibling species therefore can be analysed by comparing differences in the mtDNA sequences. Fortunately, it is not necessary to examine the complete mtDNA genome of organisms (the mammalian mitochondrion is approximately 17,000 bp in size). This is because certain regions of the mtDNA have been identified to be hypervariable. One example of such a hypervariable region is the cytochrome oxidase *b* (cyt *b*) gene. These hypervariable regions can then be selectively amplified from a DNA extract containing both nuclear DNA and mtDNA (separation and purification of mtDNA from nuclear DNA is not required) by PCR and subjected to sequence analysis. Loss or addition of nucleotide bases is compared between populations to determine phylogenetic relationships.

The objective of this exercise is to selectively amplifiy a region (approx. size 500 bp) from the mitochondrial cyt *b* gene of a mammalian species by PCR, using total DNA (genomic) extracts, and primers specific to mitochondrial cyt *b* gene.

Stock solutions:

10x PCR buffer : 15 mM $MgCl_2$, 100 mM Tris.HCl, pH = 9.0 (25^0C), 500 mM KCl, 1% TritonX-100, 2 mg/ml BSA or gelatin

5 mM mixture of each deoxynucleotide triphosphate (dNTP - A,T,G,C)

Primers : L14724 (5'CGAAGCTTGATATGAAAAACCATCGTTG3'),
H 15149 (5'GCAGCCCCTCAGAATGATATTTGTCCTC3')
50 µM stock solution, 10 µM working solution

Taq DNA polymerase (Appligene oncor) 5u/µl

PCR reaction mixture:

	Volume per R^n	Final Concentration
10x PCR buffer containing $MgCl_2$	7.5 µl	1x
dNTP (5 mM) solution	0.8 µl	53 µM
Primer (10 uM) L 14724	1.5 µl	0.2 µM
H 15149	1.5 µl	0.2 µM
DNA (200 ng) dissolved in sterile dist.water	1.0 µl	
Sterile distilled water	57.7 µl	
Total volume	70 µl	

Enzyme mix (added after step 1 in the PCR programme (Hot start)

Taq DNA polymerase (Appligene)	0.3 µl (1.5 u)
St. distilled water	4.7 µl
Total volume	5.0 µl

Total reaction volume 75 µl

Preparation of a master mix for simultaneous amplification of samples

If the number of samples to be amplified = 5 ; calculate the volume of each reagent for 5.5 samples:

10x PCR buffer containing $MgCl_2$	7.5 x 5.5 = 41.25 µl
dNTP (5 mM) solution	0.8 x 5.5 = 4.4 µl
Primer (10 uM) L 14724	1.5 x 5.5 = 8.25 µl
H 15149	1.5 x 5.5 = 8.25 µl
Sterile distilled water	57.7x 5.5 = 317.35 µl

Mix thoroughly. Add 69.0µl to each reaction vial.

Add DNA (200 ng) = 1 µl

Taq DNA polymerase mix (sample number = 5)
Taq DNA polymerase 0.3 x 5.5 = 1.65 µl
sterile distilled water = 25.85 µl
Enzyme mix (5 µl) added after step 1 in the PCR programme (Hot start)

PCR programme for mtDNA amplification:

1. Hot start 94.0^{0}C – 5 min (Taq DNA polymerase absent)
   (initial denaturation of template)

2. 88. 0^{0}C – 1 min – pause the reaction and add Taq DNA polymerse

3. 94. 0^{0}C – 1.30 min
   (denaturation)

4. 53.0^{0}C – 1.15 min
   (primer annealing)

5. 72. 0^{0}C – 1.30 min
   (chain extension)

6. Repeat steps 3-5 x 40 cycles

7. 72.0^{0}C – 3.00 min
   (final extension)

8. 4.0^{0}C (for an indefinite time)
   (samples stored at 4. 0^{0}C until removal.

9. Switch off the PCR machine.

10. Store the PCR products at –20.0^{0}C

1% (w/v) agarose gel in 1 x TBE buffer
Load 8-10 µl of PCR product in 1x gel loading buffer (2 µl of 5x buffer for 8 µl of sample)
Load a DNA marker (0.5 µg Lambda DNA - HindIII digest.
Stain the gel with ethidium bromide and observe under UV.

2) Multiplex -PCR based typing of humans using short tandem repeat (STR) DNA markers

Identification of humans based on differences at the level of DNA, or DNA fingerprinting , is now a widely accepted tool in the forensic investigations. DNA fingerprinting began with the use of minisatellite DNA probes that detect variable number of tandem repeat (VNTR) loci. A subgroup of the VNTR loci is the short tandem repeat (STR) loci, also known as microsatellite loci. These are composed of highly polymorphic tandemly repeated sequences of 1-7 bp in length, which are well distributed in the human genome. STR alleles are small in size, typically less than 350 bp in length, and are amenable to amplification by PCR and size fractionation by polyacrylamide gel electrophoresis. DNA typing by STR analysis has the advantage of requiring only a small amount of DNA, and works well in situations where the DNA may be degraded. Further, alleles can be determined precisely with the help of an allelic ladder. By simultaneous amplification of several STR loci (multiplex PCR), the power of discrimination of the method can be significantly enhanced.

The objective of this exercise is to simultaneously amplify and analyse three STR loci, HUMCSF1P0, HUMTPOX, and HUMTHO1, of Sri Lankan human DNA isolates using three sets of primers in a multiplex PCR assay. The expected allele sizes of the three loci: HUMTH01 – 179-203; HUMTPOX – 224-252; HUMCSF1PO – 291-327 bp.

Stock solutions:

10x STR buffer : 15 mM $MgCl_2$, 100 mM Tris.HCl, pH = 9.0 (25°C), 500 mM KCl, 1% TritonX-100, 2 mM mixture of each deoxynucleotide triphosphate (dNTP - A,T,G,C)

Primers : 10 x CTT (2.5 µM of each primer (total of six primers, two for each STR locus)

Taq DNA polymerase 5u/µl

PCR reaction mixture

	Volume per R^n	Final Concentration
10x STR buffer	2.5 µl	1x
Primer pairs (2.5 uM – 10x) CTT	2.5 µl	0.25 µM
DNA (200 ng) dissolved in sterile dist.water	2.5 µl	
Sterile distilled water	12.5 µl	
Total volume	20.0 µl	

Enzyme mix (added after step 1 in the PCR programme (Hot start)

Taq DNA polymerase (Appligene)	0.15 µl (0.75 u)
10xSTR buffer	0.5 µl
St. distilled water	4.35 µl
Total volume	5.0 µl

Total reaction volume 25 µl

Preparation of a master mix for simultaneous amplification of many samples:

No. of samples to be amplified – 5 ; calculate the volume of each reagent for 5.5 samples:

10x STR buffer 2.5 x 5.5 = 13.75 µl
10 x Primers CTT 2.5 x 5.5 = 13.75 µl
Sterile distilled water 12.5 x 5.5 = 68.75 µl 68.75 µl

Mix thoroughly. Add 17.5 µl to each reaction vial.

Add DNA (200 ng) = 2.5 ul

Taq DNA polymerase mix
Taq DNA polymerase 0.15 x 5.5 = 0.825 µl
10xSTR buffer 0.5 x 5.5 = 2.75 µl
sterile distilled water = 4.35 x 5.5 = 23.925 µl
Enzyme mix (5 µl) added after step 1 in the PCR programme (Hot start)

PCR programme for amplification of STR loci

1. Hot start 96.0^{0}C – 2 min (Taq DNA polymerase absent)
   (initial denaturation of template)

2. 88. 0^{0}C – 1 min – pause the reaction and add Taq DNA polymerse

3. 50 seconds (ramp) to 94. 0^{0}C – 1.0 min
   (denaturation)

3. 30 seconds (ramp) to 64.0^{0}C – 1.0 min
   (primer annealing)

4. 15 seconds (ramp) to 70. 0^{0}C – 1.5 min
   (chain extension)

5. Repeat steps 3-5 x 10 cycles

7. 45 seconds (ramp) to 90.0^{0}C – 1.0 min
   (denaturation)

8. 26 seconds (ramp) to 64^{0}C 1.0 min
   (primer annealing)

10. 15 seconds (ramp) to 70^{0}C 1.5 min
    (chain extension)

11. Repeat steps 7-9 x 20 cycles

11. 4.0^{0}C – samples stored at 4. 0^{0}C until removal.

12. Switch off the PCR machine, and store the PCR products at –20.0^{0}C

Visualization of PCR amplified products by agarose gel electrophoresis

2% agarose gel in 1 x TBE buffer
Load 8-10 µl of PCR product in 1x gel loading buffer (2 µl of 5x buffer for 8 µl of sample)
Load a DNA marker (0.5 µg Lambda DNA - HindIII digest.
Stain gel with ethidium bromide and observe under UV.

PCR OPTIMIZATION STRATEGIES

The best way to understand PCR is to consider the reaction components and how they combine to produce the best results. The components required for PCR are:

1. a target DNA template for amplification
2. a heat stable DNA polymerase
3. one or more pairs of primers (oligonucleotides) complementary to the termini of template DNA region to be amplified
4. deoxynucleotide triphosphates, a reaction buffer containing Mg^{+2} and cosolvents such as gelatin, glycerol, BSA, DMSO etc.,
5. a programmable heating block with reaction vessels.

In principle, each physical and chemical component of PCR can be modified to produce a potential increase in yield, specificity and sensitivity. Reproducibility and fidelity are two other important factors needed to be considered during the optimization process.

CRITICAL FACTORS FOR SUCCESSFUL PCR

Type of Thermal Cycler – must accurately and reproducibly maintain the PCR incubation temperatures, change from one temperature to another (ramp) over a definable time, arrive at a selected temperature without significant over or under-shoot, and cycle between the temperatures repeatedly and reproducibly.

Type of reaction tubes:
The reaction tubes affect the heat transfer from thermal cycler to the reaction mixture. It is necessary to use reaction tubes that are designed for PCR and which fit precisely in to the wells of machine.

Thermal cycling profile and cycle number:
Initial denaturation (recommended :95 °C, 1-2 minutes): The DNA must be completely denatured, otherwise the template will try to "snap-back" preventing efficient primer annealing and extension or leading to "self-priming which can lead to false positives.

Primer annealing temperature : needs to be optimized for high specificity. If the temperature is too high, no annealing; if too low, non-specific annealing will occur. PCR optimization assays should start with annealing temperatures approx. 5°C above the calculated melting temperature (Tm)

Calculation of melting temperature (Sambrook *et al.*, 1989 , *in Molecular Cloning , Coldspring Habor Laboratory, NY)*

$T_m = [^0C] = 81.5 + 16.6 9log [J+]) + 0.41(\%G+C)-600/L - 0.63 (\%FA)$
[J+} = concentration of monovalent cations (usually 50-60mM)
L = length of oligonucleotide
% (G+C) = G/C content of oligonucleotide
FA = Formamide

A more simple formula:

$T_m = 2^0C . (A+T) + 4^0C . (G+C)$

Primer extension: normally carried out at 72^{0}C, the optimum temperature for *Taq* DNA Polymerase.
Usually, for fragments shorter than 500 bp – 20s extension and for fragments up to 1.2Kb – 40s. However, these times may vary depending on the source and batch;Taq DNA polymerase can add 60 bases per second at 72^{0}C!)

Denaturation step during cycling: Denaturation at 95^{0}C for 20 – 30 seconds is usually sufficient, however, this step must be adapted to the tubes and the PCR machine used.

Cycle number: 25-35 cycles are usually recommended. Increased cycle numbers could increase the non-specific products.

Final extension: usually after the last cycle the tubes are held at 72^{0}C for 5-15 minutes to promote completion of partially extended products.

Heat stable DNA Polymerase:
Taq DNA polymerase is commonly used in PCR assays.
Source – Isolated from thermophillic eubacterium, *Themus aquaticus,* strain which lacks Taq 1 restriction enzyme.
A single polypeptide chain – ~95kD, highly processive 5'->3' polymerase but lacks 3'->5 exonuclease activity. The error rate is therefore higher than *E.coli* DNA polymerase I. ($2 \times 10\text{-}^4$ errors/base)
The Enzyme is most active at pH = 9 and temperatures around 75°C.
The Enzyme has a half life of less than 5 minutes at 100^{0}C. However, the half life upto 40 minutes at 95^{0}C.
For most assays optimum amount will be between 0.5-2.5 units. However, this can vary depending on the supplier of Taq DNA polymerase and the particular batch of enzyme used. Increased enzyme concentrations can sometimes lead to decreased specificity.

Other heat stable DNA polymerases:

Tth DNA polymerase has very efficient reverse transcriptase activity in addition to polymerase activity and therefore used in RT-PCR assays. Pwo DNA polymerase: has 3'-5' exonuclease (proof reading) activity. Thermal stability is better than Taq DNA polymerase. Recommended to be used in applications where PCR product is used for sequence analysis.

Magnesium chloride concentration:

Taq DNA polymerase prefers $MgCl_2$ as a metallic cofactor; increases the melting temperature of double stranded DNA and forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. For most assays, optimum will be between 0.5-mM – 5mM, usually 1.5mM. $MgCl_2$ is recommended. The concentration of free Mg^{+2} depends on the concentration of compounds that bind the ion, including dNTP, free pyrophosphate, and EDTA.

dNTP concentration:

The nucleotide concentration usually recommended is 200µM of each nucleotide. Imbalanced dNTP mixtures will reduce Taq fidelity. dNTPs also reduce free Mg^{+2} and thereby interfere with polymerase activity and decreases primer annealing.

Detergents and other additives:

The non ionic detergent Tween 20, dimethysulfoxide(DMSO), gelatin, glycerol, ammonium sulphate, have been reported to enhance the activity of Taq DNA Polymerase.

Primer sequences:

In most PCR applications it is the sequence and the primer concentrations that determine the overall assay success. Generally, the primers should not have any internal secondary structures, balance distribution of G/C and A/T rich domains, no complementarity between 3'ends to minimise the primer dimer formation. Typically PCR primers contain 40-75% of GC content and have 14-40 nucleotides. Primer concentrations between 0.1-0.5µM are generally optimal. Higher concentrations – promote mispriming; lower – low yield.

Reaction overlay :

Mineral oil is placed on top of the reaction mixture to prevent evaporation during PCR. A layer of paraffin wax is sometimes placed for the same purpose as well as to keep reaction components separate (paraffin is a solid at 55^0C) during the initial denaturation step so that the nonspecific binding of primers during this step is avoided (Hot start-PCR). Certain PCR protocols add the enzyme after the initial denaturation step, to avoid the extension of misprimed template prior to the initial denaturation step.

Template:
The primer-template ratio strongly influences the specificity of PCR. The purity of the DNA also influences the outcome of PCR assay – degradation of DNA, accumulation of inhibitory compounds.

Contamination:
Trace amounts of DNA contaminants could serve as templates for amplification – false positives.

Take steps to avoid all sources of contaminating DNA:
Laboratory benches, equipment and pipettes could be contaminated by previous preparations.
Cross contamination between samples
Products from previous PCR amplifications: PCR product decontamination with Uracil DNA glycosylase (UNG); PCR amplifiaction is performed with dUTP instead of dTTP. Carry-over contamination can then be avoided by treating with UNG prior to PCR amplification of new sample. Any old PCR product would then be degraded.

General rules for a PCR Laboratory:
Set up physically separated working places for Pre-PCR and post-PCR work. If possible perform PCR work in a fume hood equipped with UV. Store under the fume hood, a microfuge, set of pipettes (positive displacement), special tips (containing filter), tubes and gloves which are used only for PCR. When pipetting DNA avoid making aerosols that could carry contaminants. Have your own set of PCR reagents and solutions used only for PCR. Store the reagents in small aliquots. Always include a negative control with all reaction components except the template DNA.

References:

1. PCR Applications Manual, Boehringer Mannheim
2. PCR Technology Edited by Henry A. Erlich, Stockton Press.
3. Methods in Molecular Biology (volume 15): PCR Protocols, edited by Bruce A. White

Tutorial

1. Following buffers and reagents are available in the Laboratory:
   10 X Taq DNA polymerase incubation buffer without $MgCl_2$, 15mM $MgCl_2$
   dNTP mix (5mM), primer 1 (10µM), primer 2 (10uM)DNA (25ng/µl).

How would you prepare a PCR reaction mixture (75 µl) containing Taq incubation buffer(1x), dNTP (100µM), each primer(0.4µM) and DNA (50ng), using the above stock solutions and reagents?

2. Find the final concentration of each component in the following PCR reaction mixture:

	Volume per R^n	Final Concentration
10x PCR buffer containing 15mM $MgCl_2$	7.5 µl	?
dNTP (5 mM) solution	2.0 µl	?
Primer 1 (10 µM)	1.8 µl	?
Primer 2 (10µM)	1.8 µl	?
DNA (200 ng) dissolved in sterile dist.water	0.5 µl	—
Sterile distilled water.	?	—
Enzyme mix 1.5u/5ul	5.0 µl	—
Total Volume	75 µl	

3. How would you perform a PCR assay to determine the optimum concentration of $MgCl_2$?

4. If you find that the specificity of a PCR assay is low (ie. high yield of non-specific products), what measures can you take to reduce the yield of non specific products?

Dr.Maya B. Gunasekera, Department of Chemistry, University of Colombo, Colombo 3.

Extraction and purification of DNA of 100bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer using QIAquick Gel Extraction Kit (using a microcentrifuge)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QX1 to 1 volume of gel (100 mg ~ 100 µl).
3. Incubate at 50^0 C for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 minutes during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to buffer QX1 without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. **(Optional):** Add 0.5 ml of Buffer QX1 to QIAquick column and centrifuge for 1 minute.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 minute.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at ≥10,000 x g (~ 13,000 rpm).
12. Place QIAquick column into a clean 1.5 ml microfuge tube.
13. To elute DNA, add 50 µl of 10 mM Tris-HCl, pH 8.5 or H_2O to the center of the QIAquick column and centrifuge for 1 minute at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick column, let stand for 1 minute, and then centrifuge for 1 minute.

Removing and Purifying DNA from Agarose gels using Geneclean 11 kit, BIO 101, Inc.

1. Excise band from agarose gel.
2. Add 3 volumes of NaI stock solution.
   Incubate at 45 - 55^{0}C to dissolve agarose. (For agarose with TBE: add 1/2 volume of TBE Modifier and 4.5 volumes of NaI to given volume of agarose).
3. Add GLASSMILK suspension. Incubate for 5 minutes.
4. Pellet GLASSMILK/DNA complex for 5 seconds. Remove supernatant and set aside.
5. Wash pellet 3 times with NEW WASH.
6. Elute DNA into water or low-salt buffer.

biotin
-fluorescein

Labelled DNA fragment called
a DNA probe

Preparation of a DIG probe using PCR

PCR products labeled with PCR DIG labelling mix can be used as hybridization probes that can be detected with non-radioactive detection system. Probe labelling requires much less template than other probe labelling methods , because the PCR can amplify enough labelled probe from a few nanograms of genomic DNA or linearized plasmid DNA.

PCR Labelling protocol

1. Add the following components to a sterile microcentrifuge tube. (Place the tube on ice during pipetting.)

	Volume per R^n	Final conc.
PCR buffer with 15 mM $MgCl_2$	5.0 µl	}
25mM $MgCl_2$	2.0 µl	}2.5 mM
10x PCR DIG mix	5.0 µl	200µMdNTP
(containing 2mM of dATP,dCTP,dGTP;		
1.3mMdTTP; 0.7mMDIG-11-dUTP		
(alkali-labile:pH7.0)		
Primer M13 universal (5µM)	1.0 µl	0.2 µM
Primer M13 reversal (5µM)	1.0 µl	0.2 µM
Template DNA	1.0 µl	
Sterile distilled Water	30.0 µl	
Total volume	45.0 µl	

Enzyme mix [added after step 1 in the PCR programme (Hot start)]

Enzyme mix	0.75 µl (1.0 u)
Sterile distilled Water	4.25 µl
Total volume	5.0 µl

Total reaction volume 50 µl

2. Mix the reagents

3. PCR programme

Initial denaturation of templates	Pause: add enzyme mix	Cycling for 29 cycles	Extension step	Hold step
94°C for 5 min	88°C for 1 min	94°C 45 sec. 55°C 1.0 min 72°C 2.0 min	72°C for 7 min	4°C

4. Analysis of PCR products

Analyze 7.0µl of amplified mixture by agarose gel electrophoresis.
Due to multiple incorporation of DIG-dUTP during the PCR process the molecular weight of the PCR product is increased significantly compared to the unlabelled probe.

DNA Extraction with Chelex-100

Sample: 3-300µl of whole blood , or 9-25mm^2 portion of bloodstained material.

Procedure.

1. Add the sample to a sterile 1.5 ml microcentrifuge tube containing 1ml of sterile, de-ionized water.
2. Gently mix the samples and incubate at room temperature for 30 minutes. Mix occasionally by inversion or gentle vortexing.
3. Centrifuge the samples at top speed (15,000 x g) for 2 minutes at room temperature in a microcentrifuge.
4. Carefully remove all but 20-30µl of the supernatant from each sample and discard. If the sample is a bloodstain, leave the fabric in the tube with the pellet.
5. Add 5% Chelex-100 to a final volume of 200µl. Note: 5% Chelex-100 quickly settles out of solution. Remove portion from a beaker with a stir bar continuously stirring the solution.
6. Incubate the samples at 56°C for 20 minutes.
7. Vortex the samples on high speed for 5-10 seconds.
8. Incubate the samples at 100°C for 8 minutes.
9. Vortex the samples on high speed for 5-10 seconds.
10. Centrifuge the samples at top speed (15,00x g) for 2 minutes at room temperature in a microcentrifuge.
11. The DNA, which is in the supernatant, is now ready for amplification. We recommend using 2.5µl of the supernatant in a 25µl reaction volume.
12. Store the remainder of the sample at 2-8°C or at -20°C. Before using stored sample, repeat the centrifugation step 10.

PCR amplification of Short Tandem Repeats (STR)

Samples: a) DNA from blood stained cotton fabric
b) DNA from fresh Blood

STR Loci: Multiplex PCR- CSF1PO, TPOX, THO1
Monoplex PCR- TPOX

Master Mix

	for each reaction	for 8 reactions
10X incubation buffer	2.5µl	x 8.5 = 21.25µl
2mM dNTP mix	2.5µl	x 8.5 = 21.25µl
2.5µM Primer mix*	2.5µl	x 8.5 = 21.25µl
25mM $MgCl_2$	1.5µl	x 8.5 = 12.75µl
Sterile Distilled H_2O	1.0µl	x 8.5 = 8.5µl
Volume of master mix in each reaction	10.0µl	
Template DNA	10.0µl	
Taq DNA polymerase (diluted)	5.0µl	
Total volume of each reaction	25.0µl	

* For multiplex PCR add 2.5µl of Primer mix containing 3 primer pairs, for monoplex PCR add 2.5µl of Primer mix containing one primer pair.

Taq DNA Polymerase dilution for multiplex PCR reactions:
Concentration of Taq DNA Polymerase for each multiplex reaction = 0.75 units

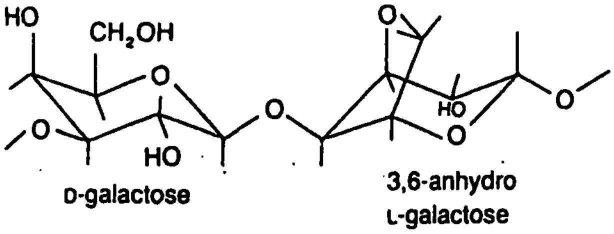
Taq DNA polymerase(5units/µl)	1.5µl
10X incubation buffer	4.5µl
$MgCl_2$	2.5µl
H_2O	36.5µl
Total	= 45.0µl

Taq DNA polymerase dilution for monoplex PCR reactions:
Concentration of Taq DNA polymerase for each monoplex reaction = 0.50 units

Taq DNA polymerase (5units/µl)	1.0µl
10X incubation buffer	6.0µl
$MgCl_2$	3.5µl
H_2O	49.5µl
Total =	60.0µl

arose Gel Electrophoresis

Agarose, which is extracted from seaweed, is a linear polymer whose basic structure is



Commercially available agarose is not completely pure; it is contaminated with other polysaccharides, salts, and proteins. The amount of contamination varies from batch to batch of agarose and from manufacturer to manufacturer. These differences can affect both the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions. Because of the great increase in demand during the past 10 years, most manufacturers now prepare special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

Some manufacturers also sell chemically modified forms of agarose that gel and melt at low temperature without significant deterioration in the strength of the hardened gel. Such chemically modified agaroses are used chiefly for preparative electrophoresis of DNA and for digestion of DNA with restriction enzymes in situ. Special grades of low-gelling-temperature agarose that can be used to analyze very small fragments of DNA (10–500 bp) are also available from some manufacturers. Gels made with agarose of this type have a greater resolving power than gels made with normal agarose, but the resolution obtained from these gels still cannot compare with the resolution obtained from polyacrylamide gels. Furthermore, since these gels contain a high concentration of agarose (4–10%), DNA fragments eluted from the gel are frequently contaminated with inhibitors that prevent further enzymatic manipulation.

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The rate of migration is determined by a number of parameters, which are discussed on the following pages.

Factors Affecting the Rate of DNA Migration in Agarose Gels

MOLECULAR SIZE OF THE DNA

Molecules of linear double-stranded DNA, which tend to become orien
an electric field in an end-on position (Fisher and Dingman 1971; Aa
Borst 1972), migrate through gel matrices at rates that are inversely p
tional to the $\log_{10}$ of the number of base pairs (Helling et al. 1974) (
6.1). Larger molecules migrate more slowly because of greater frictiona
and because they worm their way through the pores of the gel less effic
than smaller molecules.

AGAROSE CONCENTRATION

A linear DNA fragment of a given size migrates at different rates th
gels containing different concentrations of agarose. There is a linear rel

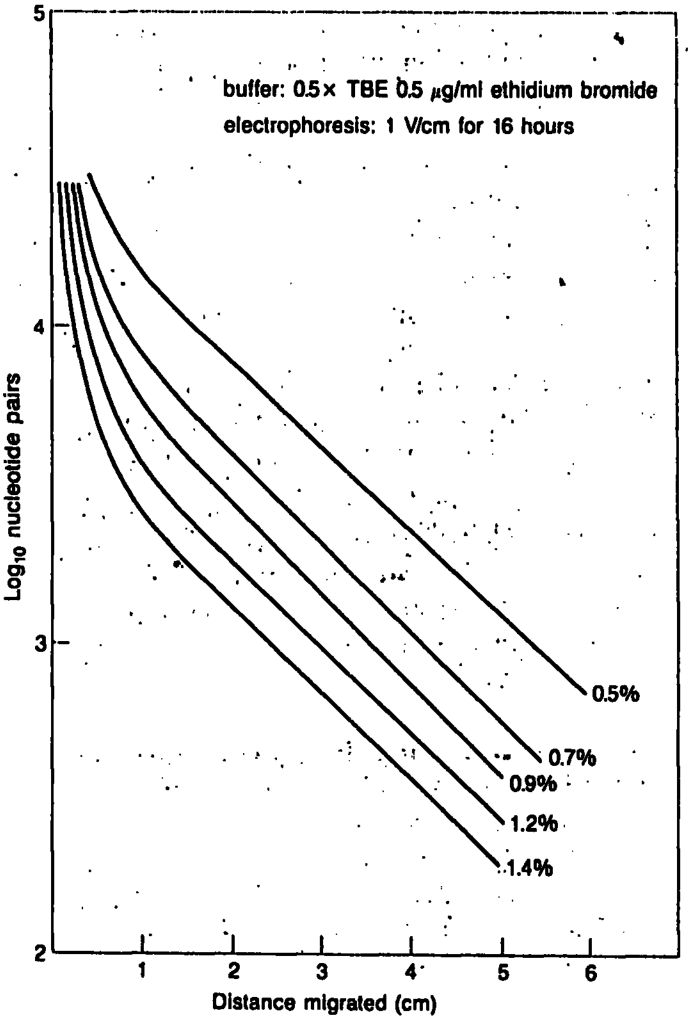


FIGURE 6.1
The relationship between the size of DNA and its electrophoretic mobility.

TABLE 6.1 Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5–60
0.6	1–20
0.7	0.8–10
0.9	0.5–7
1.2	0.4–6
1.5	0.2–3
2.0	0.1–2

ship between the logarithm of the electrophoretic mobility of DNA (μ) and the gel concentration (τ), which is described by the equation:

$$\log \mu = \log \mu_0 - K_r \tau$$

where μ_0 is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant that is related to the properties of the gel and the size and shape of the migrating molecules. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules (see Table 6.1).

ATION OF THE DNA

Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs of the same molecular weight migrate through agarose gels at different rates (Thorne 1966, 1967). The relative mobilities of the three forms depend primarily on the agarose concentration in the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA (Johnson and Grossman 1977). Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed.

An unambiguous method for identifying the different conformational forms of DNA is to carry out electrophoresis in the presence of increasing quantities of ethidium bromide. As the concentration of ethidium bromide increases, more of the dye becomes bound to the DNA. The negative superhelical turns in form I molecules are progressively removed, the radii of the molecules increase, and their rate of migration decreases. At the critical free-dye concentration, where no superhelical turns remain, the rate of migration of form I DNA reaches its minimum value. As still more ethidium bromide is added, positive superhelical turns are generated, the DNA molecules become more compact, and their mobility increases rapidly. Simultaneously, the mobilities of form II and form III DNA decrease differentially due to charge neutralization and the greater stiffness imparted to the DNA by the ethidium bromide. For most preparations of form I DNA, the critical concentration of free ethidium bromide is in the range of 0.1 μg/ml to 0.5 μg/ml.

APPLIED VOLTAGE

At low voltages, the rate of migration of linear DNA fragments is proport to the voltage applied. However, as the electric field strength is raise mobility of high-molecular-weight fragments of DNA increases differen Thus, the effective range of separation in agarose gels decreases a voltage is increased. To obtain maximum resolution of DNA frag greater than 2 kb in size, agarose gels should be run at no more than 5

Distance is measured as the shortest path between the electrodes and is not mere length of the gel itself.

DIRECTION OF THE ELECTRIC FIELD

DNA molecules larger than 50–100 kb in length migrate through aga gels at the same rate if the direction of the electric field remains const However, if the direction of the electric field is altered periodically, the molecules are forced to change course. Because larger molecules of DNA longer to realign themselves to the new direction of the field, pulsed-field electrophoresis can be used to fractionate populations of extremely la molecules of DNA (up to 10,000 kb). Pulsed-field gel electrophoresi discussed in more detail beginning on page 6.50.

BASE COMPOSITION AND TEMPERATURE

The electrophoretic behavior of DNA in agarose gels (in contrast to acrylamide gels [Allet et al. 1973]) is not significantly affected by eithe base composition of the DNA (Thomas and Davis 1975) or the temperatu which the gel is run. Thus, in agarose gels, the relative electropho mobilities of DNA fragments of different sizes do not change between 4°C 30°C. In general, agarose gels are run at room temperature. However, containing less than 0.5% agarose and low-melting-temperature agarose are rather flimsy, and it is best to run them at 4°C, where they are stron

PRESENCE OF INTERCALATING DYES

Ethidium bromide, a fluorescent dye that is used to detect DNA in aga and polyacrylamide gels (Sharp et al. 1973), reduces the electropho mobility of linear DNA by about 15%. The dye intercalates between stac base pairs, extending the length of linear and nicked circular DNA molec and making them more rigid.

Ethidium bromide is a carcinogen and should be handled with care. solutions containing ethidium bromide should be decontaminated be disposal (see pages 6.16–6.17).

COMPOSITION OF THE ELECTROPHORESIS BUFFER

The electrophoretic mobility of DNA is affected by the composition and strength of the electrophoresis buffer. In the absence of ions (e. electrophoresis buffer is omitted from the gel by mistake), electrical con tance is minimal and DNA migrates very slowly, if at all. In buffers of ionic strength (e.g., if 10× electrophoresis buffer is used by mist electrical conductance is very efficient and significant amounts of heat are generated. In the worst case, the gel melts and the DNA denatures.

Several different buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5–7.8) (Table 6.2). Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature.

For historical reasons, TAE is the most commonly used buffer. However, its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis (the anode becomes alkaline, the cathode acidic). Replacement of the buffer or recirculation between the two reservoirs is therefore advisable when carrying out electrophoresis for long periods of time at high current. Both TPE and TBE are slightly more expensive than TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate approximately 10% faster through TAE than through TBE or TPE, but the resolving powers of these systems are almost identical, with the exception that the resolution of supercoiled DNAs is better in TAE than in TBE.

The most commonly used buffer for electrophoresis of denatured single-stranded DNA is 50 mN NaOH, 1 mM EDTA (alkaline electrophoresis buffer; see Table 6.2). As discussed below, agarose cannot be melted in the presence of NaOH. Therefore, the agarose must be melted in water before the concentrated NaOH/EDTA solution is added.

BLE 6.2 *Commonly Used Electrophoresis Buffers*

ffer	Working solution	Concentrated stock solution (per liter)
-acetate AE)	1×: 0.04 M Tris-acetate 0.001 M EDTA	50×: 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
-phosphate PE)	1×: 0.09 M Tris-phosphate 0.002 M EDTA	10×: 108 g Tris base 15.5 ml 85% phosphoric acid (1.679 g/ml) 40 ml 0.5 M EDTA (pH 8.0)
-borate[a] BE)	0.5×: 0.045 M Tris-borate 0.001 M EDTA	5×: 54 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0)
aline[b]	1×: 50 mN NaOH 1 mM EDTA	1×: 5 ml 10 N NaOH 2 ml 0.5 M EDTA (pH 8.0)

recipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid blems, store the 5× solution in glass bottles at room temperature and discard any batches that develop a cipitate.

BE was originally used at a working strength of 1× (i.e., a 1:5 dilution of the concentrated stock) for rose gel electrophoresis. However, a working solution of 0.5× provides more than enough buffering er, and almost all agarose gel electrophoresis is now carried out with a 1:10 dilution of the concentrated k.

BE is used at a working strength of 1× for polyacrylamide gel electrophoresis (see page 6.39), twice the ngth usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for acrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them ften considerable. 1× TBE is required to provide adequate buffering power.

line electrophoresis buffer should be freshly made.

Apparatuses Used for Agarose Gel Electrophoresis

Because agarose gel electrophoresis is both forgiving and adaptable, m successful configurations and sizes of electrophoresis tanks have been signed during the last 15 years. The choice among these different desig largely a matter of personal preference. The most commonly used config tion is the horizontal slab gel, which was invented by Walter Schaffner a superior to the old-fashioned vertical gels in several respects:

- Low agarose concentrations can be used because the entire gel is supp from beneath
- Gels can be cast in a wide variety of sizes
- The gels are very simple to load, pour, and handle
- The apparatus is durable and inexpensive to construct or buy

Horizontal slab gels are usually poured on a glass plate or plastic tray can be installed on a platform in the electrophoresis tank. In a few des the gel is poured directly on the platform. Electrophoresis is carried out the gel submerged just beneath the surface of the buffer. The resistan the gel to the passage of electric current is almost the same as that o buffer, and so a considerable fraction of the applied current passes alon length of the gel.

Among the factors to be considered when buying an electrophoresis are:

- *It should be easy to examine the gel by ultraviolet light during the* When the gel is poured on a glass plate or plastic tray, it can easi removed, examined, and reinstalled at any stage. Most of the plastic currently supplied by commercial manufacturers are translucent t traviolet light, eliminating the need to transfer the gel from its suppo examination. When the gel is poured directly on a platform withi tank, however, it may be more difficult to remove the gel from the tank during and at the end of the run.
- *The apparatus should be supplied with a variety of combs to gen different numbers of wells of different sizes.* It is an advantage if the g accommodate two rows of wells, with at least 16 wells in each row. makes it possible to analyze many samples of DNA simultaneously.
- *The apparatus should be fitted with a lid containing shielded elec connections.* It should be impossible to remove the lid while electric cu is flowing through the system. The tank should be designed to allow replacement of corroded or broken connections and the platinum electr
- *The apparatus should be fitted with outlets to allow electrophoresis buf be removed easily and completely.* In many older designs, the buffer removed by inverting the tank over a sink. This is not acceptable whe buffer contains ethidium bromide.
- *The outlets should be designed to allow circulation of buffer betwee anodic and cathodic chambers.* This is important when RNA is analyz electrophoresis.

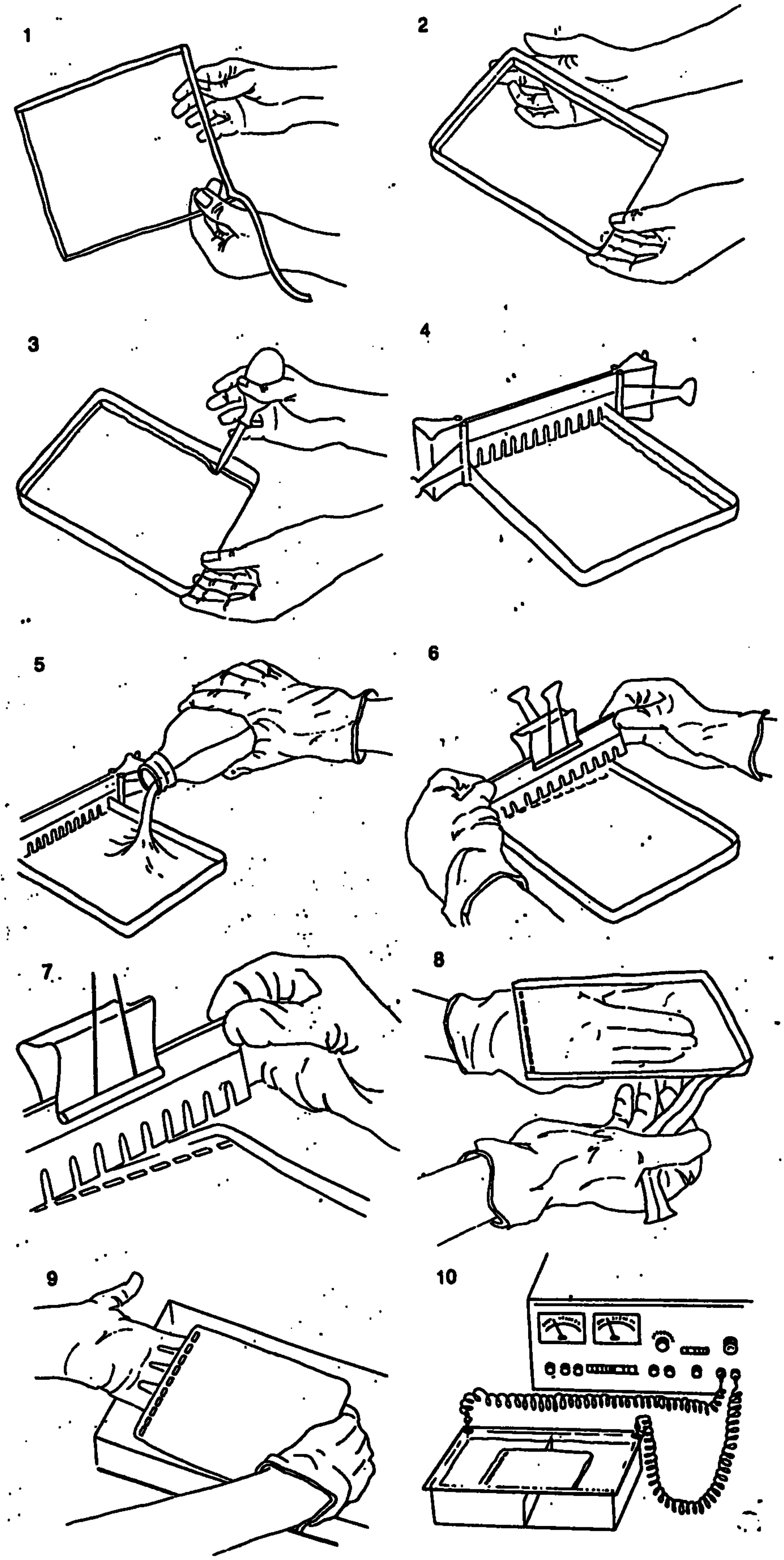


FIGURE 6.2
Pouring a horizontal agarose gel.

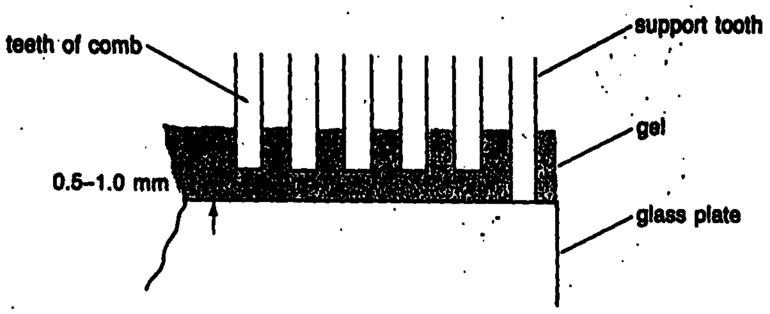


FIGURE 6.3
Diagram of a comb used to form slots in horizontal agarose gels.

base of the well may tear when the comb is withdrawn, allowing the sample to leak between the gel and the glass plate.

Some combs are designed with two outer teeth slightly longer than the internal teeth. When the comb is positioned above the plate, it is supported on the outer teeth; the inner teeth are automatically lifted clear of the glass plate (Figure 6.3). The disadvantage of this design is that the wells made by the outer teeth cannot be used during the subsequent electrophoresis.

6. Pour the remainder of the warm agarose solution into the mold. The gel should be between 3 mm and 5 mm thick. Check to see that there are no air bubbles under or between the teeth of the comb.

When preparing gels that contain low concentrations of agarose (<0.5%), first pour a supporting gel (1% agarose) without wells. Allow this gel to harden at room temperature on the glass plate or plastic tray. Then pour the lower-percentage gel directly on top of the supporting gel. This reduces the chances that the lower-percentage gel will fracture during subsequent manipulations (photography, processing for Southern hybridization, etc.). Make sure that both gels are made from the same batch of buffer and that ethidium bromide is added to both gels or to neither.

7. After the gel is completely set (30–45 minutes at room temperature), carefully remove the comb and autoclave tape and mount the gel in the electrophoresis tank.

Gels cast with low-melting-temperature agarose and gels that contain less than 0.5% agarose should be chilled to 4°C and run in the cold room.

8. Add just enough electrophoresis buffer to cover the gel to a depth of about 1 mm.

9. Mix the samples of DNA with the desired gel-loading buffer (Table 6.3). Slowly load the mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or, if you have a very steady hand, a pasteur pipette.

Gel-loading buffers are usually made up as sixfold concentrated solutions. (See Table 6.3 for the preparation of standard 6× buffers.) Tenfold concentrated solutions can also be prepared if needed.

The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their sizes. The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is about 2 ng in a

0.5-cm-wide band (the usual width of a slot). If there is more than 500 ng of DN a band of this width, the slot will be overloaded, resulting in trailing and smear a problem that becomes more severe as the size of the DNA increases. When si populations of DNA molecules (e.g., bacteriophage λ or plasmid DNAs) are analyzed, 100–500 ng of DNA should be loaded per 0.5-cm slot. When the sa consists of a very large number of DNA fragments of different sizes (e.g., restri digests of mammalian DNA), however, it is possible to load 20–30 μg of DNA pe without significant loss of resolution.

The maximum volume of solution that can be loaded is determined by the di sions of the slot. (A typical slot [0.5 cm × 0.5 cm × 0.15 cm] will hold about 37. However, to reduce the possibility of contaminating neighboring samples, it is be make the gel a little thicker or to concentrate the DNA by ethanol precipit rather than to fill the slot completely.

For most purposes, it is not necessary to use a fresh pipette tip for every sampl long as the tip is thoroughly washed with buffer from the anodic chamber bet samples. However, if the gel is to be analyzed by Southern hybridization or if b of DNA are to be recovered from the gel, it is sensible to use a separate pipette ti every sample.

Marker DNAs of known size (which can be purchased from commercial sou should be loaded into slots on both the right and left sides of the gel. This mak

TABLE 6.3 Gel-loading Buffers

Buffer type	6× Buffer	Storag tempera
I	0.25% bromophenol blue 0.25% xylene cyanol FF 40% (w/v) sucrose in water	4°C
II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 400; Pharmacia) in water	room te
III	0.25% bromophenol blue 0.25% xylene cyanol FF 30% glycerol in water	4°C
IV	0.25% bromophenol blue 40% (w/v) sucrose in water	4°C
V	*Alkaline loading buffer* 300 mN NaOH 6 mM EDTA 18% Ficoll (Type 400; Pharmacia) in water 0.15% bromocresol green 0.25% xylene cyanol FF	4°C

These gel-loading buffers serve three purposes: They increase the density of the sa ensuring that the DNA drops evenly into the well; they add color to the sample, th simplifying the loading process; and they contain dyes that, in an electric field, move t the anode at predictable rates. Bromophenol blue migrates through agarose gels approx ly 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. B phenol blue migrates through agarose gels run in 0.5× TBE at approximately the same r linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at ap mately the same rate as linear double-stranded DNA 4 kb in length. These relationshi not significantly affected by the concentration of agarose in the gel over the range of 0 1.4%.

Which type of loading dye to use is a matter of personal preference. However, bromo green should be used as a tracking dye in alkaline gels because it displays a more vivid than bromophenol blue at alkaline pH.

easier to determine the sizes of unknown DNAs if any systematic distortion of the gel should occur during electrophoresis.

When measuring the sizes of unknown DNAs, it is important that all samples be applied to the gel in the same buffer. The high concentrations of salt in certain restriction enzyme buffers (e.g., *Bam*HI or *Eco*RI) retard the migration of DNA and distort the electrophoresis of DNA in the adjacent wells.

10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 1–5 V/cm (measured as the distance between the electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and, within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel.

During electrophoresis, the ethidium bromide migrates toward the cathode (in the direction opposite to that of the DNA). Extended electrophoresis can remove much of the ethidium bromide from the gel, making detection of small fragments difficult. If this occurs, restain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 μg/ml) as described on page 6.15.

The presence of ethidium bromide allows the gel to be examined by ultraviolet illumination at any stage during electrophoresis. However, some people feel that sharper bands of DNA are obtained when the gel is run in the absence of the dye. In this case, after electrophoresis is completed, stain the gel by soaking it for 30–45 minutes in a solution of ethidium bromide (0.5 μg/ml) as described on page 6.15.

11. Turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide was present in the gel and electrophoresis buffer, examine the gel by ultraviolet light and photograph the gel as described on page 6.19. Otherwise, stain the gel with ethidium bromide as described on page 6.15 and then photograph.

Solutions containing ethidium bromide should be decontaminated by one of the methods described on pages 6.16–6.17 before they are discarded.

Optimization of a PCR protocol.

Objective:

Determination of optimal conditions for PCR amplification of the NADH dehydragenase subunit 5 (ND5) region of mammalian mitochondrial DNA.

Primers used: L 13525 (5'GCTGAGARGGNGTRGGAATCATRTC3')
H 14127 (5'CCTATTTTTCGRATRTCTTGTTC3')
N=(A,C,T,G) R=(A,G)
50 µM stock solution, 10 µM working solution

General PCR protocol:

	Volume per R^n	Final conc.
10x buffer	5.0 µl	1x
25mM $MgCl_2$	4.0 µl	2.0mM
dNTP(2mM)	2.5 µl	100 µM
Primer (10 µM) L 13525	1.0 µl	0.2 µM
H 14127	1.0 µl	0.2 µM
DNA (400 ng) dissolved in sterile dist. Water	3.0 µl	
Sterile distilled Water	28.5 µl	
Total volume	45.0 µl	

Enzyme mix [added after step 1 in the PCR programme (Hot start)]

Taq DNA polymerase (Appligene)	0.2 µl (1.0 u)
Sterile distilled Water	4.8 µl
Total volume	5.0 µl

Total reaction volume 50 µl

Optimization strategy:

1. Variation of the concentration of $MgCl_2$ and the pH of reaction buffer.
2. The optimal buffer condition is selected and tested in the presence of different additives.

Step1:

1. Preparation of a master mix for 5 reactions:

dNTP(2mM)	2.5 x 5.5 = 13.75 µl
Primer (10 µM) L 13525	1.0 x 5.5 = 5.5 µl
H 14127	1.0 x 5.5 = 5.5 µl
Sterile distilled Water	32.5 x 5.5 = 178.75 µl
DNA (400 ng) dissolved in sterile dist. Water	3.0 x 5.5 = 16.5 µl

2. Mix thoroughly. Add 40.0 µl of master mix to each reaction vial.
3. Add 5.0 µl of the buffers to the reaction tube as follows and mix thoroughly.

Tube	1	2	3	4	5
Buffer	N	O	P	K	C
$MgCl_2$	1.5 mM	2.0 mM	2.5 mM	2.0 mM	2.0 mM
pH	9.2	9.2	9.2	8.9	8.3

4. Prepare PCR reaction mixtures for positive control (use general PCR procedure) and negative control (without DNA sample).
5. Add 5.0 µl of enzyme mix to each tube after step 1 in the PCR programme (Hot start)

Enzyme mix:

Taq DNA polymerase (Appligene)	0.2 x 7.5 = 1.5 µl
Sterile distilled Water	4.8 x 7.5 = 36.0 µl

PCR programme

Initial denaturation of templates	Pause: add enzyme mix	Cycling for 40 cycles	Extension step	Hold step
94^{0}C for 5 min	88^{0}C for 1 min	94^{0}C 1.30 min 53^{0}C 1.15 min 72^{0}C 1.30 min	72^{0}C for 4 min	4^{0}C

6. Use 10 µl of PCR product for agarose gel analysis.

Step2:

1. Preparation of a master mix for 6 reactions:

Optimal buffer from step1	5.0 x 6.5 = 32.5 µl
dNTP(2mM)	2.5 x 6.5 = 16.25 µl
Primer (10 uM) L 13525	1.0 x 6.5 = 6.5 µl
H 14127	1.0 x 6.5 = 6.5 µl
Sterile distilled Water	17.5 x 6.5 = 113.75 µl
DNA (400 ng) dissolved in sterile dist. Water	3.0 x 6.5 = 19.5 µl

2. Mix thoroughly. Add 30.0 µl of master mix to each reaction vial.
3. Add different additives to the reaction tube as follows and mix thoroughly. (Place tubes on ice during pipetting)

Tube	St.dist. water	DMSO (100%)	Glycerol (50%)	Gelatine (1%)	$(NH_4)_2SO_4$ 500mM
1	15 µl				
2	10 µl	5µl (5%)			
3	-	5µl (5%)	10 µl (5%)		
4	10 µl			5 µl (0.05%)	
5	3 µl		10 µl (5%)		2 µl (10mM)
6	9 µl				6 µl (30mM)

4. Prepare PCR reaction mixture for the negative control.
5. Add 5.0 µl of enzyme mix to each tube after step 1 in the PCR programme (Hot start)

Enzyme mix:

Taq DNA polymerase (Appligene)	0.2 x 7.5 = 1.5 µl
Sterile distilled Water	4.8 x 7.5 = 36.0 µl

6. Use 10 µl of PCR product for agarose gel analysis.

J.M.D.T. EVERARD, CRI, LUNUWILA

Molecular Biology Applications in Plant Breeding and Bio-diversity conservation

In 1998 the U.S. growers planted 45 million acres of genetically engineered crops, primarily corn, soybeans, cotton and potatoes. These transgenic "smart crops" can produce their own insecticides, or withstand broad-spectrum herbicides such as Roundup or Liberty. Some say these developments signal the coming of age of the most profound technological revolution since the advent of computer technology.

Plant breeding as a science has benefited heavily from both qualitative and quantitative genetic theories as well as agricultural statistics but less benefited from past advances in molecular biology until last few years. The principles in genetics have been applied to crop variety improvement with great success for many years. Several crop species, notably corn, wheat and tomato, have been used as model genetic systems because of their central importance to food production.

Since the beginning of this decade, virtually all progress in both breeding and model genetic systems has relied on a phenotypic assay of genotype. Phenotype being a function of the heritability of the trait, factors like the environment, polygenic and quantitative inheritance, or partial and complete dominance the efficiency of a selection scheme or genetic analysis based on such often confuses the expression of a genetic trait. Many of the complications of a phenotype-based assay can be mitigated through direct identification of genotype with a DNA-based diagnostic assay. For this reason, DNA-based genetic markers are being integrated into several plant systems since the beginning of this decade and due to remarkable early accomplishments a number of plant biotechnology-related organizations from both government and industry are showing tremendous interest in many such applications.

Conservation of bio-diversity is another widely shared concern among plant breeders as genetic resources are endangered due to heavy mechanization, deforestation, urbanization, and so on and by exclusive use of high performance varieties. The conservation of natural genetic variation is important not only for ethical and aesthetic reasons but also to ensure that the earth's living resources may be used even more efficiently and sustainably in agriculture, forestry, food production and other industries. The significance of genetic diversity is often discussed with reference to sustainable agriculture of staple food species, because breeders rely on a supply of genes from related wild species to develop new characteristics, such as resistance to pest and diseases. Major collections of important crop plants are held in international centres around the world, which have been set up as repositories of the bio-diversity available for each species. These collections are a valuable source of useful genes for plant breeders. Workers at many of these centres face problems in the efficient conservation, maintenance and exploitation of germplasm, because of the lack of true genetic information about the accessions that they hold.

Efficient management of plant genetic resources depends to a large extent on the availability of information on the genetic diversity of accessions that are often gathered on data recorded for a number of identifiable and measurable plant attributes. The description of morphological, reproductive and agronomical traits has been the mainstay of germplasm characterization at the outset of bio-diversity conservation programmes. Here again phenotypic assay of genotype confounds true assessment of genetic resources.

Modern Molecular Biology provides a suite of powerful tools with enormous potential for screening and evaluation of bio-diversity. Molecular techniques for characterization of germplasm are advantageous in that, any part of a plant can be used for assessment of the entire genome, and their Mendelian mode of inheritance and environment-independent expression. In addition they are fast, less expensive, and reliable and in conjunction with numerical analysis they are very effective in management of gene banks. Particularly they are helpful in duplicate identification, estimation of diversity, monitoring of genetic erosion and for making better use of the germplasm collections in the identification of core populations.

Molecular Basis of Genes

The application of molecular techniques basically lies in their ability to locate genes of interest to chromosomes. Location of genes to positions on specific chromosomes (gene mapping) is important to map the genes controlling quantitative traits, QTL, and to understand the effect of linkage between QTL on the means and variances of quantitative traits and their response to selection.

An individual chromosome contains a single molecule of DNA with a constant length for any given chromosome in a species but varies between chromosomes. Typically, they each consist of something of the order of 10^7 - 10^8 base pairs (bp) or 10^4 - 10^5 kilo-base pairs (kbp). A typical structural gene, coding for a polypeptide chain, is between 1 - 2 kbp long, and only about 10% of the genome is actually coding, much of the rest being spacer DNA (non-coding), a chromosome probably contain 1,000 - 10,000 genes. Fortunately, the fact that the chromosome is a linear molecule means that the genes need to be mapped in only dimension. Any attempt to locate and study the gene controlling quantitative traits requires the use of independent and easily recognizable genetic marker loci as flags to identify the regions of chromosome containing the QTL. Such markers have to exist in two or more allelic forms at the DNA level, which can be distinguished so that precise genotypes at all marker loci can be identified.

Most genes are concerned with the production of polypeptides, each gene defining the primary structure of one of those molecules. They are called structural genes for this reason and a great majority of them are located on the chromosome in the nucleus. There are structural genes present in cytoplasmic organelles such as mitochondria and chloroplasts as well. Although the genes are in the nucleus, the polypeptides are synthesized in the cytoplasm by ribosomes and, once produced, they form proteins either alone or together with other polypeptides produced by other genes. In their turn, proteins have a variety of key roles acting as enzymes, hormones, transporter molecules, storage and structural elements as well as regulating the action and expression of other genes. Their roles are so fundamental to life that genetical variation that disrupts protein structure often produces a drastic change in the phenotype of the organism.

Each structural gene typically consists of a length of chromosome approximately 1000 - 2000 bp long. At one end it has a promoter which is the binding site for an enzyme, a DNA dependent RNA polymerase, which will transcribe the DNA code of the gene into RNA. At the far end of the DNA is a terminator, which tells the enzyme where to stop transcribing. The RNA produced by this process is called mRNA, and it carries a complementary copy of the DNA code of that particular gene from the nucleus to the cytoplasm where the message is read and translated by ribosome into a linear molecule of amino acids, a polypeptide chain. However, the RNA from some genes is used directly as RNA, either as building blocks for ribosome (rRNA) or for the transfer RNAs (tRNA) that carry the amino acids to the ribosomes. Some DNA sequences are never transcribed yet are essential for the control and regulation of other genes for control of transcription and replication. Within many genes of eukaryotes, there may exist one or more intervening sequences of non-coding DNA known as introns in order to distinguish them from the coding parts or exons. The function and origins of these are not known, although they appear to be tightly conserved in size and position over long periods of evolutionary time. These introns are transcribed along with the exons but are cut out before translation.

Although the processes of DNA replication are very accurate and constantly being checked for errors, it is inevitable given the vast number of bases being replicated, that over generations errors will occur, i.e. there is mutation at the base level. Mutations caused by changes in base sequences by either deletion or insertion of nucleotide lead to DNA polymorphism. The number of possible single base variants of a gene, alleles or allelomorphs could potentially exist in a large number but in practice only a small number exist in a population. If a DNA change in base do not cause a change in the amino acid sequence it is called a same-sense mutation and such will not have any effect on the individual. A mutation result in a different amino acid is called a mis-sense mutation and the consequences of such mutants are very variable, it could be negligible at one extreme and lethal at the extreme. The situation where two or more allelic forms exist at frequencies greater than expected by mutation pressure alone is called genetic polymorphism. Mutations can also occur by the introduction of a terminator codon (non-sense) which result in complete stop of translation. Clearly consequence of this is to produce an inactive protein.

The effect of base addition or deletion at the DNA level is to put the triplet reading frame out of sequence and cause frame shift mutations. They result in a non-sense sequence of amino acids being built up, followed sooner or later by premature stop codon. They are therefore, likely to behave like non-sense mutations.

Thus we can see that mutation at the base level of a gene can have a variety of effects from the almost inconsequential same-sense to the drastic non-sense mutation, depending on where it occurs and what the changes are. The so-called major mutations involve base changes which result in non or mis functioning polypeptides, while the variation that underlies quantitative traits and other polymorphisms probably consists of the same-sense and innocuous mis-sense mutations plus regulatory mutants.

Molecular Genetic Markers

It is possible to detect mutations at levels; the gross phenotype, charge changes in the substituted amino acids (isozymes i.e. the rate of migration of proteins in response to electrophoresis), the primary structure of the protein (the amino acid sequence) and the DNA sequence itself. Until 1980's it was difficult to look specifically at the DNA sequence of different individuals directly and for many reasons this is the most useful level at which to work. Only about 10% of the DNA is coding DNA and hence looking at structural genes limits the variation available for analysis. Emergence of new DNA assay procedures has made it increasingly easy to look at variation in the DNA in both coding and non-coding regions. Essentially these techniques involve breaking the DNA into small fragments, using restriction enzymes coupled with DNA blot hybridization or selective DNA amplification using the polymerase chain reaction.

Plant DNA isolation

General Considerations: Application of various DNA assay procedures in plants relies increasingly on the ability to isolate DNA from various tissues. This is particularly acute in plants because of the difficulty in isolating intact DNA. Numerous problems may be encountered during DNA isolation from plant tissue. These include DNA degradation due to the presence of either native DNases or secondary plant compounds, co-isolation of viscous polysaccharides, polyphenols and other secondary plant compounds that cause damage to DNA and/or inhibit restriction endonucleases and DNA polymerases. DNA preparations often tend to be brownish due to the oxidation of polyphenols to quinone compounds. Quinones in turn are powerful oxidizing agents that damage proteins and DNA. As a consequence yields of high molecular weight DNA from plants are often poor, and there is no single protocol which is optimally suited for each plant species.

Different kinds of experiments demand different levels of DNA purity. Relatively pure high molecular weight DNA is required for RFLP assays while for PCR of specific target DNA templates require rather crude preparations. Accordingly, hundreds of different DNA isolation methods have been described that nevertheless relies on few basic principles. The focus of this section is to discuss two methods that have been developed to overcome the problems associated with DNA isolation in plants.

How plant tissue is collected and stored prior to DNA isolation may considerably influence the results. Freshly harvested young leaf is the best choice while when secondary compounds are present flower petals is a good alternative. For short periods of time, fresh tissue may be stored on ice. Storage for longer periods of time requires freezing, lyophilization or rapid drying by means of silica-gel or anhydrous calcium chloride.

There are two commonly used techniques for isolation of plant DNA, CTAB buffer technique and Protein precipitation technique. These methods differ each other in the detergent used to solubilize the cellular membranes and in the means of separating DNA from proteins. They also differ in their equipment requirements, the quality of DNA isolated and their yield. All these techniques successfully extract DNA from fresh or freeze-dried plant tissue. Apart for these techniques there are also methods for rapid isolation of plant DNA.

CTAB buffer techniques: One of the most widely used techniques for isolating plant DNA, particularly for small samples, is based on the CTAB buffer techniques. CTAB (Cetyltrimethylammonium bromide or Hexa-decyltrimethylammonium bromide Sigma H5882) is a cationic detergent, which solubilizes membranes, and form a complex with DNA. These techniques capitalize on the remarkable ability of CTAB to bind with DNA and RNA when the salt (NaCl) concentration is 0.7 M or above and precipitate nucleic acids when the salt concentration is below 0.4M.

Most commonly quoted, first use of this method comes from Murray and Thompson, (1980)[1]. Subsequently numerous modifications to this method was reported and to mention a few, Taylor and Powell (1982)[2], Saghai-Maharoof et. al., (1984)[3]. Rogers and Bendich (1985)[4]. Doyle and Doyle (1990)[5] and Weising and Kahl (1996)[6]. The advantages of this method is that extensive preparation of the plant tissue is not required and it is adaptable to numerous types of tissue, including leaves, roots, seeds, embryos, endosperm, pollen and suspension cultures. In addition it can accommodate a wide

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[1] Murray, M. G. and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8: 4321- 4326

[2] Taylor, B. and Powell, A. (1982). Isolation of plant DNA and RNA. Focus 4:3 : 4-5

[3] Saghai-Maharoof, M.A., Soliman K.M., Jorgensin, R.A. and Allard, R.W. (1984). *Proc. Nat.Acad. Sci. USA* 81: 8014 - 8018

[4] Rogers, S.O. and Bendich, A.J. (1983). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* 5: 69-76

[5] Doyle, J.J. and Doyle, J.N. (1990). Isolation of plant DNA from fresh tissue. *Focus* 12 : 13-15

[6] Weising, K. and Karl G. (1997) Hybridization based microsatellite fingerprinting of plants and fungi. In DNA markers Protocols, Applications and overviews. G. Caetano-Anolles and P.M. Greshoff (eds.). Willy VCH, New York

range of sample sizes from milligram quantities of herbarium, mummified, or fossil tissue to many grams of freshly harvested tissue.

Murray and Thompson's CTAB Protocol

1. Homogenization: Fresh or lyophilized tissue is ground to a fine powder (For soft tissue use mortar and pestle and small glass beads / clean sand, for fibrous tissue grind in the presence of liquid Nitrogen, for hard tissues mechanical grinder such as Warring blender is necessary
2. Disperse dry powder gently in extraction buffer [1%CTAB, 0.7M NaCl, 10mM EDTA, 50mM Tris pH 8.0 and 1% β-mercaptoethanol (added just before use) until solution becomes viscous without clumps. Typically tissue:buffer ratio is 1:3.
3. Incubate for 20-30 min at 50-60 OC with occasional gentle mixing. Temperature throughout should be kept above 15 OC to prevent precipitation of CTAB
4. Extract with equal volume of chloroform:isoamyl (24:1) and centrifuge 13,000 x g , 10 min and repeat with aqueous phase.
5. Reduce the NaCl concentration 0.7 to 0.35 M by adding an equal volume of 1%CTAB, 10mM EDTA, 50mM Tris-HCl pH 8.0. After 30 minutes recover the precipitate by low speed centrifugation, 2000 x g, 5 min.
6. Dissolve CTAB-DNA/RNA complex in 2 ml of 1.0 M CsCl (density 1.12 g/ml), 50mM Tris-HCl (pH 8.0), 10 mM EDTA and 200 μg/ml ethidium bromide. Heat the solution briefly to 60 OC to speed dissolution.
7. Place the solution in a ultra centrifuge tube and add 2.4 ml of 6.6 M CsCl (density 1.82 g/ml), 50mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1% sarkosyl and mix thoroughly to perform a crude density gradient.
8. Centrifuge 58,000 rpm 2-3 hours (ultra-centrifuge TV-865 rotor) and visualize DNA under long wave UV illumination. Remove DNA with a large bore syringe and transfer to a fresh tube.
9. Fill the tube with 4.5 M CsCl (density 1.55 g/ml), 50mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% sarkosyl and 100 μg/ml ethidium bromide.
10. Centrifuge 58,000 rpm 6 hours (ultra-centrifuge TV-865 rotor) and recover DNA with a syringe.

Comments: a) Typically 20 - 70 μg of DNA can be expected / 100 mg of dry weight of powder b) A260/A230 = 2.2 - 2.5, A260/A280 = 1.8 - 1.9 c) accessible to endonucleases and polymerases d) DNA is intact

Doyle and Doyle's CTAB Protocol

1. Homogenization is same as above.
2. Incubate dried powder at 50-60 ^{o}C in hot CTAB isolation buffer [2%CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris pH 8.0 and 0.2% β-mercaptoethanol (added just before use)]
3. Extract proteins in chloroform.isoamyl alcohol
4. Precipitate CTAB-DNA complex in isopropanol.
5. Wash DNA pellet and redisslove in TE.
6. Purify pellet by phenol extraction if necessary

Depending on the species, additional purification steps may be necessary to remove RNA, polysaccharides, polyphenols and other contaminating substances. Usually for RNA removal, RnaseA treatment is carried out while ammonium acetate precipitation is carried out for removal of polysaccharides Wiezing and Karl, 1997).

The detrimental influence of polyphenols and their oxidation products may be counteracted by different strategies: a) Inclusion of polyphenol absorbents such as bovine serum albumin (BSA) and soluble polyvinylpyrrolidone (PVP) in the isolation buffer b) inclusion of phenol oxidase inhibitors such as diethyldithiocarbomic acid (DIECA) in the isolation buffer c) inhibition of polyphenol oxidation by including anti-oxidants such as sodium bisulfite, cysteine ascorbic acid or high concentrations of β-mercaptoethanol in the isolation buffer.

Polysaccharides make DNA preparations highly viscous and inhibit the activity of restriction enzymes. Strategies to remove polysaccharides include; a) purifying the crude DNA preparations by passage through ion exchange columns, b) precipitation of DNA with polyethylene glycol leaving polysaccharides in the supernatant, c) exploiting the differential ethanol precipitation of DNA from aqueous solutions containing different salt concentrations d) treatment of DNA preparations with pectic enzymes. More general strategies to purify crude DNA preparations from undesired contaminants include ultra-centrifugation in cesium chloride density gradients and preparative electrophoresis in low melting agarose. Though cesium chloride density gradients is expensive and time consuming it is the method of choice for high quality DNA.

Protein Precipitation Techniques

This procedure is an alternative to standard method of isolating total DNA (CTAB Method). This is based on precipitating cellular proteins and polysaccharides in potassium acetate prior to precipitating the nucleic acids (e.g. Dellaporte, 1983[7]).

Protocol:

1. Homogenization (same as above)
2. Add 6 volumes of extraction buffer [2%SDS, 500mM NaCl, 50mM EDTA, 100mM Tris pH 8.0, 1% PVP and 0.1% β-mercaptoethanol (added just before use)]
3. Incubate at 65 OC for 20 min.
4. Add 2 ml of 5M potassium acetate, pH 6.5, shake and chill for 5 min
5. Spin 2000 x g, 20 min at 4 OC
6. Pipette supernatant and add isopropyl alcohol (0.6 - 0.8 volumes) and centrifuge 20,000 x g, 15 min at 4 OC
7. Purify the pellet accordingly

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[7] Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation Version II *Plant Molecular Biology Reporter* 1 : 19-21

Restriction Fragment Length Polymorphism (RFLP) analysis

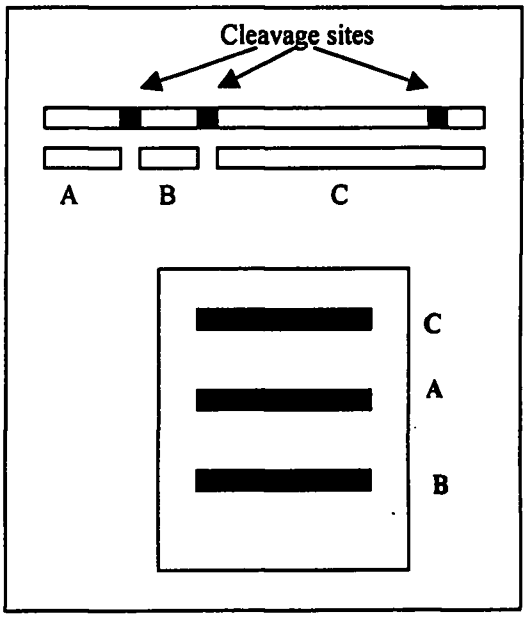
The basis of RFLP technique is use enzymes extracted from bacteria called restriction endonucleases to break DNA into small fragments. The bacteria use these enzymes as part of their defense mechanism against viruses, bacteriophages, which attack them and the enzyme functions by identifying viral DNA and cutting it. This defense is based on the enzyme recognizing particular 4 - 6 bp lengths of unmethylated DNA which are unique to the virus and do not occur in the bacterium. For example, a restriction enzyme called *EcoRI* comes from the bacterium Escherichia coli and searches out unmethylated double stranded DNA with the sequence

↓
5'   G-A-A-T-T-C   3'
3'   C-T-T-A-A-G   5'
↑

which it cuts at the point indicated by the arrows. A very wide range of restriction enzymes have now been isolated from different bacteria each cleaving a different DNA sequence.

Given the very large number of base pairs in genome, any one of these short sequences is likely to occur by chance, very frequently, Thus, a given set of six bases such as that recognized by *EcoRI* will have an expected frequency of 1 in 4^6 , i.e. 1 in 4096 randomly drawn sets of six bases. Since a single chromosome may contain 10^8 bases, such a sequence could occur some 24,000 times. DNA sequences are not entirely random over the whole of their length, but clearly the likely number of occurrences of a given site is large. Multiply this by the number of different enzymes and the potential cleavage sites across any genome are enormous. Moreover, these sites do not have to be within structural genes, indeed since most DNA is non-coding, most sites are likely to be between genes.

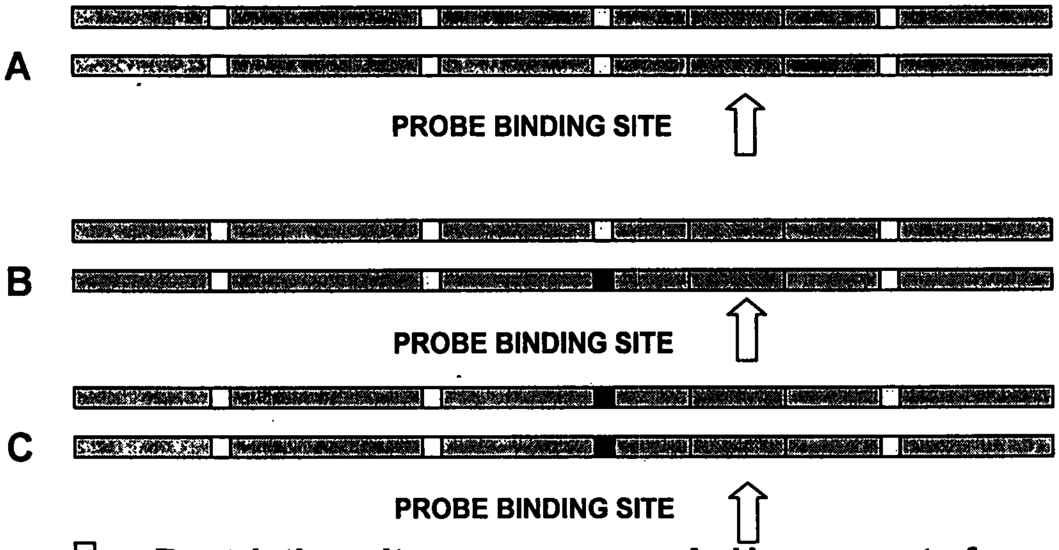
If DNA is digested with a specific restriction enzyme, it will be cut into large number of fragments, called restriction fragments, of different length, depending on the distance between the sites along the DNA. If these fragments are subjected to electrophoresis, they will be separated according to their length into bands, the shortest fragment travelling farthest as shown in the box.



These fragments can be transferred to a special nylon membrane by a process known as Southern blotting in order to stabilize them and fix their position. In practice, if this were done with total genomic DNA, there would be so many different types of fragments, that, were the gel to be treated with a DNA stain such as ethidium bromide, one would simply observe a smear without recognizing individual bands.

Therefore, it is necessary to have a label that will specifically identify particular bands. Such specific labels can be produced by isolating individual small fragments of DNA and replicating tem in a source of radioactive bases. If these labeled DNA fragments called 'probes' are spread over the smear of DNA restriction fragments under defined conditions, they will attach by complementary base pairing to any fragment that contains the same sequence. If the probes are made from the same DNA as the restriction

RESTRICITON FRAGMENT (RFLP) ANALYSIS



☐ Restriction site

■ Restriction site mutated

A: Homozygote for ☐

B: Heterozyote for ☐

C: Homozygote for ■

RFLPs behave like co-dominant Mendelian genes

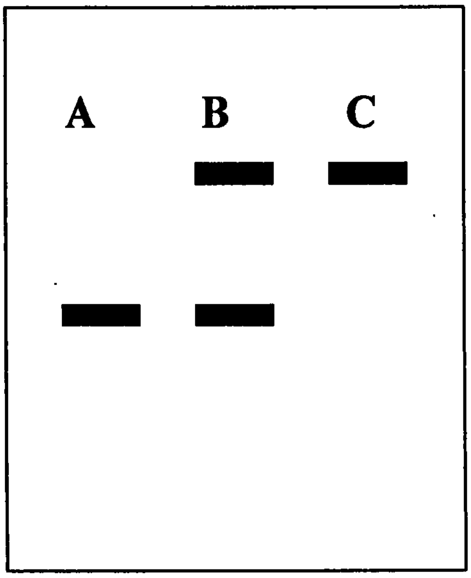
Possible causes

Deletion in the restriction site

Insertion in the restriction site

Creation of new site by deletion/insertion at some other place

Insertion of a large fragment between restriction sites



fragments then at least one fragment , i.e. that contain the sequence of chromosome from which the probe was originally made, will hybridize with it. However, there may be other fragments, which contain the same or similar sequences , as well. The presence of the probe labeled band(s) can be identified by either by autoradiographic or fluorescence techniques. By using different probes, different bands can be identified. Because there has been considerable conservation of DNA sequences during evolution, it is possible to use probes obtained from one species to identify restriction fragments in another and there are now a wide variety of useful probes available.

The value of this technique in genetical analysis lies in its ability to identify polymorphism at the DNA base level in a population. The most striking feature of RFLPs is its ability to detect heterozygotes making it a co-dominant marker.

Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR based technique applied for detecting DNA polymorphisms among individuals. This technique relies on the fact that whereas the standard PCR requires two different oligonucleotides whose base composition is fixed by the sequence of the fragment to be amplified. RAPDs require only the presence of a single randomly chosen oligonucleotide. Under the annealing conditions used, this single oligonucletide acts as both a forward and a reverse primer. An individual RAPD primer does have a defined sequence however, but this sequence is usually chosen at random. There is, therefore, an almost infinite number of oligonucletides, which could be chosen as RAPD primers.

Although the size of the RAPD can vary from 8 - 50 bp, the commercially available primers are 10 bp and most researchers tend to use primer of this size. Although the sequence of any primer can be random, it is usual to chose primers with an average GC content 50% and which lack multiple GC at or close to 3' end of the primer. The quality of RAPD primer can affect the banding pattern obtained, it is therefore, essential to use oligonucleotides from a reputable source. Sets of primer produced by OPERON Technologies Inc, USA has a good reputation for RAPD primers. If possible the same batch of primer should be used throughout a particular set of experiments.

Individual RAPD primers are able to hybridize to several hundred sites within the target DNA, however, not all of these hybridizations lead to the production of a PCR fragment. In order for this to happen it is necessary for the primer to anneal at two sites on opposite strands within 2 kb or less within each other. When these conditions are met, exponential amplification can occur and a fragment generated. Conditions are normally chosen whereby the number of fragments generated is between 1 and 20. This ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly. The full power of the RAPDs technology is further realized when it is used to screen genomic DNA from individuals for variation in sequence. These sequence variation may result in a change in the pattern of amplification products following agarose gel electrophoresis..

This makes RAPDs a powerful technique not only for screening populations for sequence diversity but also, when used in conjunction with bulk segregant analysis, for generating molecular markers from specific regions of the genome without any requirement for a detailed map.

The protocol given below is a slight modification of the original protocol of Wlliams et al., 1990 which has been successfully used on genomic DNA prepared from a large number of plants including coconut.

METHOD

1. Add the components of the reaction to the bottom of a 0.5 ml microcentrifuge tube in the following order.

Reagent	Amount to add	Final concentration
10 x PCR buffer	2 µl	1x
25mM $MgCl_2$	1.6 µl	2 mM
2mM dNTP	1.0 µl	100 µM
Taq Polymerase	0.25	1U
Primer[1]	1.0 µl	5 pM
DNA		25-50 ng
Sterile distilled water		To make the volume 20 µl

Mix well and give a pulse in a microfuge. Add approximately 20 µl of mineral oil and place in the thermocycler.

2. Programme the thermocycler to run following cycles

Step I	Denaturing	94 °C	1 minute
StepII	Annealing	36 °C	1 minute
Step II	Extension	72 °C	2 minutes

3. Mix 3 µl of loading dye to 10 µl of the PCR mix and check on 1.5% agarose in 0.5 x TBE

Important notes

Any researcher new to RAPD technique should bear in mind following notes before starting work.

1. Genomic DNA prepared by most of common protocols is suitable substrate for RAPDs. Variability in the quality and quantity of the DNA, however, the most common cause of variation in RAPD profile between experiments. Therefore, the concentration of DNA should be accurately determined.
2. RAPD reactions may produce slightly different results with different makes of thermocyclers. There are two reasons for this; a) each make of PCR machine has a distinct tnermal profile during cooling and heating cycles b) the read display on most machines does not truly the conditions in the block. It is appropriate to use a machine, which has a block with no more than 0.1 °C variation during cooling and heating cycles. Perkin Elmer 9600 is ideal for RAPD work.
3. The exact $MgCl_2$ variation can be varied from 2mM to 6 mM. The optimum concentration should be determined for each new species using a selection of random primers.
4. Most commercial preparations of Taq polymerase work very well with RAPD-PCR but different brands the enzyme can produce different results.

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[1] OPERON primer is supplied as a quantity 5000 pM of dry powder in a 1.5 ml eppendorf and when it is dissolved in 1000 µl each µl will have 5 pM.

5. Due to their ease of use high percentage agarose (2-4%) agarose are the method of choice for separation of RAPD bands. However, 4-8% polyacrylamide gels are an alternative because their resolving power is around 50 - 200 bp is greater.
6. EtBr is a powerful mutagen. Wear protective gloves to avoid contamination.
7. UV light is extremely harmful to skin and eyes. Always wear a full face UV protector.
8. When several DNA samples are to analysed use a master mix of all of the components for the region except DNA and / or primer and oil. This procedure not only saves time but also reduces the amount of variation, which can result from pipetting small volumes.
9. Detailed analysis of individual amplified fragments has shown that the failure to amplify specific fragments in some individuals when compared with others of the same species can result from as little as single base change. Because of the nature of RAPD technique almost all RAPD bands behave as dominant markers, thus it is not possible to determine whether a DNA fragment is amplified from a locus, which is homozygous or heterozygous. In order to determine this it is necessary either to carry out progeny testing or to clone the RAPD fragment for use as standard RFLP probe.
10. One of the major problem in RAPDs is scoring bands. The main problem is in deciding on the presence of weakly amplifying fragments that do not occur in all tracks on the gel. Although it is not possible to avoid this problem altogether, scoring only those bands, which have an intensity above a chosen threshold, can reduce its effect on the analysis of data. It is important to remember that it is better to exclude suspicious data from the data base than to try and increase the total amount of data.

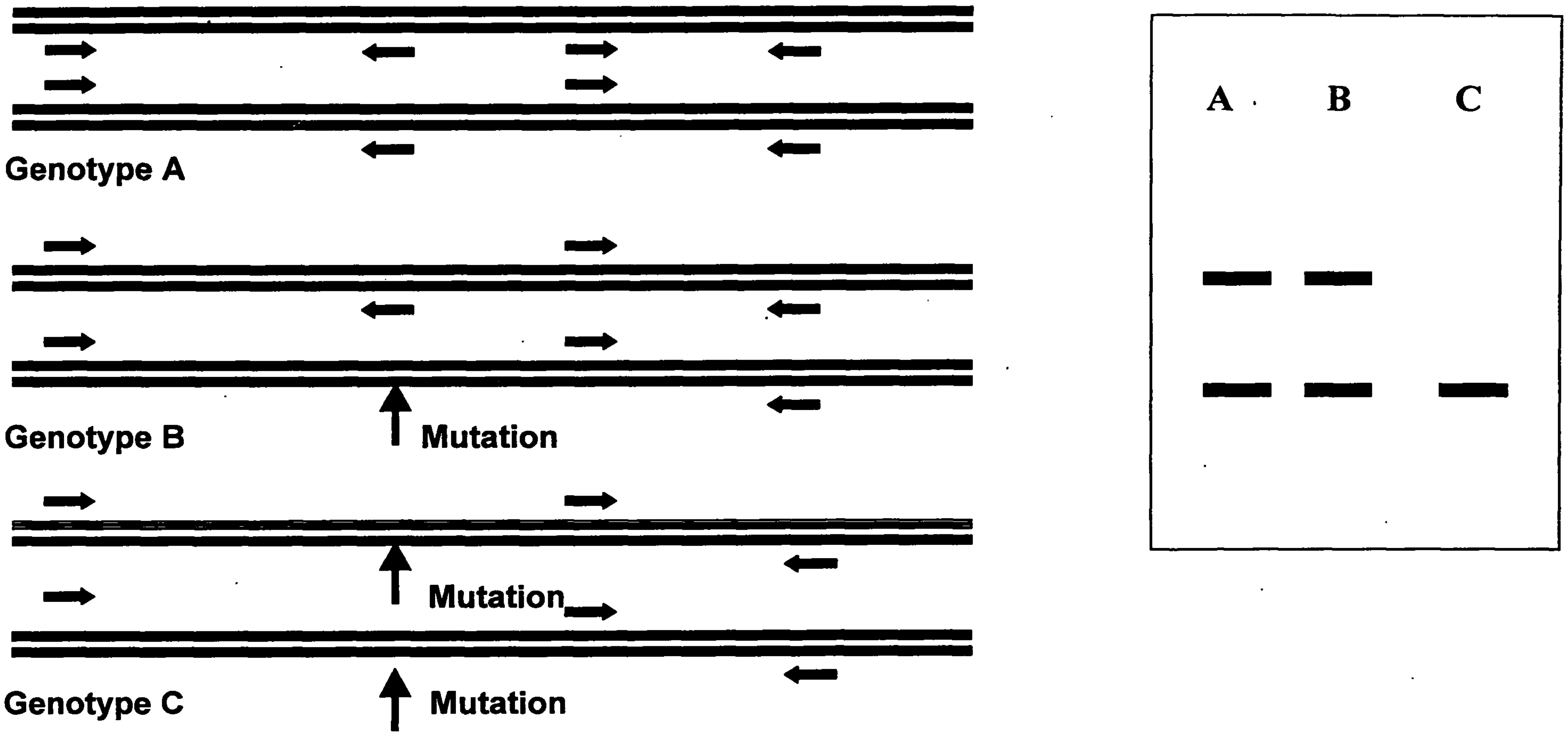
Analysis of RAPD data

RAPDs data could be used to study genetic relationships among samples. This starts with the construction of a matrix specifying character-state of each marker for each sample. A sample will usually be DNA from an individual or bulk from number of individuals (to represent an accession or taxon). Marker state may be binary, presence or absence of a band scored as 1 or 0. This sample x marker matrix of character states is then commonly used to construct sample x sample matrix of pair-wise genetic distances. There are several ways of calculating genetic distance (or similarity), but a commonly used index is Nei's genetic distance (= $2N_{AB}/(N_A+N_B)$, N_{AB} = bands common to A and B, N_A = Total bands in A, N_B = Total bands in B).

There are two ways of analyzing the resulting distance matrix. A). Principle Coordinate Analysis to produce 2 or 3-D scatter plots of the samples. Aggregations of samples in such a plot reveal sets of genetically similar material. B) Produce a dendrogram linking together in clusters samples that are more genetically similar to each other than to samples in other clusters. Clusters are linked to each at progressively lower levels of similarity until all the samples being analysed are included in a single cluster.

There are a number of software for analyzing these data; NTSYS, SAS, BIOSYS, RAPDistance (ftp:/life.anu.au/pub/molecular_biology/software/rapd103.zip)

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS



RAPD behave like dominant Mendelian genes. Possible causes: Deletion/insertion in the primer binding site, Creation of new site by deletion/insertion at some other place, Insertion of a large fragment between binding sites.

Applications of Molecular Markers in Plants

1. Generation of genetic maps
2. Mapping of simple traits
3. Mapping of quantitative trait loci
4. Mapping of mutations
5. Characterization of transformants
6. Genetic diagnosis for plant breeding
7. Population genetics
8. Molecular taxonomy and evolution
9. Identification of individuals/clones
10. Germplasm characterization
11. Identification of proprietary germplasm
12. Analysis of herbarium samples
13. Estimation of genome size

DNA markers are being applied to a wide variety of problems central to plant genome analysis. Although each marker system is characterized by a unique combination of advantages and disadvantages the choice of a marker system is dictated to a significant extent by the application. Factors consider in choosing a marker system include the amount of available plant material or DNA, the quality of the DNA, the availability of public collections of DNA markers for the species being examined, the resources and skills available to develop new markers or to use existing markers, cost on a per marker or per locus basis and any restrictions on the use of radioactive materials.

Comparison of DNA Marker Systems

Marker systems can be classified conveniently according to two criteria, information content and multiplex ratio. The relative information content of any two classes of markers may be evaluated by comparing the mean heterozygozity in a given set of individuals for a number of randomly chosen markers in each of the marker classes. Heterozygozity is the probability , P, that two gametes chosen at random from a population will carry different alleles at a locus

$$P = 1 - \varepsilon p^{2}_{n}$$

p_n is the allele frequency of allele n in the population

The higher the heterozygozity the greater the chance that a polymorphism can be detected.

Multiplex ratio is the number of different genetic loci that may be simultaneously analysed per experiment (per gel lane). The following table compares features of commonly used marker systems.

	RFLP	RAPD	SSR	AFLP
Principle	Restriction endonuclease digestion. Blotting. Hybridization.	DNA amplification with random primers	PCR of simple sequence repeat regions	PCR of a sub set of restriction fragments from extended adapter primers.
Nature of polymorphisms	Single base changes. Insertions. Deletions	Single base changes. Insertions. Deletions	Repeat length changes	Single base changes. Insertions. Deletions
Abundance in the genome	High	Very high	Medium	High
Level of polymorphism	Medium	Medium	High	Medium
Dominance	Co-dominant	Dominant	Co-dominant	Mixed
Multiplex ratio	1-2	5-20	1	30-100
DNA amount required	2-10 μg	10-25 ng	50-100 ng	1-2 μg
DNA sequence information requirement	No	No	Yes	No
Radioactive detection	Yes	No	Yes	Yes
Development cost	Medium	Low	High	Medium
Start up cost	High	Low	High	Medium

Comparison of DNA marker systems

ISOLATION OF PLANT DNA

Many protocols are available for extraction and isolation of genomic DNA and the method you use will depend on specific requirements. This protocol (Mini prep method) is a rapid micro-scale method for isolation of plant DNA. The DNA produced is of moderately high molecular weight and serve as a satisfactory substrate for most restriction endonucleases and is suitable for genomic blot analysis and PCR applications. In addition to the rapidity and convenience of mini preps, which permit a large number of samples to be processed in just a few hours, the small amount of tissue required (less than 1.0 grams) allows for molecular analysis of plants at a very young stage. The Mini prep DNA yields from leaf tissue of most species tested to date are typically 50-100 µg per gram of tissue, greater than 50 kb, and remarkably uniform from sample to sample.

The first Mini prep procedure that was reported for maize isolation (Deilaporta *et al.*, Maize Genetics Corporation Newsletter, 1993) was adopted from a procedure commonly used for yeast DNA preparation (Davis *et al.*, 1980). Since this report, numerous personal communication have demonstrated that the mini prep procedure or modification thereof, can be applied to most plant species tested. For example, the method has been successfully used on *Nicotinia tabacum, N. plumberginifolium, N. sylvestris, Lycopersicon, Amaranthus sp, Glycine max, Petunia hybrida* etc. Several modifications have been applied by these investigations in order to extend the application of mini prep procedures to other plant species. The selection of a particular protocol depends to a large degree on the plant species used. However, the procedure reported here is suitable for most situations.

PROCEDURE

1. Weigh 1g of leaf tissue (use only the leaf blade), freeze in liquid Nitrogen and grind to a fine powder in a 3" mortar and pestle, (use a small amount of acid washed sand). Transfer powder into a 30ml Oak Ridge tube.

2. Add 15ml of Extraction Buffer (EB) : 100mM Tris (pH 8.0), 50mM EDTA (pH 8.0), 500mM NaCl, 10mM Mercaptoethanol. After initial grinding, add 10ml of EB followed by the remaining 5ml.

3. Add 1.0 ml of 20% SDS, mix thoroughly by vigorous shaking, and incubate tubes at 65^0 C for 10Mins.

4. Add 5.0 ml of 5M potassium acetate, shake the tube vigorously and incubate at 0^0 C for 20 mins, using ice and water. (most proteins and polysaccharides are removed as a complex with the insoluble Potassium dodecyl sulfate precipitate).

5. Spin the tubes at 25,000xg for 20 mins. Pour the supernatant through a piece of cheesecloth in a clean 30 ml tube containing 10ml isopropanol. Mix and incubate tubes (at -20^0 C) for 30 mins.

5. Pellet the DNA at 20,000xg for 15 mins. Gently pour off supernatant and lightly dry pellets by inverting the tubes on paper towels for 10 mins.

7. Re-dissolve the DNA pellets with 0.7ml of 50mM Tris, 10mM EDTA(pH 8.0). Transfer the solution to an Eppendorf tube. Spin the tubes in a microfuge for 10 mins to remove insoluble debris.

8. Transfer the supernatant to a new Eppendorf tube and add 75µl of 3M Sodium acetate and 500µl of Isopropanol. Mix well and pellet the clot of DNA for 30 seconds in a microfuge. Re-dissolve in 700µl of 10mM Tris,1mM EDTA (pH 8.0) {1x TE pH 8.0 buffer}. Precipitation from 0.3M Sodium acetate using relatively small amounts of Isopropanol (about 0.6 volumes) has been reported to separate high molecular weight DNA from polysaccharides (Marmur, 1961). The Sodium acetate also yields a tight, fibrous precipitate that is easily washed and dried. The DNA will dissolve readily if allowed to re-hydrate at 40° C for 1 hour followed by light vortexing.

9. Treat the sample with Rnase A (50µg/ml) at 37° C for 15 mins and then add proteinase K (50µg/ml).

10. Extract the sample two times with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).

11. Transfer the aqueous phase to fresh Eppendorf tube and add 500µl of Isopropanol and 75 µl of 3M NaAc.

12. Mix well and pellet the clot of DNA.

13. Wash the pellet with 80% Ethanol and Re-dissolve in 100µl of 1x TE(pH 8.0) buffer.

14. Run a sample of the DNA in an agarose gel for confirmation.

15. Quantify the DNA by measuring the absorbance at 260nm.

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WORKSHOP ON "PCR TECHNOLOGY AND ITS APPLICATIONS'

LABORATORY HANDOUTS

Athula Perera
W.L.C.Ubayasena

LABORATORY HANDOUT NO: 01

EXTRACTION OF GENOMIC DNA of SUGARCANE FOR PCR BASED RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)

INTRODUCTION

Genomic DNA can be obtained from plant, animal and micro-organism at any time during development. This genomic DNA represents, DNA which contain a copy of every gene from that particular organism.

During genomic DNA isolation from an organism numerous problems may be encountered, such as viscous polysaccharides, polyphenols and other secondary plant compounds which courses damage to DNA and/or inhibit restriction enzymes and DNA polymerases. Milligan 1992, and Weising *et al* 1995a reviewed that, there are hundreds of different protocols for the isolation of genomic DNA, nevertheless rely on few basic principles. Selection of a suitable protocol is depend on the kind of experiments which demands different level of DNA purity. At the same time rapidness, simpleness and the inexpensively of the DNA isolation protocol are also considered.

As One of the main advantages of RAPD techniques is that it requires minute amount of template DNA which need not to be ultra pure in terms of protein contamination, the protocol modified by Tai and Tanskeley (1991) for genomic DNA extraction is used in this demonstration.

Reagents

a. 1 M	Tris buffer (pH=8)
b. 0.5 M	EDTA
c. 10%	Sodium dodecyl sulfate (SDS)
d. 7.4 M	Ammonium acetate
e. 5 M	Potassium acetate

f. Extraction Buffer (for 100ml)

i.	1M Tris buffer (pH=8) =	10 ml
ii.	0.5 M EDTA =	10 ml
iii.	NaCl (~3%) =	2.922 g
iv.	10% SDS =	25 ml
v.	Sodium bisulphite =	0.38 g*

*= Add fresh each time

g. TE buffer

   i. 10 mM Tris-HCL
   ii. 1 mM EDTA, (pH7.4)

h. Isopropernol
i. Ethanol

Apparatus

i. Mortar and pestle
ii. Centrifuge
iii. Water Bath
iv. Pipettes

Procedure

1. Cut the sugarcane tops and trim the leaf blades to obtain immature leaf roll tissues and immerse in liquid nitrogen.

2. Grind the frozen leaf roll tissues(1-2 g) in a cold mortar in the presence of liquid nitrogen to form fine powder.

3. Collect the leaf powder in to 15 ml centrifuge tube and add 0.7 ml of pre-heated (65^{0}C) extraction buffer and incubate in 65^0 C for 15 minutes.

4. Add 250µl of 5M potassium acetate and leave for 20-40 minutes on ice.

5. Spin the tubes at 13,000 RPM for 5 minutes.

6. Take supernatant and add an equal volume of isopropernol.

7. Spin at 8000 rpm for 5 minutes if you can see the DNA, if not spin at 13,000 RPM for 5 minutes and discard the supernatant.

8. Wash the pellet in 70% ethanol and air dry.

9. Add 300 µl of TE buffer and resuspend the DNA pellet (can put at 65^{0}C if necessary to aid resuspention).

10. Add 150 µl of 7,4 M ammonium acetate and mix thoroughly and centrifuge at 13000 rpm for 5 minutes.

11. Transfer supernatant to new 2.5 ml tube and add equal volume of isopropenol to precipitate DNA (incubate if necessary at -20°C).

12. Spin at 13000 rpm for 5 minutes and discard the supernatant.

13. Wash the DNA pellet 70 % and 100% ethanol respectively and dissolve in 100µl of TE buffer.

LABORATORY HANDOUT NO: 02

DNA QUANTIFICATION (BY AGAROSE GEL ELECTROPHORESIS)

Preparation of agarose gel

Reagents

- i. Electrophoresis buffer
  - a. TBE buffer : for 1 litre (10 x stock) 108g Tris-base, 55.0g boric acid, 9.3g EDTA, final pH 8.2-8.4.
  - b. TAE buffer : for 1 litre (10 x stock) 48.4g Tris-base, 11.4 ml glacial acetic acid, 20 ml 0.5M EDTA (pH 8.0), final pH 7.6.
- ii. Agarose
- iii. Ethidium bromide

Apparatus

- 1. Electrophoresis apparatus with all accessories
- ii. UV transilluminator
- iii. Camera system for gel documentation

Procedure

1. Assemble the gel mould.

2. Add the weighed amount of agarose to the measured volume of electrophoresis buffer needed to fill the mould.

3. Heat until the agarose has dissolved in a microwave oven.

4. Cool the melted agarose solution to about 50^{0}C, pour into the mould and immediately place the sample well-forming comb in position.

5. When the gel has completely cooled and set (30-40 min), remove the comb and place the gel in the electrophoresis apparatus.

6. Add sufficient buffer to fill the electrode chambers and cover the gel with a depth of about 1 mm.

7. load 2 µl aliquots of all DNA samples along with the three concentrations of lambda Hindy iii digest molecular weight marker in to the wells.

8. Run the electrophoresis at 5.6 V/cm of gel length for 1 hour.

9. Stained the gel in 0.5 µg/ml ethidium bromide solution for 20 min and destained the gel in water for 10 min.

10. Visualise the under UV and photograph

11. Compare the intensities of the extracted DNA bands with the bands of the molecular weight marker and calculate the DNA concentrations.

LABORATORY HANDOUT NO:03

RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) PROTOCOL

PRINCIPLE

The PCR based RAPD analysis is conceptually simple. Nanograme amounts of total genomic DNA are subjected to PCR using short synthetic oligonucliotides of random sequence. The amplification protocol differs from the standard PCR conditions in that only a single random oligonucliotide primer is employed and no prior knowledge of the genomic subjected to analysis is required.

The amplification products are resolved on agars gels and polymorphism's serve as dominant genetic markers.

Reagents

i. Taq DNA polymerase (2u/µl)
ii. PCR buffer (10 X)
iii. dNTPs (100mM) (dATP, dCTP, dTTP, dGTP)
iv. $MgCl_2$ (100mM)
v. Nano pure Water
vi. 10-mer primers
vii. electrophoresis buffer
viii. Gel loading buffer
ix. Ethidium Bromide solution
x. 1 kb Ladder

Apparatus

i. Thermal cycler
ii. Micro pipette
    a. 0.5-10 µl variable volume pipette
    b. 2-20 µl variable volume pipette
    c. 10-100 µl variable volume pipette
iv. Electrophoresis apparatus with all accessories
v. UV transilluminater
vi. Gel documentation system (Polaroid camera system)

Procedure

1. Dilute extracted genomic DNA samples to 5 ng/µl with DD/water.

2. Prepare cocktail mixture of PCR reactants adequate for all the sample (allow one sample for pipetting errors).

   Ex. Number of test samples = 10
   Negative control = 1
   Positive control = 1

   Sub total 12
   For pipetting errors 1

   Total number of Samples 13

   In this example have to prepare cocktail mixture adequate for 13 samples.

3. Pipette PCR reactant in to 0.5 ml PCR tube

Reactant	Volume for * 1 sample	Volume required for 13 samples
a. PCR buffer (10 x)	2 µl	26 µl
b. $MgCl_2$ (100mM)	0.5 µl	6.5 µl
c. dNTPs (2mM each)	2 µl	26 µl
d. Primer	1 µl	13 µl
e. BSA	0.2 µl	2.6 µl
f. Taq DNA polymerase	0.3 µl	3.9 µl
g. Template DNA	3 µl	-----
h. Water	11 µl	143 µl
	-------	------
	20 µl	221 µl

* These amounts will be vary depending on the concentration of the stock solution and the reaction conditions.

4. Put 17 µl of the prepared cocktail mixture in to labelled 0.5 ml PCR tubes.

5. Add 3 µl of water in to 1 tube (negative control).

6. Add 3 µl of template DNA (5 ng/µl) in to the PCR tubes.

7. Mix well and spin for a second and load in to the thermal cycler.

8. Set the thermal Program of the thermal cycler with a suitable amplification condition.

Ex. Initial Denaturation	1 minute	at	92°C
30 cycles of:			
Denaturation	40 seconds	at	92°C
Annealing	1 minute	at	48°C
Extension	1.5 minute	at	72°C
Final extension	10 minute	at	72°C

Hold at 4°C forever

9. Prepare agaroge gel (1.5 %(W/V)) containing ethidium bromide with the concentration of 0.5µg/ml as explained by handout no. 02.

10. Mix 15-20 µl of PCR products with 3 µl of gel loading buffer and load in to the gel wells.

11. Load 1 kb ladder in to the 1st well of the gel.

12. Run the gel at 5.6 V/cm for 2 hours.

13. See the bands under UV light and photographed.

RANDOM AMPLIFICATION OF POLYMORPHIC DNA

RAPD

Athula Perera
Lasantha Ubayasena

Laboratory Procedures

Random Amplification of Polymorphic DNA
RAPD

RAPDs is a PCR-based technique which uses one (or even two) primers of arbitrary sequence (usually 8 - 10 bases) to amplify anonymous stretches of DNA. These are then separated and visualized by gel-electrophoresis. A characteristic feature of RAPDs is that *nothing* is known about the identity of the amplification products. But they can be used as markers in assessing genetic diversity and for selection in plant breeding programmes.

RAPD technology was developed in 1990 by Williams et.al, to detect genetic polymorphisms. In this technique, the PCR is used to amplify DNA with random primers. Using PCR, a random primer of arbitrary nucleotide sequence binds to the DNA at two different sites on opposite strands of the DNA template. If the primer sites are located at a close amplifiable distance from each other, the result is a DNA product by a thermocyclic reaction.

The amplification products can be observed in denaturing polyacrylamide gels or agarose gels.

The most important sequences of the primer are the first 5 nucleotides from its 5' end. This is so because of Taq polymerase binding behaviour. Taq binds to the 3' end of the template. The balance sequences can be flexible, and as such can cause mismatches, thereby weakening the binding of the primer to the template at high temperatures. Therefore, it is preferable to use lower annealing temperatures when using RAPDs compared to primers of specific target sequences.

```
5' ....G C T A C G C T G G...3' Template
                <---C G A C C...5' Primer
```

Primers need to be at least 10 bp in length to ensure proper annealing.

Characteristic features of RAPDs

* Number of fragments

Each single primer normally amplifies many different fragments and therefore is a fast method for detecting polymorphisms. Most of the RAPD primers available produce 6 - 12 fragments, although some primers will fail to amplify any fragment of some genomes.

- Simplicity

RAPD technique does not require hybridization with radioactive material and autoradiography. Only very small quantities of target DNA are required. The primers are relatively cheap to purchase.

- Dominant markers

RAPD primers either amplify a segment of DNA or it does not. Therefore, in scoring the bands in a gel, a presence / absence (+/-) score is given. This means that homozygotes and heterozygotes cannot be distinguished.

- Reproducibility

RAPD amplification is sensitive to the different conditions of a PCR reaction, and hence changes can occur to some of the amplified fragments. Nevertheless, reproducible results can be achieved if proper standardization of the reaction conditions are carried out.

Analysis

Since the primers used in the RAPDs technique are random / arbitrary, nothing is known about the identity of the amplification products, unless information is available from pedigree analysis. Therefore, the following facts have to be considered in analyzing the amplification products of RAPDs.

- RAPD markers are dominant

- Co-migration - same band, same DNA fragment ?

Different DNA fragments amplified by RAPDs may be of the same size (identical molecular weights). Therefore, the presence of a band of identical molecular weight in different individuals is not evidence, *per se*, that the individuals share the same (homologous) DNA fragment.

- Co-migration - one band, one fragment ?

A single band on a gel can consist of different amplification products. The electrophoresis techniques used can separate DNA quantitatively (i.e. according to size) but they cannot separate equal-sized fragments qualitatively (i.e. according to base sequence).

PCR with arbitrary primers

There are several forms of PCR that use random primers.

(i) RAPDs (Williams et. al., 1990)- most common.

(ii) DAF - DNA Amplification Fingerprinting.

- Higher primer concentration.
- Shorter primers (5-8 nucleotides).
- Two - temperature cycle compared to 3 in RAPDs.
- Produces very complex banding patterns.

(iii) AP - PCR - Arbitrarily Primed PCR

- Amplification is in 3 parts.
- High primer concentration used in first cycle.
- Primers are of variable length.

(iv) MAAP - Multiple Arbitrary Amplicon Profiling.

Includes all of the above.

RAPDS

- FAST - quick method to detect polymorphisms.
- SIMPLE - not technically demanding.
- CHEAP - low unit cost.
- NO USE of radioisotopes

The RAPDs technique has been used to determine DNA diversity in a range of crops including beans (Haley et.al., 1994), rice (Ko et.al., 1994), cassava (Marmey et.al., 1994), coffee (Orozco-Castillo et.al., 1994), tomato (Rus-Kortekaas et.al., 1994), sugarcane (Harvey et.al., 1996) and *Saccharum spontaneum* (Al-Janabi et.al., 1993; Sobral and Hunycutt, 1993). RAPD markers have been used for distinguishing plant species and cultivars such as the cereals (Ko et.al., 1994), *Festuca* (Valles et.al., 1993; Stammers et.al., 1995), *Hordeum* (Gonzales and Ferrer, 1993), mustard (Jain et.al., 1994), oil palm (Shah et.al., 1994), cocoa (Wilde et.al., 1992) and apple (Koller et.al., 1994), for genetic mapping (Williams et.al., 1990; Torres et.al., 1993; Yu and Pauls, 1993), for genetic stability analysis of tissue cultured plants (Valles et.al., 1993), and for assessing genetic diversity in coconut (Rhodes et.al., 1995).

PROTOCOL 1. (Pammi et.al., 1994)

The amplified products are separated on 5% denaturing polyacrylamide gels and stained with silver nitrate.

REACTION COCKTAIL

Use DNA of concentration 4 ng/μl

(i) Pipette 2.4 μl of the template DNA into labeled PCR tubes

Prepare the reaction cocktail in a 1.5 ml microcentrifuge tube.

Add the components in the order listed.

■ Sterile water	4.815 μl
■ 10 x Stoffel buffer	4.5 μl
■ $MgCl_2$	4.5μl
■ dATP	4.5 μl
■ dGTP	4.5 μl
■ dTTP	4.5μl
■ dCTP	4.5μl
■ Primer	5.76 μl
■ Taq DNA polymerase	0.225μl
Reaction total	37.8 μl

This cocktail contains volumes necessary for 3 reactions. Always an extra reaction (3rd in this case) is considered to compensate for pipetting errors.

Mix the contents of the tube by gently pipetting up and down two times.

(ii) Pipette 12.6 μl from the cocktail into the PCR tube containing the template DNA.

Mix by gently pipetting up and down two times.

(iii) Place the 0.2 ml PCR tubes in one PCR rack. Place in thermocycler.

AMPLIFICATION CONDITIONS.

(a) Sorghum

Initial heating	1 minute	at 92^0C	
Denaturation	40 seconds	at 92^0C	30 cycles
Annealing	1 minute	at 48^0C	
Extension	1.5 minutes	at 72^0C	
Final extension	10 minutes	at 72^0C	
Hold	4^0C forever		

(b) Rice

Initial heating	1.5 minutes	at 92^0C	
Denaturation	1 minute	at 92^0C	30 cycles
Annealing	1 minute	at 48^0C	
Extension	1.5 minutes	at 72^0C	
Final extension	10 minutes	at 72^0C	
Hold	at 4^0C Forever.		

PROTOCOL:2

(Rice)

REACTION COCKTAIL

(for a single reaction.)

10 x PCR buffer	1.5 µl
25 mM $MgCl_2$	1.5 µl
1 mM dATP	1.5 µl
1 mM dGTP	1.5 µl
1 mM dCTP	1.5 µl
1 mM dTTP	1.5 µl
Primer	1.5 µl
Taq polymerase	0.075 µl
Water	2.425 µl
5 ng/ µl DNA template	2.0 µl
Total volume	15.0 µl

*** Same PCR conditions as in Rice-1**

PROTOCOL: 3

(Onions)

REACTION COCKTAIL

For a single reaction.

■ Sterile water	14.3 µl
■ 10 x PCR buffer	2.5 µl (1x)
■ 4mM Primers	2.0 µl (400 µM)
■ dNTPs (10 mM)	2.0µl (200 µ M)
■ 25 mM $MgCl_2$	2.0 µl (2 mM)
■ Taq DNA polymerase	(5 U/µl) 0.2 µl (about 1 Unit)
Total volume	23 µl

Add 23 µl of the cocktail to each PCR tube containing 2 µl of template DNA.

AMPLIFICATION CONDITIONS.

Denaturation	5 minutes	at 94°C	
Denaturation	1 minute	at 94°C	35 cycles
Annealing	45 seconds	at 54°C	
Extension	2 minutes	at 72°C	
Final extension	10 minutes	at 72°C	

Hold at 4°C forever

PROTOCOL: 4

(Lisa, M ., et al., 1994).

REACTION COCKTAIL

- 20 ng of genomic DNA (template DNA)
- 1 µM each of the primer primer (2 µM in the case of single -primer)
- 200 µM each of dATP, dCTP, dGTP and dTTP
- 50 mM KCl
- 2.5 mM $MgCl_2$
- 10 mM Tris-HCl (pH 9.0)
- 0.1% Triton x-100
- 1 unit of Taq polymerase.

Total reaction volume - 20 µl

AMPLIFICATION CONDITION

Predenaturation	1 minute	at 94°C	
Denaturation	2 minutes	at 94°C	45 cycles
Annealing	1 minute	at 32°C	
Extension	3 minutes	at 72°C	
Hold	7 minutes	at 72°C.	

PROTOCOL: 5

(Susan, E.. W., *et al.*, 1993)

REACTION COCKTAIL

- 50 ng template DNA
- 0.2 µM primer
- 200 µM each of dATP, dCTP, dGTP, and dTTP
- 1U of Taq polymerase in 1 x Parr reaction buffer made up to a final concentration of 2 mM $MgCl_2$
- The reaction mixture is over laid with 100 µl of mineral oil

  Total reaction volume - 100 µl

AMPLIFICATION CONDITION

Preliminary denaturation	3 minutes	at 94°C	
Denaturation	1 minute	at 94°C	45 cycles
Primer annealing	1 minute	at 33°C	
Extension	2 minutes	at 73°C	

- For final extension, hold the reaction mix for 5 min. at 73°C and allow to cool to room temperature.

PROTOCOL: 6

(Pammi. S., *et al.*, 1994).

Using radioactive d CTP.

REACTION COCKTAIL

- 9.6 ng of template DNA
- 3 µM of primer (10 mer primers)
- 0.38 units of Taq polymerase
- 2.5 mM $MgCl_2$
- 11.1 x 10^4 Bq° of (^{32} p) d CTP
- 100 µM dGTP, dATP, dTTP and 6µM dCTP

Total reaction volume - 15 ml

AMPLIFICATION CONDITIONS

Denaturation	1 minute	at 92°C	30 cycles
Annealing	40 Second	at 48°c	
Extension	1.15 min	at 72°C	

After first cycle, use 40 Sec denaturation time.

Hold 15 min. at 72°C for final extension.

PROTOCOL: 7

(King , I. P., *et al.,* 1993).

REACTION COCKTAIL

- 25 ng of template DNA
- 100 µM each of dATP, dCTP, dGTP, and dTTP
- 200 µM oligonucleotide primer
- 0.8 units of Taq polymerase in 10 mm Tris Hcl (pH 8.3)
- 1.5 mM $MgCl_2$
- 50 mM KCl
- 100 µg / ml gelatine
- 0.05% v/v Tween 20
- 0.05% v/v Nonidet P-40.
- Overlay the reaction with mineral oil prior to amplification.

Total reaction volume - 50 µl

AMPLIFICATION CONDITIONS

Denaturation	1 minute	at 94°C	45 cycles
Annealing	1 minute	at 36°C	
Extension	2 minutes	at 72°C	
Final extension	5 minutes	at 72°C	

Note : After final extension, allow it to cool to 4°C

PROTOCOL: 8 (Jhu-Jhu, L., *et al.*, 1996).

REACTION COCKTAIL

- 1 mM Tris-HCl (pH 8.3)
- 6 mM KCl
- 2.75 mM $MgCl_2$ $6H_2O$
- 100 µ M dNTPs
- 0.8 µ M of arbitrary decamers
- 80 ng template DNA
- 1.8 Units of taq polymerase
- Mineral oil.

Total reaction volume - 25 µl

AMPLIFICATION CONDITION

Denaturation	1.2 minutes	at 94°C	35 cycles
Annealing	1.2 minutes	at 34°C	
Extension	2.3 minutes	at 72°C	
Incubate	10 minutes	at 72°C. for final extension.	

PROTOCOL: 9

(Kleinhofs . A., *et al.*, 1993).

REACTION COCKTAIL

- 50 ng DNA
- 12.5 Pmoles (1 μM) primer
- 100 μM dNTPs
- 0.5 U Taq polymerase
- 1 x Buffer. (promega 10 x =

  100 mM Tris pH 9.0

  500 mM KCl

  15 mM $MgCl_2$

  0.1% Gelation

  1% Triton x - 100
- Cover the reaction with two drops of mineral oil

  Total reaction volume 12.5 μl

AMPLIFICATION CONDITION

Denaturation (initial)	1 second	at 94°C	40 cycles
Denaturation	30 seconds	at 92°C	
Annealing	2 minutes	at 36°C	
	3 minutes ramp to 72°C		
Extension	2 minutes	at 72°C	

- Store the reaction at 10°C until used.

PROTOCOL: 10 (Sumanasinghe,. V. A., *et al.*, 1997).

REACTION COCKTAIL

- 1 ng of template DNA
- 8 µl of premix consisting of 5.4 µl of sterilized demineralized distilled water.
- 1 of 10 x buffer
    - 10 mM Tris-HCl (pH 8.3)
    - 50 mM KCl
    - 2 mM $MgCl_2$
    - 0.001% (w/v) Gelatine
- 0.2 mM each of dATP dCTP, dGTP, dTTP
- 0.2 µl of 25 mM $MgCl_2$
- 2 µ M of random primer
- 0.2 units of Taq DNA polymerase
- mineral oil.

Total reaction volume -8µl

AMPLIFICATION CONDITIONS

Denaturation	1 minute	at 93°C	45 cycles
Annealing	2 minutes	at 35°C	
Extension	3 minutes	at 72°C	

Finally hold the reaction 7 minutes at 72°C

PROTOCOL: 11

(Tao, J. M., *et al.*, 1993).

REACTION COCKTAIL

- 67 mM Tris-HCl (pH 8.8)
- 16.6 mM $(NH_4)_2SO_4$
- 0.45% (v/v) Triton - x-100
- 200 µg /ml gelatine
- 3.5 mM $MgCl_2$
- 125 µM dNTPs
- 0.2 µM oligonucleotide primer
- 25-50 ng genomic DNA
- 1.4 units of Taq polymerase.

Total reaction volume - 25 µl

AMPLIFICATION CONDITION

Denaturation	1 minute	at 94°C	40 cycles
Annealing	1 minute	at 37°c	
Extension	1 minute	at 72°C	

PROTOCOL: 12

(Russel J. R., *et al.*, 1993).

REACTION COCKTAIL

Template DNA	5 µl
dNTPs	2.5µl
(2 mM dATP, dCTP,dGTP ,and dTTP)	
10 x Taq polymerase buffer (NBL)	2.5 µl
Oligonucleotide primer	2.5 µl
1 Unit of Taq polymerase (NBL)	
Sterile distilled water	12.3 µl
Mineral oil	

AMPLIFICATION CONDITION

Denaturation	1 minute	at 92 °C	45 cycles
Annealing	1.5 minutes	at 35 °C	
Extension	2 minutes	at 72 °C	

Hold at 4 °C forever.