



INTERNATIONAL ATOMIC ENERGY AGENCY

Mutant Germplasm Characterization using Molecular Markers

A Manual

**Prepared by the
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture**



**VIENNA, 2002
ISSN 1018-5518**

T R A I N I N G C O U R S E S E R I E S

19

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The originating Section of this publication in the IAEA was:

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MUTANT GERMLASM CHARACTERIZATION USING MOLECULAR MARKERS
A MANUAL

IAEA, VIENNA, 2002

IAEA-TCS-19

ISSN 1018-5518

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Printed by the IAEA in Austria
October 2002

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FOREWORD 1

FOREWORD

Plant biotechnology applications must not only respond to the challenge of improving food security and fostering socio-economic development, but in doing so, promote the conservation, diversification and sustainable use of plant genetic resources for food and agriculture. Nowadays the biotechnology toolbox available to plant breeders offers several new possibilities for increasing productivity, crop diversification and production, while developing a more sustainable agriculture. This training course focuses on one of the most promising set of techniques used in modern crop improvement programmes, i.e. on molecular markers. These are rapidly being adopted by plant breeders and molecular biologists as effective and appropriate tools for basic and applied studies addressing biological components in agricultural production systems. Their use in applied breeding programmes can range from facilitating the appropriate choice of parents for crosses, to mapping/tagging of gene blocks associated with economically important traits (often termed “quantitative trait loci” (QTLs)). Gene tagging and QTL mapping in turn permit marker-assisted selection (MAS) in backcross, pedigree, and population improvement programmes, thereby facilitating more efficient incremental improvement of specific individual target traits. And through comparative genomics, molecular markers can be used in ways that allow us to more effectively discover and efficiently exploit biodiversity and the evolutionary relationships between organisms.

Substantial progress has been made in recent years in mapping, tagging and isolating many agriculturally important genes using molecular markers due in large part to improvements in the techniques that have been developed to help find markers of interest. Among the techniques that are particularly promising are Restriction Fragment Length Polymorphism (RFLPs), Amplified Fragment Length Polymorphism (AFLPs), Random Amplified Polymorphic DNA (RAPDs), Microsatellites and PCR based DNA markers such as Sequence Characterized Amplified Regions (SCARs) or Sequence Tagged Sites (STS). These techniques help in direct selection of many desired characters simultaneously using F2 and back-cross populations, near isogenic lines, doubled haploids and recombinant inbred lines.

During the last decade the world of classical Mendelian genetics has entered a new age, namely that of genomics, which means the study of structure of genes and their function. A great deal of DNA sequence information is now available in particular from model species such as rice and *Arabidopsis*, but the functions of the derived genes are mostly unknown. Concentrated research efforts are therefore being made to fill this so-called “phenotypic gap”. Induced mutations combined with molecular marker technology are playing an important role in this field, leading to a reinforced demand for mutagenized plant material in which certain characters have been changed due to knockout mutations of the responsible genes. Using molecular and genetic tools a mutated character can then be associated with a DNA sequence of previously unknown function. Recent reports on the homology of genes and the gene order between for instance the grass genomes (synteny) suggest that the knowledge acquired will also be useful for identification and isolation of genes from under-utilised crops.

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FOREWORD 2

This manual on selected molecular marker techniques was prepared using the handouts and other materials distributed to participants of the FAO/IAEA Interregional Training Course on "Mutant germplasm characterisation using molecular markers" that was organised by the Plant Breeding and Genetics Section of the Joint FAO/IAEA Division of Nuclear Applications in Agriculture and the FAO/IAEA Agriculture and Biotechnology Laboratory. The FAO/IAEA Division wishes to acknowledge lecturers for their willingness to participate in the preparation of the manual. The handouts, protocols and publications provided during the course were prepared and selected by J. Bennetzen (USA), K. Devos (UK), G. Kahl (Germany), U. Lavi (Israel), M. Mohan (ICGEB) and S. Nielen (FAO/IAEA). Participants gained experience in many molecular marker techniques with special emphasis on genomic DNA isolation, restriction analysis of genomic and plasmid DNA, gel electrophoresis, Southern transfer of genomic DNA, non-radioactive DNA hybridization, silver staining, RFLP, AFLP, SSR, ISSR and RAPD analysis, and retrotransposon-based marker systems. The manual was compiled by P. Gustafson (USA) and B. Forster (UK) with the assistance of M. Mohan and S. Nielen. The final editing and composition was done by M. Maluszynski and S. Nielen.

This first issue of the manual is in a loose-leaf format in the expectation that other molecular marker systems and protocols will be added. We would very much appreciate suggestions and comments, which could further improve and enrich its contents. Correspondence should be addressed directly to M. Maluszynski, Plant Breeding and Genetics Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, P.O. Box 100, Vienna, Austria, Fax: +43 1 26007; email M.Maluszynski@iaea.org. All proposals will be carefully considered by the team of editors mentioned above and, if suitable, added to the manual.

The manual will also be available on the FAO/IAEA Joint Division web site <http://www.iaea.org/programmes/nafa/d2>. A hard copy with attached CD-ROM will be distributed, free of charge, to interested scientists from FAO and IAEA Member States. Requests for the manual should be sent by those interested to S. Nielen, Plant Breeding and Genetics Section, Joint FAO/IAEA Division of Nuclear Application in Agriculture, P.O. Box 100, Vienna, Austria, Fax: +43 1 26007 or by email: S.Nielen@iaea.org. An individual request is necessary as we plan to have all user's addresses in order to distribute new additions and supplements. On request all cited publications except books, for scientists or students from developing countries can be provided. Requests should be sent to the address given above.

The Joint FAO/IAEA Division is grateful to M. Gale (UK) and R. Adlam (UK) for the review of the final version of protocols.

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LIST OF ACRONYMS 1

LIST OF ACRONYMS

AFLP	A mplified F ragment L ength P olymorphism
CAPS	C leaved A mplified P olymorphic S equences
EST	E xpressed S equences T ag
IPCR	I nverse P olymerase C hain R eaction
IRAP	I nter- R etrotransposon A mplified P olymorphism
ISSR	I nter- S imple S equences R epeat amplification
PCR	P olymerase C hain R eaction
RAPD	R andom A mplified P olymorphic D NA
REMAP	R etrotransposon- M icrosatellite A mplified P olymorphism
RFLP	R estriction F ragment L ength P olymorphism
SCAR	S equences C haracterized A mplified R egion
SNP	S ingle N ucleotide P olymorphism
SSCP	S ingle S tranded C onformation P olymorphism
SSR	S imple S equences R epeat
STS	S equences T agged S ite

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INTRODUCTION 1

1. INTRODUCTION

Molecular markers have already played a major role in the genetic characterization and improvement of many crop species. They have also contributed to and greatly expanded our abilities to assess biodiversity, reconstruct accurate phylogenetic relationships, and understand the structure, evolution and interaction of plant and microbial populations. Molecular markers systems reveal variation in genomic DNA sequence. The first generation of molecular markers, RFLP, were based on DNA-DNA hybridisation and were slow and expensive. The invention of the polymerase chain reaction (PCR) to amplify short segments of DNA gave rise to a second generation of faster and less expensive PCR-based markers, which are the main focus of this review. It is already clear, however, that the technology, and future editions of this publication, will continue to move on as new detection systems are developed in the search for evermore efficient and cost effective markers for the breeders of the 21st century.

Restriction fragment length polymorphisms (RFLPs) were the first generation of hybridization-based markers with substantial impact in agricultural biotechnology. These were subsequently followed by amplification-based technologies derived from the polymerase chain reaction (PCR). Notably, the use of arbitrary oligonucleotide primers in the amplification reaction facilitated the study of previously uncharacterized genomes. The most common of these PCR-based DNA techniques are Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR) and Randomly Amplified Polymorphic DNA (RAPD). Other techniques have also been developed and are widely used in genetic analysis.

Molecular markers are being used extensively to investigate the genetic basis of agronomic traits and to facilitate the transfer and accumulation of desirable traits between breeding lines. A number of techniques have been particularly useful for genetic analysis. For example, collections of RFLP probes have been very versatile and important for the generation of genetic maps, construction of physical maps, the establishment of syntenic relationships between genomes, and marker assisted breeding. Numerous examples of specific genes that have been identified as tightly linked to RFLP markers are available for the improvement of specific agronomic traits in almost all major crops. Specific examples include viral, fungal and bacterial resistance genes in maize, wheat, barley, rice, tomatoes and potatoes. Additional examples include insect resistance genes in maize, wheat and rice as well as drought and salt tolerance in sorghum. These markers often used in conjunction with bulked segregant analysis and detailed genetic maps, provide a very efficient method of characterizing and locating natural and induced mutated alleles at genes controlling interesting agricultural traits. Markers have also been used to identify the genes underlying quantitative variation for height, maturity, disease resistance and yield in virtually all major crops. In particular, the PCR-based techniques have been useful in the assessment of biodiversity, the study of plant and pathogen populations and their interactions; and identification of plant varieties and cultivars. Amplified DNA techniques have produced sequence-tagged sites that serve as landmarks for genetic and physical mapping. It is envisioned that emerging oligonucleotide-based technologies derived from the use of hybridization arrays, the so-called DNA chips and oligonucleotide arrays, will become important in future genomic studies. However, many

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of these are still under development, are proprietary, or require the use of expensive equipment, and are therefore not yet suitable nor cost-effective for adequate transfer to developing countries. Clearly, the initial transfer of technology has only involved a selected group of techniques that are well established and/or seem to have a broad application (e.g., RFLP, SSR, ISSR, AFLP, RAPD, IRAP and REMAP and SNPs). However, techniques are continuously changing and evolving, so technology transfer needs to keep pace with current developments in genomics.

New developments in genomic research have given access to an enormous amount of sequence information as well as new insights on the function and interaction of genes and the evolution of functional domains, chromosomes and genomes. In this context, functional and comparative genomics can help in comparative genetic mapping and linkage analysis of useful agricultural traits. Future DNA marker techniques, such as the use of oligonucleotide arrays, are likely to be sequence based. Comparative analyses of sequence information in the growing databases now publicly available on the World Wide Web will be an invaluable resource for genetic characterisation of exotic crop germplasm. This will have an increasingly important role in prospection and conservation endeavours.

The following pages in this section present, in very synthetic and basic form, information on the more widely used marker systems; their implementation, application suitability, critical requirements and general comparison of different marker systems. The information is general in nature as many of these techniques are flexible and can be adjusted to suit various requirements.

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1.1. Marker Techniques

Marker/technique	PCR-based	Polymorphism (abundance)	Dominance
RFLP	No	Low-Medium	Co-dominant
RAPD	Yes	Medium-High	Dominant
SSR	Yes	High	Co-dominant
ISSR	Yes	High	Dominant
AFLP	Yes	High	Dominant
IRAP/REMAP	Yes	High	Co-dominant

Additional marker systems not covered in the course

Morphological	No	Low	Dominant/Recessive/Co-dominant
Protein/isozyme	No	Low	Co-dominant
STS/EST	Yes	High	Co-dominant/Dominant
SNP	Yes	Extremely High	Co-dominant
SCARS/CAPS	Yes	High	Co-dominant
Microarray		High	

1.2. Ideal Genetic Markers (highly dependent on application and species involved)

- No detrimental effect on phenotype
- Co-dominant in expression
- Single copy
- Economic to use
- Highly polymorphic
- Easily assayed
- Multi-functional
- Highly available (Un-restricted use)
- Genome-specific in nature (especially when working with polyploids)
- Can be multiplexed
- Ability to be automated

1.3. Marker Application Suitability

RFLP	Comparative maps Framework maps Genetic maps Breeding Varietal/line identification (multiplexing of probes necessary) Marker-assisted selection F ₁ identification Diversity studies Novel allele detections Gene tagging Bulk segregant analysis Map-based gene cloning
RAPD	Genetic maps F ₁ identification Varietal/line identification (multiplexing of primers necessary) Breeding Bulk segregant analysis Diversity studies Marker-assisted selection Seed testing Map-based gene cloning
SSR	Fingerprinting Varietal/line identification (multiplexing of primers necessary) Framework/region specific mapping Genetic maps F ₁ identification Comparative mapping Breeding Bulk segregant analysis Diversity studies Novel allele detections Marker-assisted selection High-resolution mapping Seed testing Map-based gene cloning

ISSR	Fingerprinting Varietal/line identification Genetic maps F ₁ identification Gene tagging Breeding Bulk segregant analysis Diversity studies Marker-assisted selection High-resolution mapping Seed testing
AFLP	Fingerprinting Very fast mapping Region-specific marker saturation Varietal identification Genetic maps F ₁ identification Gene tagging Breeding Bulk segregant analysis Diversity studies Marker-assisted selection High-resolution mapping Map-based gene cloning
IRAP/REMAP	Fingerprinting Varietal identification F ₁ identification Gene tagging Bulk segregant analysis Diversity studies Marker-assisted selection High-resolution mapping Seed testing

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Additional marker systems not covered in the course

Morphological	Genetic maps Alien gene introduction Varietal/line identification F ₁ identification Novel phenotypes Breeding
Protein and Isozyme	Genetic maps Quality trait mapping Varietal/line identification (multiplexing of proteins or isozymes necessary) F ₁ identification Breeding Seed testing
STS/EST	Fingerprinting Varietal identification Genetic maps F ₁ identification Gene tagging and identification Bulk segregant analysis Diversity studies Marker-assisted selection Novel allele detection High-resolution mapping Map-based cloning
SNP	Genetic maps F ₁ identification Breeding Gene tagging Alien gene introduction Bulk segregant analysis Diversity studies Novel allele detections Marker-assisted selection High resolution mapping

SCARS/CAPS Framework mapping
Can be converted to allele-specific probes
F₁ identification
Gene tagging
Bulk segregant analysis
Diversity studies
Marker-assisted selection
Map-based cloning

Microarray Fingerprinting
Sequencing
Transcription
Varietal identification
Genetic maps
F₁ identification
Gene tagging and identification
Bulk segregant analysis
Diversity studies
Marker-assisted selection
High-resolution mapping

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1.4. Implementation

Marker/techniques	Development costs	Running costs per data point	Portability (Lab/Crops)
RFLP	Medium	High	High/High
RAPD	Low	Low	Low/Low
SSR	High	Medium	High/Low
ISSR	Low	Low	High/Low
AFLP	Medium-High	Low	High/Low
IRAP/REMAP	High	Medium	High/Low

Additional marker systems not covered in the course

Morphological	Depends	Depends	Limited to breeding aims
Protein and isozyme	High	Medium	High/High
SCARS/CAPS	High	Medium	High/Low
STS/EST	High	Medium	Medium/High
SNP	High	Medium-Low	Unknown
Microarray	Medium	Low	Unknown

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1.5. Requirements

Marker/technique	Amount/quality of DNA	DNA Sequence Required	Radioactive detection	Gel system
RFLP	High/High	No	Yes/No	Agarose
RAPD	Low/Low	No	No	Agarose
SSR	Low/Medium	Yes	No	Acrylamide/Agarose
ISSR	Low/Medium	Yes/No	No	Acrylamide/Agarose
AFLP	Low/High	No	Yes/No	Acrylamide
IRAP/REMAP	Low/Medium	Yes	No	Acrylamide/Agarose

Additional marker systems not covered in the course

Morphological	No	No	No	None
Protein/isozyme	No	No	No	Agarose/Acrylamide
STS/EST	Low/High	Yes	Yes/No	Acrylamide/Agarose
SNP	Low/High	Yes	No	Sequencing required
Microarray	Low/High	Yes	No	None
SCARS/CAPS	Low/High	Yes	Yes/No	Agarose

1.6. Comparison of Different Marker Systems

Marker	Advantages	Disadvantages
RFLP	<ul style="list-style-type: none"> - Unlimited number of loci - Co-dominant - Many detection systems - Can be converted to SCARs - Robust in usage - Good use of probes from other species - Detects in related genomes - No sequence information required 	<ul style="list-style-type: none"> - Labour intensive - Fairly expensive - Large quantity of DNA needed - Often very low levels of polymorphism - Can be slow (often long exposure times) - Needs considerable degree of skill
RAPD	<ul style="list-style-type: none"> - Results obtained quickly - Fairly cheap - No sequence information required - Relatively small DNA quantities required - High genomic abundance - Good polymorphism - Can be automated 	<ul style="list-style-type: none"> - Highly sensitive to laboratory changes - Low reproducibility within and between laboratories - Cannot be used across populations nor across species - Often see multiple loci - Dominant
SSR	<ul style="list-style-type: none"> - Fast - Highly polymorphic - Robust - Can be automated - Only very small DNA - Co-dominant - Multi-allelic - Multiplexing possible - Does not require radioactivity 	<ul style="list-style-type: none"> - High developmental and startup costs - Species-specific - Sometimes difficult interpretation because of stuttering - Usually single loci even in polyploids

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Marker	Advantages	Disadvantages
ISSR	<ul style="list-style-type: none"> - Highly polymorphic - Robust in usage - Can be automated 	<ul style="list-style-type: none"> - Usually dominant - Species-specific
AFLP	<ul style="list-style-type: none"> - Small DNA quantities required - No sequence information required - Can be automated - Can be adapted for different uses, e.g. cDNA-AFLP 	<ul style="list-style-type: none"> - Evaluation of up to 100 loci - Marker clustering - Dominant - Technique is patented - Can be technically challenging
IRAP/ REMAP	<ul style="list-style-type: none"> - Highly polymorphic depends on the transposon - Robust in usage - Can be automated - Species-specific 	<ul style="list-style-type: none"> - Alleles cannot be detected - Can be technically challenging
<i>Additional marker systems not covered in the course</i>		
Morpho- logical	<ul style="list-style-type: none"> - Usually fast - Usually cheap 	<ul style="list-style-type: none"> - Few in number - Often not compatible with breeding aims - Need to know the genetics
Protein and Isozyme	<ul style="list-style-type: none"> - Fairly cheap - Fairly fast analysis - Protocol for any species - Co-dominant - No sequence information required 	<ul style="list-style-type: none"> - Often rare - Often different protocol for each locus - Labour intensive - Sometimes difficult to interpret
STS/EST	<ul style="list-style-type: none"> - Fast - cDNA sequences - Non-radioactive 	<ul style="list-style-type: none"> - Sequence information required - Substantially decreased levels of polymorphism

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Marker	Advantages	Disadvantages
	<ul style="list-style-type: none"> - Small DNA quantities required - Highly reliable - Usually single-specific - Can be automated 	
SNP	<ul style="list-style-type: none"> - Robust in usage - Polymorphism are identifiable - Different detection methods available - Suitable for high throughput - Can be automated 	<ul style="list-style-type: none"> - Very high development costs - Requires sequence information - Can be technically challenging
SCARS/ CAPS	<ul style="list-style-type: none"> - Co-dominant - Small DNA quantities required - Highly reliable - Usually single locus - Species-specific 	<ul style="list-style-type: none"> - Very labour intensive
Microarray	<ul style="list-style-type: none"> - Single base changes - Highly abundant - Highly polymorphic - Co-dominant - Small DNA quantities required - Highly reliable - Usually single locus - Species-specific - Suitable for high throughput - No gel system - Can be automated 	<ul style="list-style-type: none"> - Very high development and start-up costs - Portability unknown

2. DNA ISOLATION PROTOCOL

There are many DNA isolation methods. The one you use is dependent on your laboratory conditions. The one given below was used in the FAO/IAEA course and produced good quality DNA.

2.1. ISOLATION OF TOTAL DNA FROM LEAF OR OTHER PLANT MATERIAL

The method utilized in the course is suitable for isolation of high molecular weight DNA from leaf or other plant tissue. Grinding the frozen tissue in a mortar using a pestle acts to disrupt the cell walls. The extraction buffer containing the detergent cetyl-trimethylammonium bromide (CTAB) ensures that the DNA is released from the cell nuclei, and EDTA protects the DNA from endogenous nucleases. DNA is separated from proteins by chloroform extraction and is subsequently precipitated by the addition of isopropanol.

2.1.1. Materials Required

In addition to frozen tissue the following is needed. Mortar and pestle, liquid nitrogen, water bath (60°C), centrifuge for 50 ml centrifuge tubes, and a microcentrifuge

2.1.2. Method

Tissue collection

NOTE: Wear gloves, goggles, and lab coat at all times for safety and to prevent contamination.

There are as many ways to collect tissue, as there are laboratories. A suggestion is to collect only young tissue from any part of the plant. The older the tissue, the harder it is to obtain an adequate grinding for good DNA extraction. If you are collecting tissue from plants that are not in the same room as the mortar/pestle (or some form of tissue grinder that can be cooled with liquid nitrogen) and the liquid nitrogen or dry ice, then you need to take care and place the tissue in liquid nitrogen as fast as possible. If the plants are in the glasshouse or the field you should take a Styrofoam container of liquid nitrogen to the plants. You also need to take tissue-collecting containers (e.g. tubes with lids). Once you label these containers and collect the tissue, you

should immediately place them into the liquid nitrogen until you remove the tissue to the mortar for grinding. You might also find it easier to grind the tissue in the mortar if you place a small quantity of acid washed sand in the mortar. The sand helps the grinding of the tissue. Generally a finer grinding will increase your DNA yield from the extraction.

DNA extraction

NOTE: Wear gloves, goggles, and lab coat at all times for safety and to prevent contamination.

- S.*1. Add 25 μ l β -mercaptoethanol to 20ml ml 1.5% CTAB extraction buffer in an 50 ml centrifuge tube and preheat the mixture to 60°C in a water bath.
- S.2. Cool the mortar and pestle with N₂ liquid and add 6-8 g tissue to the mortar. Grind tissue quickly but carefully, to a fine powder (do not let the tissue thaw).

NOTE: If you are using smaller quantities of tissue, adjust the volumes of solutions accordingly.

NOTE: Make sure all the equipment containing tissue is maintained at -20°C using liquid nitrogen or dry ice.

NOTE: If you are using a mortar/pestle, this is also the time to add acid washed sand to help in the grinding.

- S.3. Transfer the frozen powder to the centrifuge tube containing 20 ml pre-heated CTAB extraction buffer, using e.g. a self-made spatula from filter paper dipped into N₂ liquid. Mix well and incubate with gentle agitation at 60°C for 20 minutes.

NOTE: Do not re-use the spatula. A metal one can be used only if it is thoroughly cleaned in ethanol after every use. Care is needed in order to avoid cross-contamination.

- S.4. Let the solution cool down to room temperature, then add 1 volume chloroform:isoamylalcohol. Mix well (but carefully!).
- S.5. Centrifuge for 25 minutes at 3,000 rpm 10°C.

* Step

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- [S.6. Transfer (upper) aqueous phase to a fresh tube, add 2 ml of 10% CTAB at 65°C, and mix.]
- S.7. Repeat chloroform:isoamylalcohol extraction by adding 20 ml chloroform:isoamylalcohol and shake for 20 minutes at room temperature.
- S.8. Centrifuge at 3,000rpm, 10°C for 25 minutes.
- S.9. After the second centrifugation step transfer the upper, supernatant phase to a fresh centrifuge tube, add 2/3 volume isopropanol and mix.

NOTE: After a few minutes you will see the precipitated DNA suspended in the solution. Let the precipitation continue for at least 1 hour.

- S.10. Spool the precipitate onto a glass rod or collect by centrifugation (5,000 rpm, 20 minutes).
- S.11. Drain the liquid carefully and wash the pellet with 70% ethanol (centrifuge 5 minutes at 5,000 rpm).
- S.12. Drain isopropanol and dry the pellet remaining in the bottom of the centrifuge tube (e.g. in a flow bench).
- S.13. Re-suspend pellet in 400 µl TE and transfer the dissolved nucleic acids to a 1.5 ml microcentrifuge tube.
- S.14. Add 4 µl RNase A (10mg/ml) and incubate at 65°C for 1 hour or until all the RNA is removed.
- S.15. Precipitate DNA again by adding 0.1 volume 3M sodium acetate and 0.7 volume isopropanol (leave overnight at 4°C) or 2.5 volume 96% ethanol (leave overnight at -20°C).
- S.16. Centrifuge in microcentrifuge at maximum speed for 15 minutes at 4°C.
- S.17. Wash pellet with 70% ethanol.
- S.18. Drain liquid and air-dry pellet on bench.
- S.19. Re-suspend pellet in 100 µl TE or double distilled H₂O.
- S.20. Store DNA samples at -20°C

2.2. QUANTIFICATION OF NUCLEIC ACIDS

2.2.1. Equipment Required

Spectrophotometer with Hg-lamp for providing UV-light, quartz cuvette.

Although fluorometric assays are available that offer improved sensitivity and are specific for DNA, nucleic acids are routinely quantified spectrophotometrically by measuring the absorbance at 260 nm ($A_{260\text{ nm}}$) - the aromatic rings absorb extremely strongly and rather characteristically with a peak between 255 and 260 nm. Tyrosine and tryptophan confer absorption at 280 nm to proteins. Thus the $A_{260\text{ nm}}: A_{280\text{ nm}}$ ratio of a nucleic acid extract

should exceed 1.5 if it is to be considered protein-free. If the DNA appears to be impure, remove any remaining proteins by phenol/chloroform extraction (see A.1.). Nucleic acid extracts are generally diluted 100 to 500-fold with water before assay.

Extinction coefficients for various nucleic acids yield:

[DNA]	=	$A_{260\text{nm}} \times 50 \times \text{dil. factor}$	$\mu\text{g/ml}$
[RNA]	=	$A_{260\text{nm}} \times 40 \times \text{dil. factor}$	$\mu\text{g/ml}$
[Oligonucleotides]	=	$A_{260\text{nm}} \times 30 \times \text{dil. factor}$	$\mu\text{g/ml}$

If a spectrophotometer is not available, a good estimation of the DNA quantity can be achieved by using agarose gel electrophoresis where the extracted DNA along with a dilution series of a standard DNA (i.e. DNA from phage lambda) is run in an agarose gel. After staining with ethidium bromide the gel is exposed to UV-light. By comparison of the band's intensity with each other, the unknown DNA concentrations can be estimated. However, one needs to be extremely careful to load the same amount of DNA and standards in the lanes. Without this you cannot accurately compare the samples.

A further advantage of this procedure is that DNA integrity can be checked at the same time. High quality DNA should give a sharp, high molecular weight band, whereas sheared or DNase-digested DNA results in no bands. Sheared or DNase-digested DNA should not be used for further analysis, since the fragmentation is only arbitrary.

2.3. SOLUTIONS/CHEMICALS NEEDED

- 1.5% CTAB (1000 ml)
 - CTAB (15.0 g)
 - 1 M Tris (pH 8.0) (75 ml)
 - 0.5 M Na₂EDTA (30 ml)
 - NaCl (61.43 g)
 - H₂O bring to 1000 ml

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- 10% CTAB (1000 ml)
 - CTAB (100.0 g)
 - NaCl (0.7 M) (40.95 g)
 - H₂O bring to 1000 ml
- β-mercaptoethanol
- chloroform;isoamylalcohol (24:1)
- RNase A (DNase free) (10 mg/ml)
 - Boil stock solution in a water bath for at least 10 min to destroy any DNases. It can then be kept frozen until needed
- sodium acetate (3M)
- ethanol (96%, 70%)
- TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0)

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RESTRICTION DIGESTION PROTOCOL 1

3. RESTRICTION DIGESTION PROTOCOL

Restriction enzymes are produced by various bacterial strains. In these bacterial strains they are responsible for limiting attack from certain bacteriophages. They act by cutting ("restricting") the phage DNA at a sequence-specific point, thereby destroying phage activity.

Sequence-specific cutting is a fundamental tool in molecular biology. DNA fragments can be ligated back together ("recombined") by T4 DNA ligase. Many restriction enzymes have been cloned and are available in a commercially pure form. They are named after their bacterial origin: e.g. *EcoRI* from *E. coli*.

The known restriction enzymes recognize four or six bases (eight in the case of "very rare cutters" like *NotI* and *SfiI*). Recognition sequences are almost always "palindromic" where the first half of the sequence is reverse-complementary to the second:

e.g. the *XbaI* site is

5' T C T A G A 3'
3' A G A T C T 5'

The position of the actual cut is enzyme dependent and symmetrical on the opposite strand:

5' T		A 3'
3' A	G A T C	T 5'

leaving cohesive termini (sticky ends) at the 5' end:

5' T 3'
3' A G A T C 5'

The commercially available restriction enzymes are supplied with the appropriate restriction buffers (10 x concentrated). The enzymes are adjusted to a specific activity per μl , usually 10 U/ μl . (1 Unit is the amount of enzyme needed to cut 1 μg of lambda DNA in one hour at 37°C).

A typical restriction digestion is performed using between 20 μl and 100 μl reaction volume per 5 μg and more of plant DNA. For purified plasmid DNA 2 U per μg DNA is sufficient, for plant DNA 4 U per μg should be used.

For example: digestion of 5 μg DNA in 40 μl reaction volume:

Restriction buffer (10x)	4 μl
DNA 1 $\mu\text{g}/\mu\text{l}$	5 μl
Doubled distilled H ₂ O	29 μl
Enzyme (10 U/ μl)	2 μl

Incubate for at least 1 hour at 37°C. The restriction enzyme can be inactivated by heating to 65°C for 10 minutes or by adding 1.0 μl 0.5 M EDTA.

4. RFLP

RFLP definition: The variation(s) in the length of DNA fragments produced by a specific restriction endonuclease from genomic DNAs of two or more individuals of a species (Kahl, 2001).

Restriction fragment length polymorphism (RFLP) technology was first developed in the 1980s for use in human genetic applications and was later applied to plants. By digesting total DNA with specific restriction enzymes, an unlimited number of RFLPs can be generated. RFLPs are relatively small in size and are co-dominant in nature. If two individuals differ by as little as a single nucleotide in the restriction site, the restriction enzyme will cut the DNA of one but not the other. Restriction fragments of different lengths are thus generated. All RFLP markers are analyzed using a common technique. However, the analysis requires a relatively complex technique that is time consuming and expensive. The hybridization results can be visualized by autoradiography (if the probes are radioactively labelled), or using chemiluminescence (if non-radioactive, enzyme-linked methods are used for probe labelling and detection). Any of the visualization techniques will give the same results. The visualization techniques used will depend on the laboratory conditions.

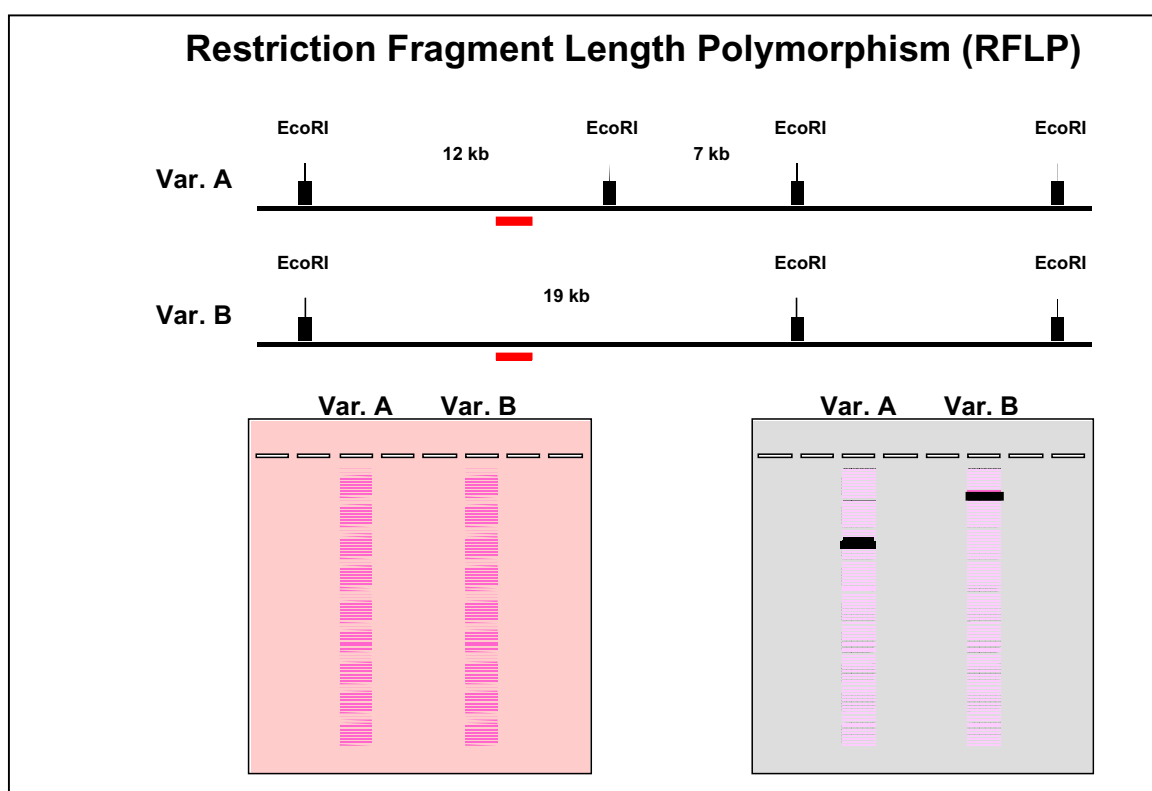


Figure 4.1. The scheme depicts enzyme digestion of DNA into fragments and their subsequent gel separation and the detection of allelic variation in varieties A and B (With permission, K. Devos).

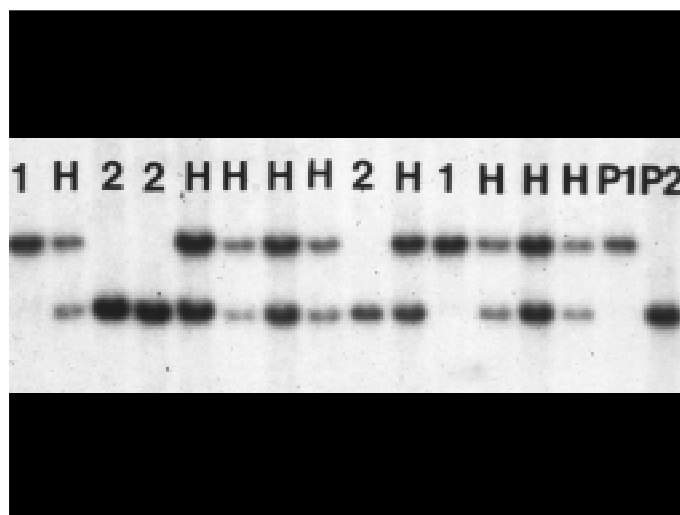


Figure 4.2. An autoradiograph detecting parent (P1 and P2) and homozygous (1 and 2), respectively and heterozygous (H) F₂ segregation (With permission, M.D. Gale).

4.1. PROTOCOL

4.1.1. Agarose Gel Electrophoresis

Agarose is a galactose-based polymer, widely used in analytical and preparative electrophoretic separation of linear nucleic acids in the size range above 100 bp. DNA applied to an agarose gel, which is exposed to an electrical field, migrates towards the anode, since nucleic acids are negatively charged. The smaller the molecules the faster they run through the gel matrix (Figures 4.1, 4.2, and 4.3). Migration is inversely proportional to the log of the fragment length. In order to determine the length of the separated fragments in the gel a molecular weight fragment ladder control is placed in a lane alongside the experimental samples. Restricted genomic DNA is usually separated in a 0.8 – 1.0 % gel whereas gels with a higher concentration of agarose (2 – 3%) are needed for separation of small DNA fragments (<500 bp).

Method: Gel preparation and running

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

The buffer for gel preparation and for filling the electrophoresis tank is 0.5xTBE.

- S*.1. Agarose powder is dissolved in buffer by slowly boiling in a microwave or water bath.
- S.2. Let the agarose cool down to 60°C (just cool enough to hold).
- S.3. Ethidium bromide (EthBr) is added to the gel at a concentration of 0.5 µg/ml before the gel is poured (alternatively the gel can be stained after electrophoresis in water containing EthBr). (*Caution: Ethidium bromide is toxic. Gloves should be worn and avoid inhalation.*)
- S.4. As the agarose is cooling down prepare the gel tray by placing tape across the ends of gel tray such that there is no leakage and so the tray will be able to accommodate the desired thickness of the gel.
- S.5. Pour the agarose-EthBr mixture into the prepared gel tray and insert combs using a comb size depending on the depth, width, and thickness of the desired well. To avoid breaking the wells when the comb is removed, leave 1mm between the comb teeth and the bottom of the gel tray. Allow the gel to solidify (20-30 minutes).
- S.6. Remove tape and place tray in gel rig. Pour enough 0.5x gel buffer into the gel rig to cover the gel, then remove combs.
- S.7. Load the DNA samples, containing the lane marker bromophenol blue dye, into the wells. Load the wells of the gel to the top. It typically takes 30 to 40 µl to fill each well.

NOTE: Do not over load the wells as that would definitely lead to DNA contamination.

NOTE: The DNA is mixed with loading buffer and dye order to facilitate the solution sinking into the gel wells. As a single band, 10 ng DNA can still be visualized with EthBr.

- S.8. Run samples into gel at 100mA for 5-10 minutes, then reduce the amperage and run at 25 mA, constant current, until the bromophenol blue dye marker has migrated almost to the end of the gel. Typically a long gel will be done after 14-16 hours.

NOTE: The following step is used only if the EthBr was not added as in step S.3. Stain each gel in 1 µg/ml EthBr (50 µl of 10 mg/ml EthBr in 500 ml dH₂O) for 20 minutes shaking gently.

* Step

- S.9. Rinse gel in ddH₂O for 20 minutes, slide gel onto a UV transilluminator and photograph. For Fotodyne PCM-10 camera with 20 x 26 cm hood and Type 667 Polaroid film use an f8, 10 second exposure. (*Caution: Wear gloves and lab coat, and UV-protective full face shield or glasses when you are exposed to the UV light of the transilluminator.*)

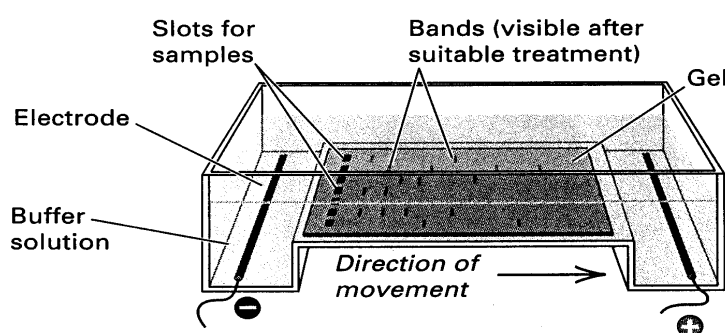


Figure 4.3. Apparatus for gel electrophoresis (Hartl and Jones, 1999).

4.1.2. Southern Blotting and Hybridization

4.1.2.1. Southern blotting

Localization of particular sequences within genomic DNA is usually accomplished by the transfer technique described by Southern (1975) and subsequent hybridization with a labelled probe. Genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a nylon membrane. The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA is hybridized to radioactive or (in our case) non-radioactive labelled DNA probes, and the positions of bands complementary to the probe can be visualized by autoradiography or alternative enzyme-linked detection systems.

Capillary transfer: In the capillary transfer method (Southern 1975), DNA fragments are carried from the gel in a flow of liquid and deposited on the surface of the nylon membrane. The liquid is drawn through the gel by capillary action that is established and maintained by a stack of dry and absorbent paper towels (see Figure 4.4).

Method: Transfer of DNA from agarose gel to a nylon membrane.

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. After taking a photograph of the gel mark the gel for orientation purposes.
- S.2. Soak the gel for 5 minutes in 0.25 M HCl for depurination.
- S.3. Soak gel 2 x 20 minutes in denaturing solution [0.4 M NaOH, 1 M NaCl] with constant, *gentle* agitation. Meanwhile prepare the transfer apparatus (see Figure 4.4).
- S.4. Discard denaturing solution and add 1M ammonium acetate to neutralize the gel (shake for 10 minutes).
- S.5. Wrap a piece of Whatman 3 MM paper around a piece of Plexiglas or a stack of glass plates to form a support that is longer and wider than the gel (an empty box for pipette tips with plain surface is sufficient). Place the wrapped support inside a large baking dish, which is then filled with the transfer buffer (20XSSC).
- S.6. Cut a piece of nylon membrane to the size of the gel along with a similar piece of 3 MM paper (do not touch the membrane, wear gloves and lab coat, and use forceps to handle membrane - otherwise it will result in background signals after detection). Wet both pieces in transfer buffer. Place the gel *face-down* on the wrapped support, and smooth out all bubbles.
- S.7. Place the nylon membrane on top of the gel and smooth out all bubbles. Cut a corner of the membrane according to the orientation cut made on the gel. Mask the surrounding 3 MM paper with Parafilm strips.
- S.8. Place the wet 3 MM piece on top of the membrane, excluding bubbles, followed by a further dry piece and then a stack of paper towels (5-8 cm high). Put a glass plate on top of the stack.
- S.9. Wrap the whole apparatus with clingfilm to reduce evaporation and weigh the stack down with a 500 g weight.
- S.10. Leave overnight for transfer - and sleep well!
- S.11. Remove the paper towels and the 3MM paper from the gel. Peel the membrane off and soak it for 5 minutes in 2XSSC to remove any pieces of agarose sticking to the filter.
- S.12. Dry the membrane on 3 MM paper for 30 minutes.
- S.13. Then fix the DNA by baking the filter (refer to manual of the nylon membrane which is used, e.g. the positively charged nylon membrane from Roche is baked for 30 minutes at 120°C).
- S.14. Proceed with hybridization of probe.

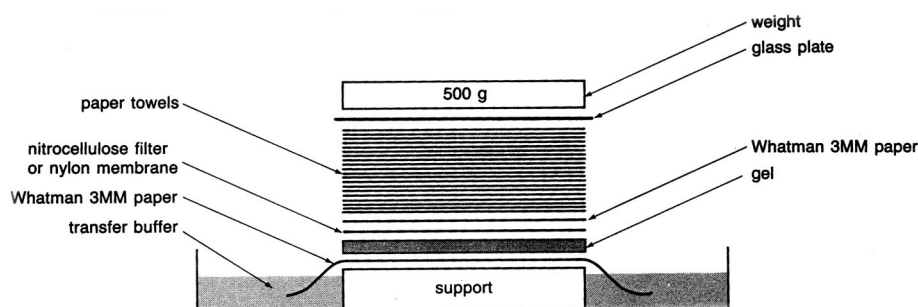


Figure 4.4. Blotting apparatus for capillary transfer of DNA (Sambrook *et al.*, 1989).

4.1.2.2. DNA:DNA hybridization using the DIG system

NOTE: The hybridization protocol used in the FAO/IAEA course was that obtained in the “Random Prime Labelling and Detection System” (RPN 3040/3041) commercially available from Amersham LIFE SCIENCE. This is a very good labelling and detection kit that comes with a step-by-step procedure. However, if you cannot obtain the Amersham kit try the following protocol, which also works well.

Most of the *non-radioactive* labelling and detection systems for nucleic acids are based on the incorporation of a nucleotide, which is linked to a haptene molecule, into the hybridization probe. The identification of the haptene molecule at the hybridization sites is facilitated by an immunological detection reaction.

In the case of the Digoxigenin system (DIG-system, Roche) the haptene is digoxigenin, a steroid exclusively occurring in the plant *Digitalis purpurea*. The molecule is linked to desoxyuracilphosphate by an 11 atoms linear spacer (Dig-[11]-dUTP), (Figure 4.5).

The DNA: DNA hybridization sites are detected by using antibodies against digoxigenin, which are conjugated to alkaline phosphatase (AP) as a reporter enzyme. By adding the colourimetric substrate NBT/X-phosphate or alternatively the chemiluminescence substrate AMPPD (CSPD[®]) the presence of the enzyme is visualized (Figure 4.6).

The main advantage of the non-radioactive system is the avoidance of radioisotopes and the associated hazards, as well as saving high costs for maintaining an isotope laboratory (e.g. for disposal of the radioactive waste). Furthermore, DIG labelled probes are much more stable. They can be stored at -20°C for more than 12 months, and the hybridization solution can be re-used several times. At the same time the sensitivity of the DIG system is comparable to that of ^{32}P labelled probes.

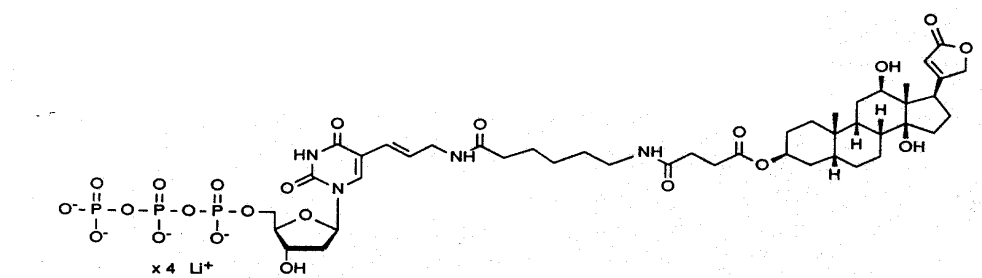


Figure 4.5. Structure of the Dig-[11]-dUTP molecule (source: DIG DNA Labelling and Detection Kit).

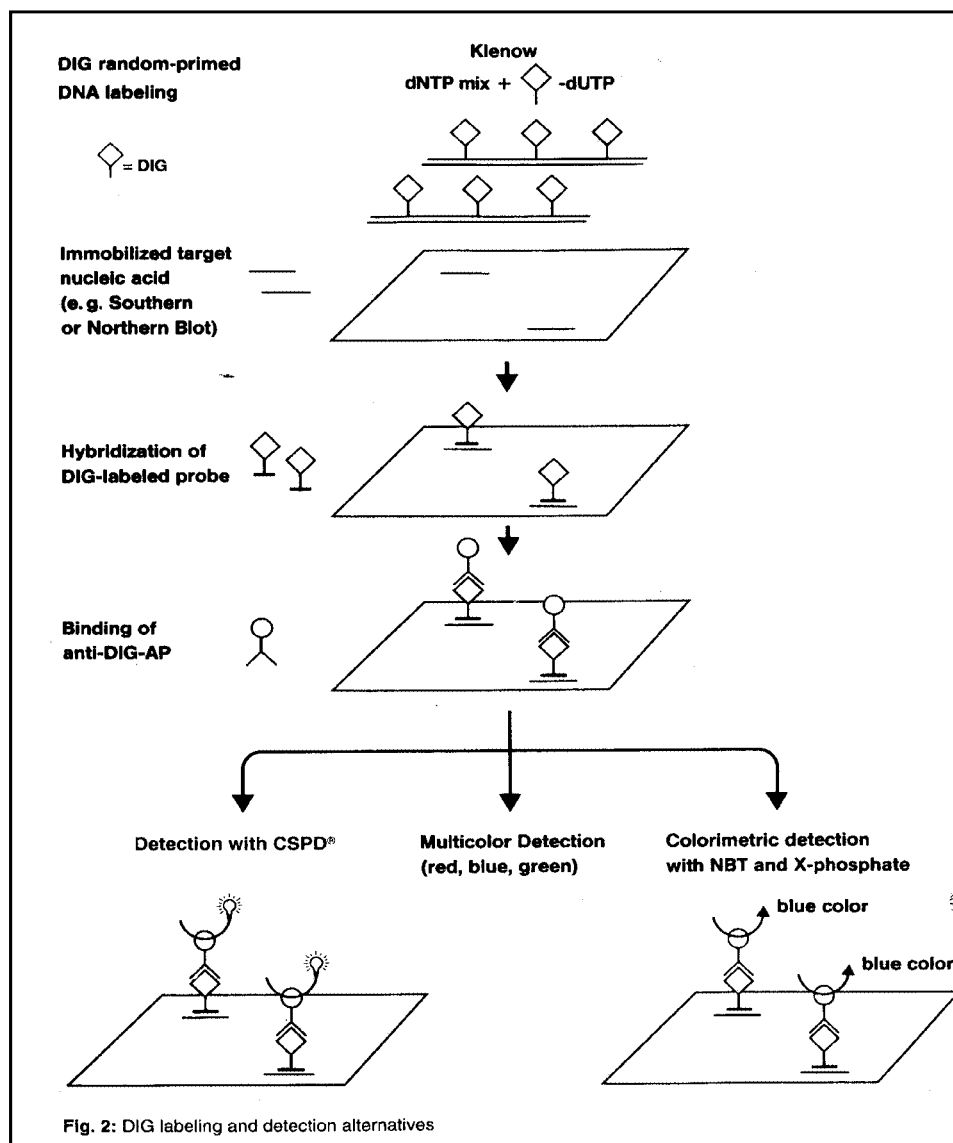


Figure 4.6. DIG labelling and detection alternatives (source: DIG DNA Labelling and Detection Kit).

4.1.2.3. Labelling the probe [random primed labelling-method after Feinberg and Vogelstein (1983) and dot blot/quantification]

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. Dilute template DNA (0.5 µg - 3 µg) to a total volume of 15 µl and denature by heating for 10 minutes in a boiling waterbath, then quickly chill on ice/NaCl.
- S.2. Add on ice: 2 µl hexanucleotide-mixture, 2 µl dNTP mixture (containing Dig-[11]-dUTP), and 1 µl Klenow enzyme (DNA polymerase).

- S.3. Mix, centrifuge briefly, and then incubate for at least 60 minutes (20 hours is better) at 37°C.
- S.4. Add 2 µl 0.2 M EDTA, pH 8.0 to stop the reaction.
- S.5. Precipitate the labelled DNA by adding 2.5 µl 4M LiCl and 75 µl pre-chilled ethanol. Mix well and leave for 2 h at -20°C.
- S.6. Spin in a microcentrifuge for 15 minutes. Wash the pellet with 50 µl cold ethanol, 70%.
- S.7. Dry the DNA pellet and dissolve in 50 µl TE-buffer.
- S.8. Dot Blot/Quantification of labelling efficiency

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

It is absolutely necessary to estimate the yield of DIG-labelled probe. If the probe concentration in the hybridization solution is too high, large background signals will appear on the blot after detection. Therefore the kit contains a DIG-labelled control DNA of known concentration. A dot blot with a dilution series of your probe and the provided control DNA makes the test. If the amount of template DNA was about 1,000 ng you can expect between 260 ng (after 1 hour incubation with Klenow enzyme) up to 780 ng (after 20 hours) of newly synthesized DIG-DNA.

- S.8.1. Cut a piece of nylon membrane and label 1 cm² squares with a soft pencil.

NOTE: Do not use an ink or ballpoint pen.

- S.8.2. Apply 1 µl of the probe dilution series (1:10, 1:100, 1:1,000) and of the control-DNA dilution series to each square on the membrane. To prepare the dilution series of the control DNA follow the scheme proposed in the kit manual (see below).
- S.8.3. Fix the DNA to the membrane by cross-linking with UV-light or baking (dependent on the type of nylon membrane used).
- S.8.4. After the spots are dry continue with the detection procedure. The colourimetric assay is the method of choice, because you can easily follow the development of the colour on the membrane.
- S.8.5. Stop the reaction as long you can see differences between the concentrations of the calibration series. For detection procedure see below (4.1.2.6).

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RFLP 10

DIG-labelled control DNA, diluted 1:5; starting concentration (1µg/ml)	Stepwise dilution in DNA dilution buffer	Final concentration (pg/µl)	Total dilution
(A) 1ng/µl	5µl/45µl	100	1:10
(B) 100pg/µl	5µl/45µl	10	1:100
(C) 10pg/µl	5µl/45µl	1	1:1,000
(D) 1pg/µl	5µl/45µl	0.1	1:10,000
(E) 0.1pg/µl	5µl/45µl	0.01	1:100,000

4.1.2.4. Hybridization (start early in the morning, unless you want to stay up all night)

Pre-hybridization:

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. The nylon membrane is inserted into a heat resistant polythene bag.
- S.2. The hybridization solution (without the probe!) is added (20 ml per 100 cm² membrane).
- S.3. Before heat-sealing the bag, air bubbles are removed by rolling a pipette over the bag, which should be placed on a sloping plane.
- S.4. Allow the sealed bag to gently shake in the water bath at 42°C for at least 1 hour.

Hybridization:

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. The pre-hybridization solution can be exchanged with the hybridization solution containing the probe (2.5 ml per 100 cm² membrane), or as an alternative, you could add the probe to the hybridization mixture directly into the bag.

- S.2. The DIG-labelled probe has to be denatured as before (see Section 4.1.2.3 Step S.1) and is subsequently added to the hybridization solution at a concentration of 40 ng/ml (for probe concentration see results of dot blot test (see Section 4.1.2.3 Step S.8.5).
- S.3. Carefully remove all air bubbles from the bag before you heat-seal it.
- S.4. Let the hybridization proceed over night (at least 14 hours) in the water bath at 42°C (with formamide-containing hybridization solution) or 68°C (without formamide) with gentle agitation. [*Caution: Formamide is harmful. Gloves should be worn.*]

NOTE: After hybridization the solution is collected at one corner of the bag by rolling a pipette over it and transferred to a reaction tube for re-use.

- S.5. The hybridization solution containing the Dig-labelled probe is stored at -20°C and can be re-used several times.

NOTE: It has to be denatured before each new application.

4.1.2.5. Washing method

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

During the washing procedure the remaining probe is diluted and washed from the membrane. In a second washing step the probe DNA, which binds unspecifically to the DNA on the blot, is removed.

It is useful to know that the stability of DNA:DNA hybrids is dependent on certain factors, such as the melting temperature (T_m) at which the probe is annealed to 50% of its exact complement. The factors influencing the T_m are included in the formula of Meinkoth and Wahl (1984):

$$T_m = 81.5^\circ\text{C} + 16.6 \log M + 0.41 (\% \text{ G} + \text{C}) - 500/n - 0.61 (\% \text{ formamide})$$
where M is the concentration (mol l^{-1}) of monovalent cations in the hybridization solution/washing solution, ($\% \text{ G} + \text{C}$) the proportion of guanine and cytosine in the probe, and n the length of the probe in base pairs.

The melting temperature T_m together with the selected hybridization and washing temperature T_a determine the conditions for annealing between probe and target DNA. This is called the stringency:

$$\text{stringency (\%)} = 100 - Mf(Tm - Ta)$$

where Mf is the "mismatch factor" (1 for probes longer than 150 bp).

Under hybridisation/washing conditions with a stringency of 100%, all DNA:DNA hybrids with less than 100% homology are resolved.

In general one can say, the lower the salt concentration in the washing solution and the higher the hybridization or washing temperature, the higher the stringency.

- S.1. The hybridization bag is opened.
- S.2. The membrane is transferred to a plastic dish. It is very important that the plastic dish has been thoroughly cleaned. Use 500ml of each solution per 100cm² membrane.

1st wash: 2 x SSC, 0.1% SDS (w/v) - 2 x 15 minutes at room temp.

2nd wash (new dish): 0.1XSSC*, 0.1% SDS (w/v) - 2 x 15 minutes at 68 °C.

NOTE: *These conditions are highly stringent. The SSC concentration in the second (stringent) wash should be increased when a probe of lower G/C content (e.g. some repetitive sequences) is used, or when you are working with heterologous probes.

- S.3. The membrane is heat-sealed in a new plastic bag for subsequent detection.

4.1.2.6. Detection

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. Wash the membrane briefly with maleic acid buffer (buffer I) to remove any residues of SDS.

NOTE: To avoid unspecific binding of the antibodies, incubate the membrane for at least 60 minutes in maleic acid/1% (w/v) blocking reagent (buffer II), (1 ml/cm²) on a shaker before adding antibody solution.

- S.2. Dilute the antibody stock solution (750 U/ml) with buffer II to 75 mU/ml (1:10,000), (0.2 ml/cm²).
- S.3. Centrifuge the antibody stock solution before adding to the membrane in order to separate any precipitates, which can lead to background spots on the filter.
- S.4. Discard buffer II and add the diluted antibody solution to the membrane.
- S.5. Remove bubbles before sealing the bag.
- S.6. Incubate for 30 minutes (no longer) at room temperature on a shaker.
- S.7. Open the bag, remove buffer, and transfer membrane to a thoroughly cleaned dish with 5 ml/cm² wash buffer (buffer I plus 0.3% (v/v) Tween[®]20).
- S.8. Wash 3 x 15 minutes with gentle agitation at room temperature.
- S.9. Transfer membrane to a clean dish with alkaline buffer (buffer III) to activate the reporter enzyme alkaline phosphatase.

NOTE: The following detection methods are independent of the method utilized in the FAO/IAEA course, and might provide useful alternatives.

Colorimetric detection:

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

The dye solution (10 ml/100 cm²) is prepared by addition of 45 µl NBT-solution and 35 µl BCIP-solution to 10 ml buffer III. The incubation takes place in the dark for up to 20 hours. Avoid any shaking since this will cause a diffuse signal. The reaction can be stopped by washing the filter in TE buffer as soon the desired bands are visible.

Chemiluminescent detection:

The chemiluminescence substrate AMPPD (or CSPD[®]) emits light after a two-step reaction. At first the molecule is de-phosphorylated by the enzyme alkaline phosphatase (AP) and in the second step the molecule decomposes and emits light. The emitted light appears as a continuous glow for more than 24 hours, and it can be documented on X-ray films. The advantages of chemiluminescence are remarkably improved sensitivity, the possibility to test different exposure times, and the facilitation of rehybridization experiments.

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

- S.1. Dilute the CSPD[®] solution in buffer III to a final concentration of 0.235 M (1:100), (1.5 ml/100 cm²).
- S.2. Place membrane on a clean transparent sheet and pipette the diluted CSPD[®] solution onto the membrane. Cover the membrane slowly with another transparent sheet to produce a uniform layer of liquid. Incubate for 5 minutes.
- S.3. Place the membrane on 3 MM paper until the liquid is evaporated from the surface (do not let the membrane dry).
- S.4. Seal the damp membrane in clingfilm and incubate for 15 minutes at 37°C.
- S.5. Expose an X-ray film to the "glowing" membrane in the dark. The exposure times needed for genomic Southern blots are between 30 minutes and 14 hours.

4.1.2.7. Membrane rehybridization method

- S.1. For repeated hybridization of a membrane previously detected by chemiluminescence, wash it in sterile H₂O for 5 minutes.
- S.2. Follow this by a 2 x 15 minutes incubation in 0.2 N NaOH, 0.1% SDS at 37°C in order to remove the bound Dig-labelled probe. After final washing in 2XSSC the filter is ready for new pre-hybridization.

4.2. REFERENCES

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- Southern, E. M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J.Mol.Biol.* **98**: 503

4.3. SOLUTIONS/CHEMICALS NEEDED

- Use only sterile-distilled water for all solutions
- 0.25M HCl. Concentrated HCl (37% (w/v) is 10 M, or 40x.
- NaCl
- Sodium citrate
- (5x) TBE per liter

TRIS base	54 g
Boric acid	27.5 g
EDTA 0.5 M	20 ml
- EDTA 0.2M
- LiCl 4M
- Ethidium bromide (EthBr)
- Antibody stock solution (750 U/ml) (Anti-Digoxigenin – alkaline phosphate) (provided in the Dig-Kit, Roche)
- Ammonium acetate 1M
- 70% Ethanol
- TE-buffer (10 mM Tris, 1mM EDTA, pH 8.0)
- CSPD solution in buffer III (alkaline buffer) (provided in the Dig-Kit, Roche)
- 0.2 N NaOH
- Maleic Acid
- Tween[®]20
- Alkaline phosphatase (AP) (provided in the Dig-Kit, Roche)
- NBT/X-phosphate (provided in the Dig-Kit, Roche)
- AMPPD (resp. CSPD[®]) (provided in the Dig-Kit, Roche)
- Hexanucleotide-mixture (provided in the Dig-Kit, Roche)
- dNTP mixture (containing Dig-[11]-dUTP) (provided in the Dig-Kit, Roche)
- Klenow enzyme (DNA polymerase) (provided in the Dig-Kit, Roche)
- Bromophenol blue Dye solution

45 µl NBT-solution
35 µl BCIP-solution
10 ml buffer III
- NBT solution

(75 mg/ml)*(BRL#95540) *Dissolved in dimethylformamide, TOXIC

- BCIP solution

(50 mg/ml)*(BRL#95541) *Dissolved in dimethylformamide, TOXIC

- Denaturing solution
 - 0.4 M NaOH
 - 1M NaCl
- Loading buffer (x10) per ml
 - Glycerol (80%) 600 µl
 - Xylene cyanol 2.5 mg
 - Bromophenol blue 2.5 mg
 - H₂O 400 µl
- Hybridization pre-hybridization solutions (100 ml)
 - 50% (v/v) Formamide (50 ml)
 - 5% (w/v) Blocking reagent (5g)
 - 5x SSC (pH 7.0) (25 ml 20xSSC)
 - 0.1% N-Lauroyl sarcosine (1 ml of 10% stock)
 - 0.02% (w/v) SDS (0.2 ml of 10% stock)
- Buffer I (Maleic acid buffer MAB)
 - 0.1 M Maleic acid (11.61 g/l)
 - 0.15 M NaCl (8.76 g/l)
 - pH 7.5
 - Autoclave
- Buffer II
 - Maleic acid/1% w/v)
 - Blocking reagent (provided in the Dig-Kit, Roche)

NOTE: It is advisable to prepare a 10 x concentrated stock solution of blocking reagent. Therefore, weigh 10 g of blocking reagent into an autoclavable flask, fill it up to ca. 90 ml with buffer I, and heat it in an 80°C water bath to dissolve the blocking reagent (needs about 1 hour). The last particles can be dissolved by briefly boiling in a microwave. Autoclave the solution!

- Wash Buffer
 - MAB + 0.3% (v/v) Tween[®]20
- Buffer III
 - 0.1 M TRIS-HCl (12.11 g/l)
 - 0.1. M NaCl (5.84 g/l)
 - pH 9.5
 - Autoclave

- 20% SDS

Dissolve 200 g sodium dodecylsulfate in ddH₂O to final volume of 1 litre.

You can use a low grade (Sigma #L5750) for hybridization washes, etc. and a better grade (Sigma #L4390) for hybridization solution, plasmid preps, stop solutions, etc.

- 20XSSC

NaCl	175.3 g
------	---------

Na-Citrate • 2 H ₂ O	88.2 g
---------------------------------	--------

Adjust pH to 7.4 with 1 N HCl

Add H ₂ O to	1 litre
-------------------------	---------

5. SSR

SSR (Microsatellite) definition: Any one of a series of very short (2-10 bp), middle repetitive, tandemly arranged, highly variable (hypervariable) DNA sequences dispersed throughout fungal, plant, animal and human genomes (Kahl, 2001).

Simple sequence repeats (SSR) or microsatellites are a class of repetitive DNA elements (Tautz and Rentz, 1984; Tautz, 1989). The di-, tri- or tetra-nucleotide repeats are arranged in tandem arrays consisting of 5 – 50 copies, such as (AT)₂₉, (CAC)₁₆ or (GACA)₃₂. SSRs are abundant in plants, occurring on average every 6-7 kb (Cardle *et al.*, 2000). These repeat motifs are flanked by conserved nucleotide sequences from which forward and reverse primers can be designed to PCR-amplify the DNA section containing the SSR. SSR alleles, amplified products of variable length, can be separated by gel electrophoresis and visualised by silver-staining, autoradiography (if primers are radioactively labelled) or via automation (if primers are fluorescently labelled) (Figures 5.1 and 5.2). SSR analysis is amenable to automation and multiplexing (Figure 5.2), and allows genotyping to be performed on large numbers of lines, and multiple loci to be analysed simultaneously. SSRs can be identified by searching among DNA databases (e.g. EMBL and Genbank), or alternatively small insert (200-600bp) genomic DNA libraries can be produced and enriched for particular repeats (Powell *et al.*, 1996). From the sequence data, primer pairs (of about 20 bp each) can be designed (software programmes are available for this).

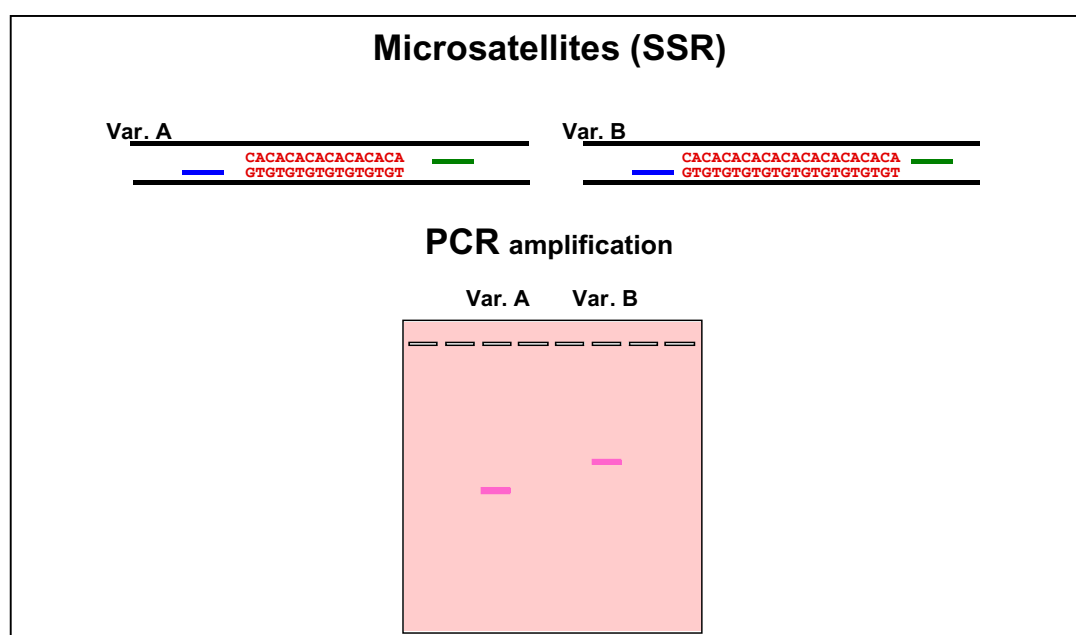


Figure 5.1. The schematic above shows how SSR variation (short A and long B) can be detected using gel electrophoresis after PCR with forward (blue) and reverse primers (green) (With permission, K. Devos).

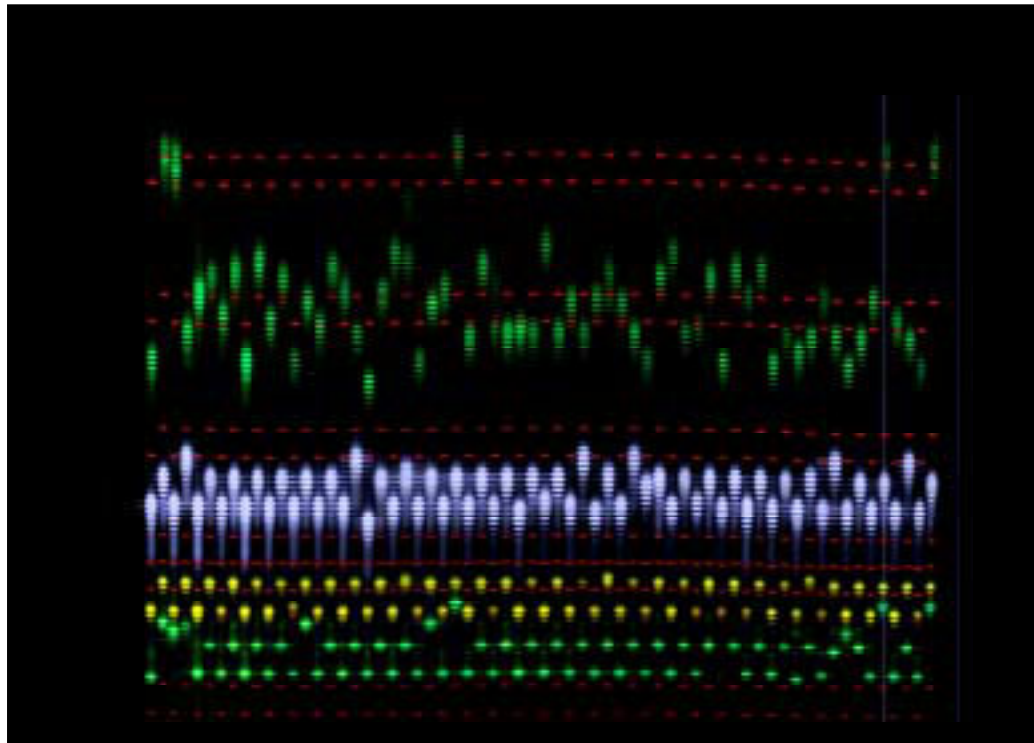


Figure 5.2. A computer image showing an example of SSR multiplexing with different colours (With permission, J. Kirby and P. Stephenson).

5.1. PROTOCOL

PCR reaction mix

Microsatellite primers are specific for each individual genome or species. It is essential to know that the primer pairs chosen will work for your given species. The example used in the FAO/IAEA course utilized four unlabelled primer pairs, two from wheat and two from pearl millet.

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

5.1.1. Prepare 30 µl Reaction Mix

S*.1. Take four sterile PCR tubes and add to each:

10 x <i>Taq</i> buffer	3 µl
dNTPs (2 mM)	2 µl
Forward primer (2 µM)	3 µL
Reverse primer (2 µM)	3 µL
<i>Taq</i> DNA polymerase (5U/µl)	0.3 µl
DNA (10 or 20ng/µl)	5 µl

*Add sterile distilled water up to 30 µl

S.2. Mix by gently tapping against the tube.

S.3. Centrifuge briefly (~14,000 rpm for 5 seconds).

NOTE: Keep all reagents and reaction mix on ice until used.

5.1.2. PCR Amplification

Place tubes in a PCR machine and amplify using a programme designed for the primers being used; an example is given below:

<i>Step 1</i>	Initial denaturing	94°C	2 minutes
<i>Step 2</i>	Denaturing	94°C	1 minute
<i>Step 3</i>	Ramp to 61°C at rate of 0.5°C per second		
<i>Step 4</i>	Annealing*	61°C	1 minute
<i>Step 5</i>	Ramp to 72°C at rate of 0.5°C per second		
<i>Step 6</i>	Extension	72°C	1 minute
<i>Step 7</i>	Cycling	repeat steps 2-6 for 30 cycles	
<i>Step 8</i>	Final extension	72°C	4 minutes
<i>Step 9</i>	Hold	4°C	

*NOTE: The annealing temperature (*Step 4*), in particular, can and does vary with primers used. Please note this when changing primers.

* Step

5.1.3. Separation of the Amplification Products in Agarose Gel

S.1. Split the contents of each tube in half (put 15 µl into a fresh tube).

NOTE: Where SSR polymorphism is large, bands can be separated in agarose gels, however small base-pair differences among alleles requires separation in polyacrylamide gels.

S.2. Add 3 µl 5 x loading buffer containing dye to first half of PCR-product.

S.3. Vortex briefly

S.4. Centrifuge briefly (14,000 rpm for 5 seconds).

S.5. Load 8 µl into a 1.2 % agarose gel.

S.6. Run gel until dark blue colour marker has run two thirds of the gel.

NOTE: Do not run the dye off the gel or you will also lose your DNA samples.

NOTE: See Section 4.1.1 of RFLP Protocol (Agarose gel electrophoresis) for details of gel preparation and running.

S.7. Stain gel with ethidium bromide (*Caution: ethidium bromide is toxic wear gloves and lab coat and avoid inhalation*).

S.8. Visualise under UV light (*Caution: wear gloves, and UV protective glasses or a shield over your face when you are exposed to the UV light of the transilluminator*).

5.1.4. Preparation of Denaturing 6% Polyacrylamide Gels

NOTE: Denaturing the samples produces single-stranded DNA, which is used for detection in polyacrylamide gels (see below). Single-stranded detection is preferred as it results in a greater clarity in band separation for detection.

S.1. Wear gloves and lab coat, and clean the bigger plate of the gel rig using Alconox and warm water. Rinse and dry the plate, clean the upper surface only with 100% ethanol. Apply Repelcote to the upper surface of the plate and spread evenly using blue roll, then wipe with more ethanol, and dry.

NOTE: Change gloves between working with bind silane and Repelcote!

NOTE: Clean everything following use, and dispose of materials carefully according to the regulations of your organization.

- S.2. Clean the smaller plate using Alconox and water (you may also need to use a razor blade to remove old bits of gel that have stuck). Rinse and dry the plate, clean the upper surface only with 100% ethanol. Apply 30 µl bind silane to the upper surface of the plate and spread evenly using blue roll, then wipe with water, and dry. Rinse twice with ethanol.
- S.3. Ensure the spacers are clean and dry. Align and sandwich between plates. Secure plates at base with clips.
- S.4. Double glove and prepare 1 litre of gel solution by adding together:
- 150 ml acrylamide/bis solution 19:1 (40%, *Caution: acrylamide is toxic*)
100 ml 10xTBE
480 g urea 8 M
Make up to 1 litre with distilled water.

NOTE: An alternative option is to use SequaGel[®]XR., which gives sharper bands (National Diagnostics, Inc.)

- S.5. Filter and keep at 4°C.
- S.6. Take 60 ml gel mix, add 30 µl TEMED (*Caution: TEMED is corrosive*) and 300 µl 10% fresh ammonium persulphate solution (*Caution: ammonium persulphate, APS, is harmful*). Mix and pour gel. Secure the plates with clips down each side.

NOTE: Gel will start to polymerize after adding APS, be prepared to move quickly.

- S.7. Insert clean dry comb, straight edge first, so that teeth lie flush to edge of large glass plate and secure with four more clips.
- S.8. Leave to polymerise for approximately 1 hour.

NOTE: Make up the developer for silver-staining while the gel is polymerising, see section 5.1.6 below

5.1.5. Polyacrylamide Gel Running Conditions

- S.1. Remove the comb from the gel and clean the well space using distilled water. Replace comb carefully, teeth first this time.

NOTE: You can only replace the comb once, so be very careful!

- S.2. Take gel and 1 litre of 1xTBE (buffer) to vertical rig. Ensure power supply is disconnected. Close the drainage screw on the side of the rig. Position the gel in the rig and secure by tightening clamps.
- S.3. Pour 500 ml 1xTBE into the top reservoir. Pour 500 ml 1xTBE into lower reservoir.
- S.4. Connect rig to power supply, switch on and pre-run at 80 watts (power limiting) for 30 minutes.
- S.5. Prepare samples by adding 15 µl of formamide dye mix to 15 µl of your PCR reaction (second half). Denature the samples for 3 minutes and place on ice (*Caution: formamide is harmful*).
- S.6. Load 1 kb marker ladder: 10 µl 1 kb ladder (50 ng/µl) + 6 µl formamide loading buffer; load 5 µl into first lane (and at convenient intervals across the gel).
- S.7. Load 5 µl of each sample into individual wells of the gel.
- S.8. Run gel for approximately 1 hour and 20 minutes at 80 watts (power limiting) or until just before the dark blue runs off the bottom of the gel. You will need to quantify the best time for your particular PCR products.

NOTE: Do not run the dye off the gel or you will also run your sample off the gel and lose it.

5.1.6. Silver-staining

NOTE: Dispose of all solutions with due care and according to the regulations of your organization.

- S.1. While the gel is polymerising, make up the developer: Dissolve 60 g sodium carbonate in 2 litres of distilled water and put at 4 °C (*Caution: sodium carbonate is toxic, gloves should be worn*).
- S.2. While the gel is running, make up the fixer (10 % acetic acid): Add 200 ml glacial acetic acid to 1.8 liters distilled water (*Caution: acetic acid is corrosive, gloves should worn*).
- S.3. Make up the silver-stain (*toxic, wear gloves*): Add 12 ml 1N silver nitrate solution in 2 litres of distilled water (*Caution: silver nitrate is corrosive, gloves should be worn*). Then add 3 ml formaldehyde (40% solution) and mix (*Caution: formaldehyde solution is toxic, Wear gloves and lab coat, and avoid inhalation*).
- S.4. Remove the gel from the rig and separate the plates. Place the gel in a tray with the fixer and leave shaking in a fume hood for 30 minutes.

NOTE: Do not pour solutions directly onto the gel as it may come off the plate!

- S.5. Pour off fixer (save) and wash gel once in water. Place on shaker for approx. 10-15 minutes or until "greasiness" has gone from the gel. Rinse again and tip water off before adding silver-stain and leave shaking for 30 minutes.

NOTE: Silver stain can be re-used up to 10 times

NOTE: The next few moves have to be followed quickly and carefully so make sure you have everything set up and ready.

- S.6. Remove gel from the silver-stain and rest it on a tray containing water (do not put it in the water yet). Dispose of spent stain according to the regulations of your organization. Rinse the box that contained the silver-stain with water.
- S.7. Immediately prior to developing the gel add 300 µl of sodium thiosulphate solution (0.1 N) and 3 ml formaldehyde (40% solution) to the pre-chilled developer (*Caution: formaldehyde solution is toxic, wear gloves and lab coat, and avoid inhalation*). Tip the developer into the box that contained the silver-stain.
- S.8. Set a timer for 10 seconds. Start the timer and quickly lower the gel into the water. Agitate several times to remove all excess silver-stain. When 10 seconds is up quickly drain the gel and place it in the developing solution.
- S.9. Agitate the gel in developer solution and, use a piece of white paper placed behind the gel to check progress of the band development. Keep an eye on the gel as it develops. Stop the reaction when bands start to appear near the bottom of the gel (i.e.: 70 bp marker on the 1 kb ladder) by adding the 2 litres of fixer saved from earlier. Agitate until bubbling ceases. Rinse gel in water for 20 minutes and leave to dry standing vertically.

NOTE: Do not run the bands off of the bottom of the gel.

- S.10. Gels can be recorded/documented using Kodak duplicating film.

S.10.1. Place glass plate upside down on the film.

S.10.2. Expose to room light for 15-17 seconds (depending on the room light intensity).

NOTE: The longer the light exposure, the brighter the film gets following development.

5.2. REFERENCES

- Cardle, L., L Ramsay, D. Milbourne, M. Macaulay, D. Marshall, and R. Waugh, 2000. Computational and experimental characterisation of physically clustered simple sequence repeats in plants. *Genetics*. **156**: 847-854
- Kahl, G., 2001. *The Dictionary of Gene Technology*. Wiley-VCH, Weinheim.
- Powell, W., G. C. Machray, and J. Provan, 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Sci.* **1**(7): 215-222
- Tautz, D., 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* **17**: 6463-6471
- Tautz, D. and M. Rentz, 1984. Simple sequences are ubiquitous repetitive components of eucariotic genomes. *Nature*. **322**: 652-656

5.3. SOLUTIONS/CHEMICALS NEEDED

- Use only sterile distilled water for all solutions.
- *Taq* buffer
- dNTPs
- Alconox
- Repelcote
- Sterile distilled water
- Primers
- *Taq* DNA polymerase (5U/μl)
- DNA (10-20ng/μl)
- 10 x loading buffer

Glycerol (80%)	600 μl
Xylene cyanol	2.5 mg
Bromophenol blue	2.5 mg
Distilled water	400 μl
- 5 x loading buffer

Glycerol (80%)	300 μl
Xylene cyanol	1.3 mg
Bromophenol blue	1.3 mg
Distilled water	400 μl
- Ethidium bromide
- Agarose
- Acrylamide
- Bis-acrylamide
- TEMED
- Ammonium persulphate
- TBE

H ₂ O	~800 ml
Tris base	108 g
Boric acid	55 g
EDTA	9.3 g
ddH ₂ O	Adjust volume to 1 litre
- 100% ethanol
- Bind silane
- Sodium carbonate
- Glacial acetic acid

- Formamide dye mix (for 1 ml)

Formamide (deionized)	950 µl
dd H ₂ O	30 µl
EDTA (0.5 M)	20 µl
Bromophenol blue	1 mg
Xylene cyanol	1 mg
Mix and store at –20°C	

6. ISSR

ISSR amplification definition: A variant of the polymerase chain reaction that uses simple sequence repeat primers (e.g. $[AC]_n$) to amplify regions between their target sequences (Kahl, 2001).

Inter-SSR (ISSR) amplification is an example (one of many) of a PCR-based fingerprinting technique. The technique exploits the abundant and random distribution of SSRs in plant genomes by amplifying DNA sequences between closely linked SSRs (Figure 6.1). The method used in the FAO/IAEA course used 3'-anchored primers to amplify regions between two SSRs with compatible priming sites (Yang *et al.*, 1996). More complex banding patterns can be achieved using 5'-anchored primers that incorporate the SSR regions in their amplification products, and by combining 3'- and 5'- primers (Zietkiewicz *et al.*, 1994).

Other methods of fingerprinting using primers complementary to SSR motifs involve using SSR specific primers in combination with an arbitrary primer (Davila *et al.*, 1999), or in combination with primers that target other abundant DNA sequences such as retrotransposons (Provan *et al.*, 1999).

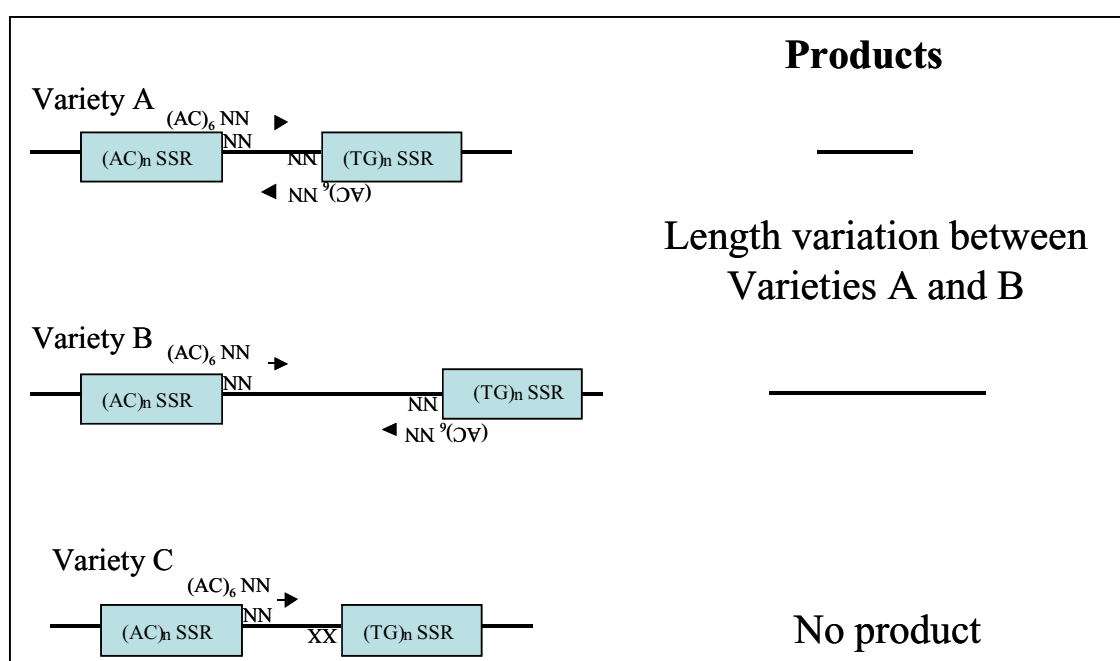


Figure 6.1. The above scheme shows how sequence variation between two SSRs results in variation in PCR products in varieties A, B and C. The figure shows variation at only one ISSR locus, amplification of all compatible ISSR loci among the genomes of a range of varieties will result in complex, fingerprinting, banding patterns.

6.1. PROTOCOL

In the example below, one of three primers given in the ISSR protocol of Yang *et al.*, (1996) is used; this produces a relatively simple fingerprint (small number of bands). In more recent applications two or more primers have been used to produce more complex banding profiles (similar to AFLP profiles).

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

6.1.1. Prepare 25 µl Reaction Mix

S*.1. Take one PCR tube and add:

10x PCR buffer	2.5 µl
Primer (100 µM)	2.5 µl
dNTPs (2mM)	2.5 µl
DNA (10-20ng/ µl)	1-2.5 µl
<i>Taq</i> DNA polymerase (5 U/ µl)	0.2 µl
Add sterile distilled water to bring volume to 25 µl	

Primers used in the FAO/IAEA course (from Yang et al.1996):

5'-CACACACACACA(G/A) (C/T)-3'

5'-CACACACACACA(G/A)G-3'

5'-AGCAGCAGCAGC(C/T)-3'

S.2. Mix by tapping bottom of tube.

S.3. Centrifuge briefly (14,000 rpm for 5 seconds)

NOTE: Keep all reagents and reaction mix on ice.

6.1.2. PCR Amplification

Place tube in a PCR machine and amplify using a programme designed for the primer(s). In this example the following programme can be used:

* Step

FAO/IAEA Interregional Training Course on Mutant Germplasm Characterization

ISSR 3

<i>Step 1</i>	Initial denaturing	94 °C	7 minutes
<i>Step 2</i>	Denaturing	94 °C	30 seconds
<i>Step 3</i>	Annealing	45 °C	45 seconds
<i>Step 4</i>	Extension	72 °C	2 minutes
<i>Step 5</i>	Cycling	repeat steps 2-4 for 32 cycles	
<i>Step 6</i>	Final extension	72 °C	7 minutes

6.1.3. Separation and Visualization of the Amplification Products

- S.1. Add 1 µl of 5x loading buffer to 6 µl of PCR sample.
- S.2. Vortex briefly.
- S.3. Centrifuge briefly (14,000 rpm for 5 seconds)
- S.4. Load sample into a non-denaturing 6% polyacrylamide gel/3M urea gel (see Section 5.1.4. of SSR protocol for preparation of 6% acrylamide gel. [Step S.4: Use 180 g urea (3M) instead of 480 g (8M)!])

6.1.4. Gel Running Conditions

- S.1. Run gel under non-denaturing condition at 12 V/cm for 10-13 hours.

NOTE: This is normally done overnight.

NOTE: Non-denaturing gels are run at low voltages and 1 x TBE to prevent denaturation of small fragments of DNA by the heat generated in the gel during electrophoresis.

6.1.5. Silver-staining

Follow Section 5.1.6 of SSR Protocol (Silver-staining).

6.2. REFERENCES

- Davila, J. A., Y. Loarce, and E. Ferrer, 1999. Molecular characterization and genetic mapping of random amplified microsatellite polymorphism in barley. *Theor.Appl.Genet.* **98**: 265-273
- Provan, J., W. T. B. Thomas, B. P. Forster, and W. Powell, 1999. *Copia*-SSR: a simple marker technique which can be used on total genomic DNA. *Genome.* **42**: 363-366
- Yang, W., A. C. De Olivera, I. Godwin, K Schertz, and J. L. Bennetzen, 1996. Comparison of DNA marker technologies in characterizing plant genome diversity: variability in Chinese sorghums. *Crop Sci.* **36**: 1669-1676
- Zietkiewicz, E., A. Rafalski, and D. Labuda, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored Polymerase Chain Reaction Amplification. *Genomics.* **20**: 176-183

6.3. SOLUTIONS/CHEMICALS NEEDED

Use only sterile distilled water for all solutions:

- *Taq* buffer
- dNTPs
- Sterile distilled water
- Primer(s)
- *Taq* DNA polymerase (5U/ μ l)
- DNA (10-20 ng/ μ l)
- 10 x loading buffer
 - Glycerol (80%) 600 μ l
 - Xylene cyanol 2.5 mg
 - Bromophenol blue 2.5 mg
 - Water 400 μ l
- 5 x loading buffer
 - Glycerol (80%) 300 μ l
 - Xylene cyanol 2.5 mg
 - Bromophenol blue 2.5 mg
 - Water 400 μ l
- Ethidium bromide
- Agarose
- Acrylamide
- Bis-acrylamide
- TEMED
- Ammonium Persulphate
- Alconox
- TBE (see 5.3)
- 100% ethanol
- Repelcote
- Bind silane
- Sodium carbonate
- Glacial acetic acid

7. AFLP

AFLP definition: Any difference between corresponding DNA fragments from two organisms A and B, that is detected by the amplified restriction length polymorphism technique (Kahl, 2001).

The amplified fragment length polymorphism (AFLP) technique combines components of RFLP analysis with PCR technology (Vos *et al.*, 1995). Total genomic DNA is digested with a pair of restriction enzymes, normally a frequent and a rare cutter. Adaptors of known sequence are then ligated to the DNA fragments. Primers complementary to the adaptors are used to amplify the restriction fragments. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualized (Figure 7.1). A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit application. Care is needed in selection of primers with selective bases.

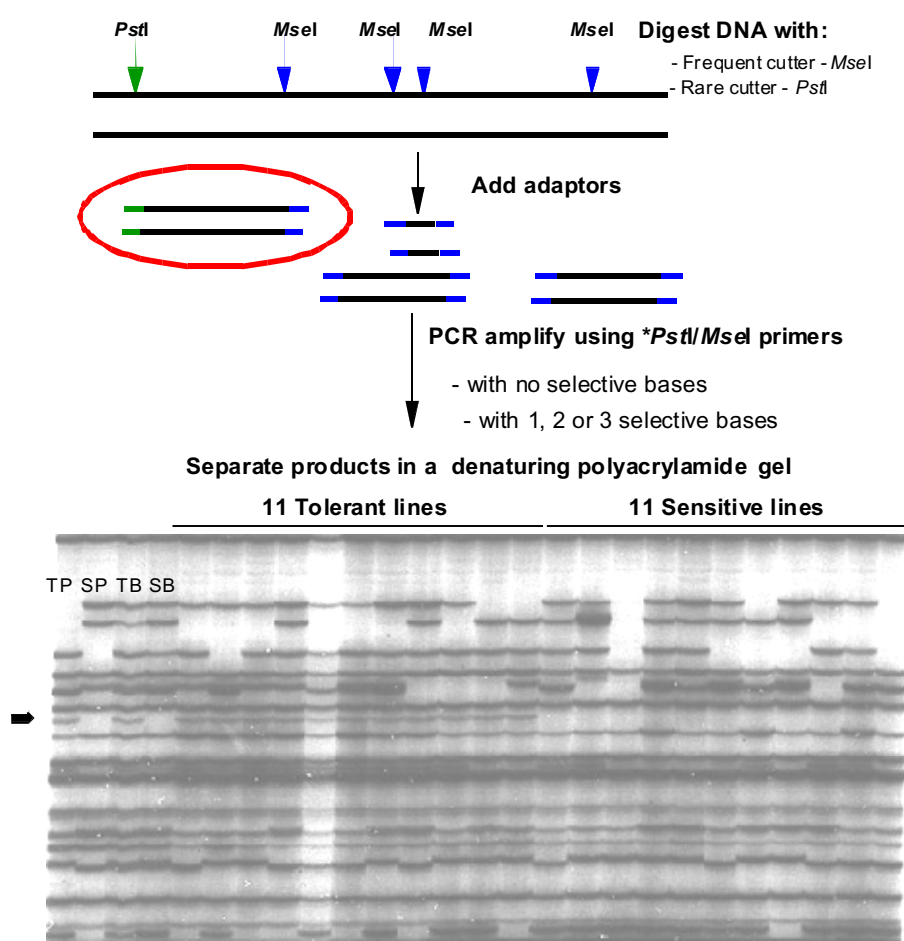


Figure 7.1. In the figure above AFLP profiles have been used in bulk segregant analysis to detect a band associated with tolerance to aluminum in rye, the arrow shows the presence or absence of a band in the tolerant (TP) and susceptible (SP) parents, tolerant (TB) and susceptible (SB) bulks, and 11 tolerant and 11 susceptible individuals (scheme and data with permission, K. Devos and Miftahudin, respectively).

7.1. PROTOCOL

NOTE: Wear gloves and lab coat at all times for safety and to prevent contamination.

7.1.1. Restriction-Ligation

Two pairs of restriction enzymes, *MseI* and *PstI*, and *MseI* and *EcoRI*, were used to digest the genomic DNA. *MseI* is a frequent cutter with a T/TAA cutting site, whereas *PstI* and *EcoRI* are 6-base rare cutters (*PstI* is methylation sensitive).

S*.1. Put on gloves (to protect yourself and the reaction mix) and add the following to a 0.5 ml Eppendorf tube:

Restriction-ligation reaction mixture

Sterile distilled water	6 µl
5x RL buffer	5 µl
Genomic DNA (20 ng/µl)	12.5 µl
Rare cutting enzyme <i>PstI</i> (5 U/µl)	0.5 µl
Frequent cutting enzyme <i>MseI</i> (5U/µl)	0.5 µl
<i>PstI</i> adaptor (5 pmole/µl)	0.5 µl
<i>MseI</i> adaptor (50 pmole/µl)	0.5 µl
rATP (10 mM)	0.5 µl
T4 DNA ligase	1 Unit

S.2. Mix by tapping the bottom of the tube.

S.3. Centrifuge briefly (14,000 rpm for 5 seconds).

S.4. Incubate 3-4 hours at a constant 37°C.

7.1.2. Pre-Amplification Mix

S.1. Put on gloves and add to a PCR tube:

Sterile distilled water	16 µl
10 x PCR buffer	2.5 µl
Restriction-ligation reaction (from 7.1.1)	2.5 µl
<i>PstI</i> primer (50 ng/µl)	0.75 µl
<i>MseI</i> primer (50 ng/µl)	0.75 µl
dNTPs (2 mM)	2.5 µl
<i>Taq</i> DNA polymerase (5U/µl)	0.2 µl

* Step

- S.2. Mix by tapping the bottom of the tube.
- S.3. Centrifuge briefly (14,000 rpm for 5 seconds).

NOTE: The *Pst*I and *Mse*I primers used in pre-amplification are non- selective in that they recognise all *Pst*I and *Mse*I priming sites.

7.1.3. PCR Pre-Amplification

This step amplifies all of the DNA fragments carrying *Pst*I and *Mse*I terminal adaptors, and provides sufficient template for subsequent selective amplification.

Place the tube in the PCR machine and amplify using the following programme:

<i>Step 1</i>	Denaturing	94 °C	30 seconds
<i>Step 2</i>	Annealing	56 °C	30 seconds
<i>Step 3</i>	Extension	72 °C	1 minute
<i>Step 4</i>	Cycling	repeat steps 1-3 for 19 cycles	
<i>Step 5</i>	Hold	4°C	

7.1.4. Check Step

It is important to check that everything has worked in the previous steps before proceeding.

- S.1. Take a 5 µl aliquot of the PCR-amplified product from 7.1.3 above and place in a fresh 0.5 ml tube, and add 1 µl 5x loading buffer.
- S.2. Vortex briefly.
- S.3. Centrifuge briefly (14,000 rpm for 5 seconds).
- S.4. Load the sample into a 1.2 % agarose gel.
- S.5. Run gel at 50V for 30 minutes.
- S.6. Visualise DNA by UV illumination (Figure 7.2). (*Caution: wear gloves, and UV protective glasses and shields over your face when you are exposed to the UV light of the transilluminator*)

NOTE: If previous steps have worked you should see a clear DNA band (Figure 7.2).

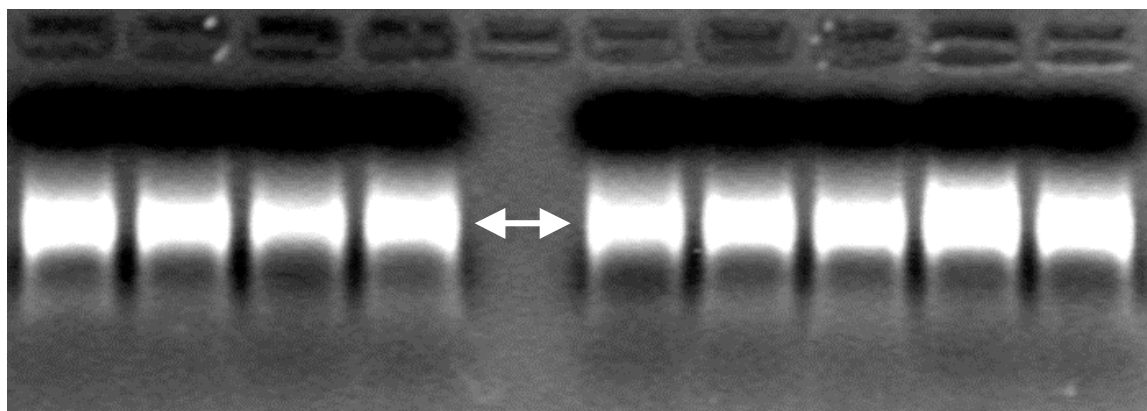


Figure 7.2. An example of a preamplification DNA (arrow) from the FAO/IAEA course.

- S.7. Dilute the remaining 20 µl sample 1:30 with T_{0.1}E buffer (20 µl sample + 580 µl T_{0.1}E buffer) and store at 4 °C (short term) or –20 °C (long term).

NOTE: It is the diluted sample (S.7.) that is used in selective amplification (7.1.5.) PCRs, and now termed ‘Test DNA’.

7.1.5. Selective Amplification PCR Mix

In this section specific subsets in the test DNA are amplified using *Pst*I and *Mse*I primers that are extended with one to three selective nucleotides.

- S.1. Put on gloves and in a PCR tube add:

Sterile distilled water	9 µl
10 x PCR buffer	2 µl
Test DNA (diluted DNA from 7.1.4, S.7.)	5 µl
<i>Pst</i> I selective primer (50 ng/µl)	0.75 µl
<i>Mse</i> I selective primer (50 ng/µl)	0.75 µl
dNTPs (2 mM)	2.5 µl
<i>Taq</i> DNA polymerase (5U/µl)	0.2 µl

- S.2. Mix by gently tapping against the tube.
S.3. Centrifuge briefly (14,000 rpm for 5 seconds).

7.1.6. Selective PCR amplification

Place tube in the PCR machine and amplify using the following programme:

<i>Step 1</i>	Denaturing	94 °C	30 seconds
<i>Step 2</i>	Annealing	65 °C (-0.7 °C/cycle)	30 seconds
<i>Step 3</i>	Extension	2 °C	1 minute
<i>Step 4</i>	Cycling	repeat steps 1-3 for 11 cycles	
<i>Step 5</i>	Denaturing	94 °C	30 seconds
<i>Step 6</i>	Annealing	56 °C	30 seconds
<i>Step 7</i>	Extension	2 °C	1 minute
<i>Step 8</i>	Cycling	repeat steps 5-7 for 22 cycles	
<i>Step 9</i>	Hold	4 °C	

7.1.7. Production of Single Primer, Linear PCR Products

NOTE: This procedure is used to avoid doubled stranded DNA fragments and results in a greater clarity of band separation.

S.1. Put on gloves and add in a PCR tube:

10 x PCR buffer	2 µl
Selective amplification DNA (produced in Step 6)	2 µl
<i>Pst</i> I selective primer (50 ng/µl)	1.5 µl
dNTPs (2 mM)	2.5 µl
<i>Taq</i> DNA polymerase (5U/µl)	0.1 µl
Add sterile distilled water to make up to 20 µl	

S.2. Mix gently by tapping the tube.

S.3. Centrifuge briefly (14,000 rpm for 5 seconds).

7.1.8. PCR Amplification to Produce Single Stranded DNA

Put on gloves and place tube from 7.1.7, step S.3. into a PCR machine and amplify using the following programme:

<i>Step 1</i>	Denaturing	94 °C	30 seconds
<i>Step 2</i>	Annealing	56 °C	30 seconds
<i>Step 3</i>	Extension	72 °C	1 minute
<i>Step 4</i>	Cycling	repeat steps 1-3 for 22 cycles	
<i>Step 5</i>	Denaturing	94 °C	30 seconds
<i>Step 6</i>	Hold	4°C	

7.1.9. Gel Electrophoresis

The single-stranded AFLPs are separated in long, denaturing polyacrylamide gels (often referred to as sequencing gels).

- S.1. Add 15 µl formamide loading buffer to the 20 µl linear amplification samples. The number of samples will be determined by the number of wells you have in your polyacrylamide gel.
- S.2. Denature for 5 minutes at 95 °C - 100 °C, and snap-cool on ice.
- S.3. Centrifuge briefly (14,000 rpm for 5 seconds).
- S.4. Run 5 µl samples in denaturing 6% polyacrylamide gels. SequaGel®XR
<http://www.nationaldiagnostics.com/electroproducts/ec842.html>

7.1.10. Silver-Staining

Follow the procedure given in the SSR Protocol (5.1.6. Silver-staining).

7.2. REFERENCES

- Kahl, G., 2001. The Dictionary of Gene Technology. Wiley-VCH, Weinheim
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau, 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**(21): 4407-4414.

7.3. SOLUTIONS/CHEMICALS NEEDED

- Use only sterile distilled water for all solutions
- 5x RL buffer
 - 50 mM TrisAc pH7.5
 - 50 mM MgAc
 - 250 mM KAc
 - 25 mM DTT
 - 250 ng/μl BSA
- Rare cutting enzyme, *Pst*I (5U/μl)
- Frequent cutting enzyme, *Mse*I (5U/μl)
- *Pst*I adaptor (5 pmole/μl)
- *Mse*I adaptor (50 pmole/μl)
- *Eco*RI adaptor (5 pmole/μl)
- rATP (10 mM)
- T4 DNA ligase
- 10 x PCR buffer
- *Pst*I non-selective primer (50 ng/μl)
- *Mse*I non-selective primer (50 ng/μl)
- *Taq* DNA polymerase (5U/μl)
- Agarose
- T_{0.1}E buffer
- *Pst*I selective primer
- *Mse*I selective primer
- dNTPs (2 mM)
- SequaGel[®] XR <http://www.nationaldiagnostics.com/electroproducts/ec842.html>

8. RAPD

RAPD definition: Any DNA segment that is amplified using short oligodeoxynucleotide primers of arbitrary nucleotide sequence (amplifiers) and polymerase chain reaction procedures (Kahl, 2001).

Random amplified polymorphic DNAs (RAPDs) are produced by PCR using genomic DNA and arbitrary primers (Welsh and McClelland, 1990; Devos and Gale, 1992). *Taq* polymerase is used to amplify DNA segments between closely spaced sequences (<2 kb) and complementary to the short random oligomers (typically 10-mers). RAPD polymorphism results from changes in the primer-binding site in the DNA sequence (Figure 8.1). PCR products can be separated by gel electrophoresis. RAPDs are a dominant marker system.

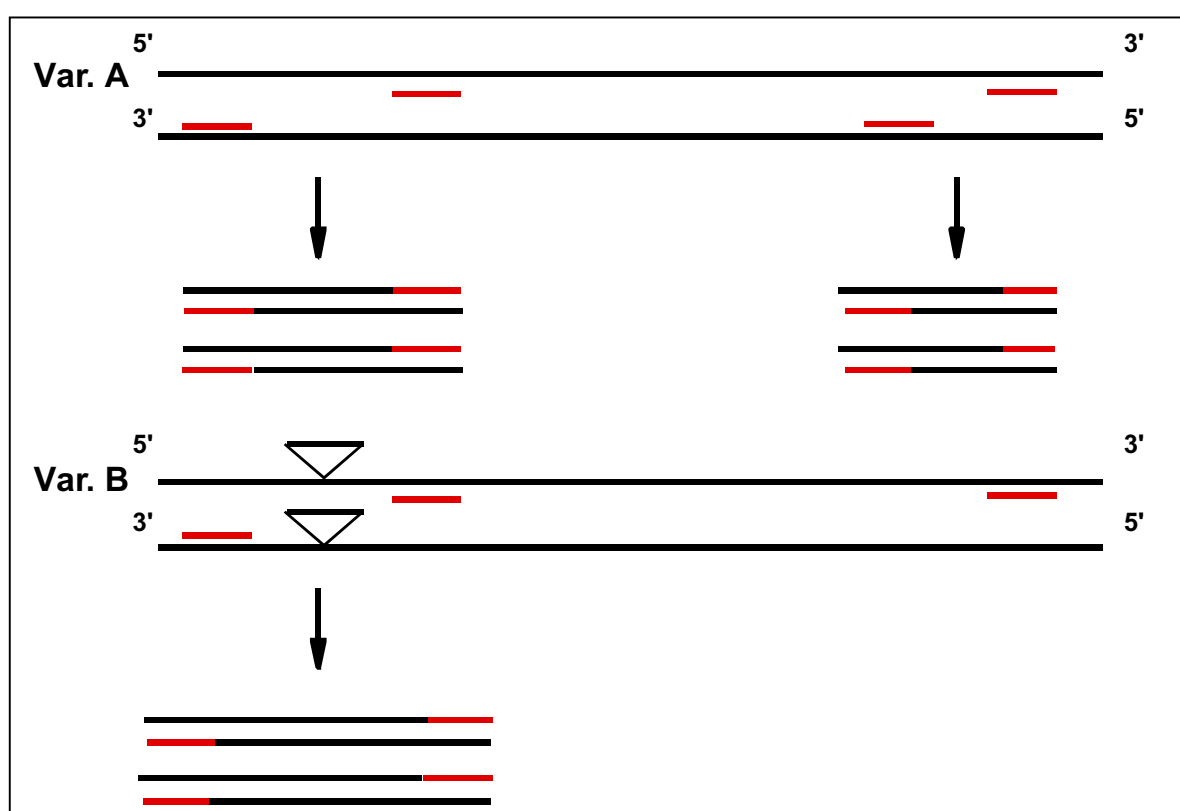


Figure 8.1. The scheme above shows RAPD variation between varieties A and B. In variety A there are 4 primer binding sites resulting in two RAPD products; variety B lacks one of the binding sites resulting in only one RAPD marker being produced. (With permission from K. Devos).

NOTE: For a general PCR Protocol see Appendix A.2.

8.1. PROTOCOL

8.1.1. Master Stock Mixture

	Conc. stock solution	Vol	Final concentration
PCR-buffer + MgCl ₂	10x (15 mM)	3 µl	1x (1.5 mM)
Primer	15 µM	0.4 µl	0.2 µM
dNTP-mix	10 mM	0.51 µl	0.17 mM
<i>Taq</i> -polymerase	5 U/µl	0.25 µl	1.25 U
ddH ₂ O		20.84 µl	
Final volume		25 µl	

S*.1. Add 25 µl of master mix to 5 µl of your DNA in a sterile tube.

NOTE: In each PCR run you conduct, include 2 samples, one of control DNA without primers (3 µl DNA), and one sample without DNA (5 µl ddH₂O).

S.2. Place all tubes in a thermocycler.

S.3. Start the thermocycler programme as listed below.

Thermocycler programme

<i>Step 1</i>	Initial denaturation	94°C	4 minutes
<i>Step 2</i>	Denaturation	93°C	20 seconds
<i>Step 3</i>	Primer annealing	36°C	40 seconds
<i>Step 4</i>	Primer extension	72°C	1 minute
<i>Step 5</i>	Recycle steps 2 - 4		45 times
<i>Step 6</i>	Final extension	72°C	4 minutes
<i>Step 7</i>	Keep samples cool	4°C	(hold)

NOTE: There are hundreds of RAPD primers available; please use the ones of your choice.

* Step

REFERENCES

- Devos, K. M. and M. D. Gale, 1992. The use of random amplified polymorphic DNA markers in wheat. *Theor.Appl.Genet.* **84**: 567-572
- Kahl, G., 2001. *The Dictionary of Gene Technology*. Wiley-VCH, Weinheim.
- Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213-7218.

9. REMAP & IRAP

REMAP definition: Any difference in DNA sequence between two genomes, detected by polymerase chain reaction-mediated amplification of the region between a long terminal repeat of a retrotransposon and a nearby microsatellite (Kahl, 2001).

The dispersion, ubiquity and prevalence of retrotransposon-like elements in plant genomes can be exploited for DNA-fingerprinting. Two DNA techniques based on retrotransposon-like elements are introduced here: IRAP and REMAP (Kalendar *et al.*, 1999). The **IRAP** (Inter-Retrotransposon Amplified Polymorphism) markers are generated by the proximity of two retrotransposons using outward facing primers annealing to their long terminal repeats (LTRs). In REMAP (**RE**trotransposon-Microsatellite Amplified Polymorphism) the DNA sequences between the LTRs and adjacent microsatellites (SSRs) are amplified using appropriate primers.

The principle of IRAP und REMAP is shown in Figure 9.1 below:

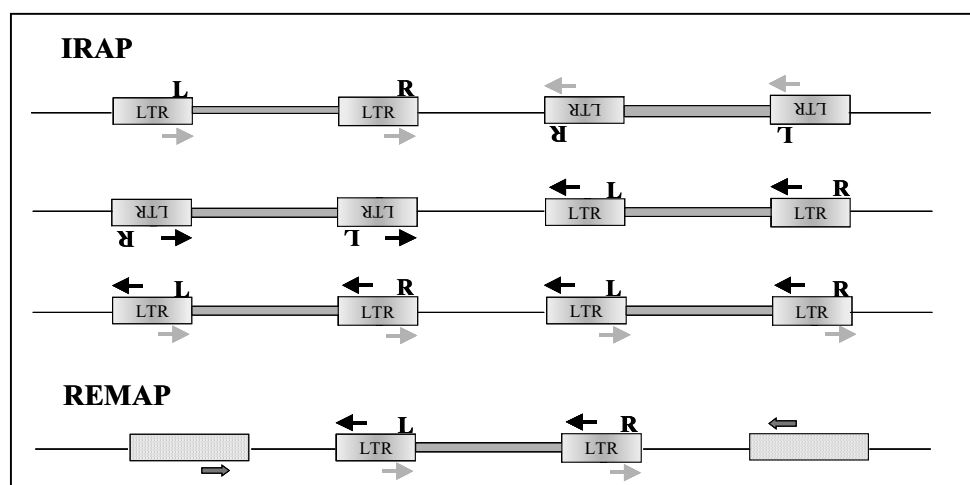


Figure 9.1. Principle of the IRAP und REMAP strategy. **IRAP:** PCR primers facing outward from the 5' (black arrows) and 3' (gray arrows) ends of LTRs will amplify intervening DNA from the retrotransposon in any of the three possible orientations (tail-to-tail, head-to-head, head-to-tail). **REMAP:** LTR primers are used together with a primer consisting of simple sequence repeats (blank boxes) (Kalendar *et al.*, 1999)

9.1. PROTOCOL

REMAP and IRAP markers are species specific. In the FAO/IAEA course the following primers for rice and barley were available and used in conjunction with rice and barley DNA.

Table 9.1. LTR primers from the rice retrotransposon Tos17 (Hirochika *et al.*, 1996), sequence and PCR annealing temperatures (T_a).

Primer	Sequence	T_a
TOS17LTR-1 (outward 3' end of LTR)	TTGGATCTTGTATCTTGTATATAC	56°C
TOS17LTR-2 (outward 3' end of LTR)	GCTAATACTATTGTTAGGTTGCAA	56°C
TOS17LTR-3 (outward 5' end of LTR)	CCAATGGACTGGACATCCGATGGG	56°C
TOS17LTR-4 (outward 5' end of LTR)	CTGGACATGGGCCAACTATACAGT	56°C

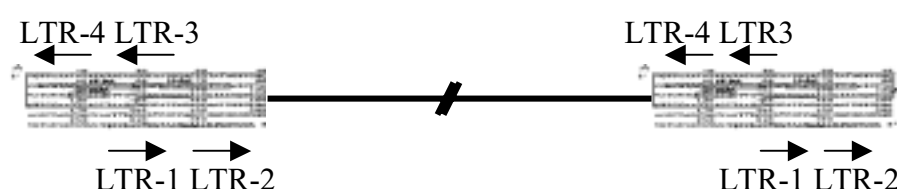
Table 9.2. LTR primers from the barley BARE-1 (Kalendar *et al.*, 1999), sequence and PCR annealing temperatures (T_a).

Primer	Sequence	T_a
BARLTR-2(LTR forward) - IRAP	CTCGCTCGCCCACTACATCAACCGCGTTTATT	60°C
BARLTR-3(LTR reverse) – IRAP/REMAP	GGAATTCATAGCATGGATAATAAACGATTATC	60°C

Table 9.3. Microsatellite (SSR) primers and PCR annealing temperatures (T_a).

Sequence	T_a
(GA) ₉ C; (CT) ₉ G; (CA) ₁₀ G	54°C
(CAC) ₇ G; (GTG) ₇ C; (CAC) ₇ T; GT(CAC) ₇	58°C

NOTE: It is very important to try different combinations of LTR- and microsatellite (SSR) primers for REMAP and LTR-primers for IRAP. Choose primers that have been derived from the species you are working with. The figure below shows you the orientation of only the TOS17-LTR-primers:



NOTE: Gloves and lab coat should be worn throughout.

9.1.1. Prepare a 50 µl Reaction Mix

S*.1. Take a sterile PCR tube and add:

10 x <i>Taq</i> buffer	5.0 µl
dNTPs (10 mM)	1.0 µl
Primer 1 (100 pmol/µl)	0.5 µl
Primer 2 (100 pmol/µl)	0.5 µl
DNA (100 ng/µl)	1.0 µl
<i>Taq</i> DNA polymerase (5 U/µl)	0.5 µl
Add ddH ₂ O to bring volume to 50 µl	

S.2. Mix by tapping against the tube.

S.3. Centrifuge briefly (14,000 rpm for 5 seconds).

9.1.2. PCR Amplification

The PCR amplification programme used for the Tos17 sequence was:

<i>Step 1</i>	Initial denaturation	94°C	2 minutes
<i>Step 2</i>	Denaturation	94°C	30 seconds
<i>Step 3</i>	Primer annealing*	T _a	30 seconds
<i>Step 4</i>	Ramp	0.5°C per second to 72°C	
<i>Step 5</i>	Primer extension	72°C	2 minutes
<i>Step 6</i>	Cycling	repeat steps 2-5 for 29 cycles	
<i>Step 7</i>	Final extension	72°C	8 minutes
<i>Step 8</i>	Hold	4°C	

* See tables above for appropriate annealing temperatures (T_a).

NOTE: In the FAO/IAEA course primer pairs TOS17LTR-1 and TOS17LTR-3 were used for rice-IRAP and BARLTR-2 and BARLTR-3 for barley-IRAP.

NOTE: TOS17LTR-1 and BARLTR-3 were also used as single primers for IRAP in rice and barley, respectively.

* Step

NOTE: For REMAP, the FAO/IAEA course used TOS17LTR-3 with a range of SSR primers in rice (see Table c), the annealing temperatures in these cases were the T_a of the SSR primers.

9.1.3. Separation and Visualization of the Amplification Products

- S.1. Place 15 µl of PCR into a fresh Eppendorf.
- S.2. Add 3 µl of 5 x loading buffer containing dye.
- S.3. Vortex briefly.
- S.4. Centrifuge briefly (14,000 rpm for 5 seconds).
- S.5. Load sample into a 2% NuSieve® agarose gel.

NOTE: NuSieve® agarose provides a good separation gel.

- S.6. Run gel for approximately 80 minutes at 80 W (power limiting) or until dark blue front has run 2/3 down the gel.

NOTE: See Section 1 of RFLP Protocol (Agarose gel electrophoresis) for details of gel preparation and running.

- S.7. Stain gel with ethidium bromide (*Caution: ethidium bromide is toxic wear gloves and avoid inhalation*).
- S.8. Visualise bands under UV light (*Caution: wear UV protective glasses and shield your face when you are exposed to the UV light of the transilluminator*).

9.2. REFERENCES

- Hirochika, H., K. Sugimoto, Y. Otsuki, H. Tsugawa, and M. Kanda, 1996. Retrotransposons of rice involved in mutations induced by tissue culture. *Proc.Natl.Acad.Sci.USA*. **93**: 7783-7788
- Kalendar, R., T. Grob, A. Regina, A. Suoniemi, and A. Schulman, 1999. IRAP and REMAP: two new retrotransposon-based DNA fingerprinting techniques. *Theor.Appl.Genet*. **98**: 704-711.

9.3. SOLUTIONS/CHEMICALS NEEDED

Use only sterile distilled water for all solutions.

- *Taq* buffer
- dNTPs
- Primers
- *Taq* DNA polymerase (5U/μl)
- DNA (10-20 ng/μl)
- 10 x loading buffer:

Glycerol (80%)	600 μl
Xylene cyanol	2.5 mg
Bromophenol blue	2.5 mg
Water	400 μl
- 5 x loading buffer

Glycerol (80%)	300 μl
Xylene cyanol	1.3 mg
Bromophenol blue	1.3 mg
Water	400 μl
- Ethidium bromide
- Agarose
- Acrylamide
- Bis-acrylamide
- TBE

10. SNPs

SNP definition: Any polymorphism between two genomes that is based on a single nucleotide exchange, small deletion or insertion. (Kahl, 2001).

Small nucleotide polymorphism (SNP) is a new marker technology originally developed in human. SNPs are the most abundant polymorphic marker with 2 – 3 polymorphic sites every kilobase (Cooper *et al.*, 1985). Originally discovered in humans, SNPs have now been developed for genotyping in plants. SNP technology is heavily dependent upon sequence data. Several methods are available for SNP detection including automated fluorescent sequencing denaturing high-performance liquid chromatography (DHPLC, Underhill *et al.*, 1996), DNA microarrays (Hacia and Collins, 1999), single-strand conformation polymorphis-capillary electrophoresis (SSCP-CE, Ren, 2001; Figure 1), microplate-array diagonal-gel electrophoresis (MADGE, Day *et al.*, 1998) and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF, Griffin and Smith, 2000). The potential of SNP technology was a topic of discussion only in the FAO/IAEA course.

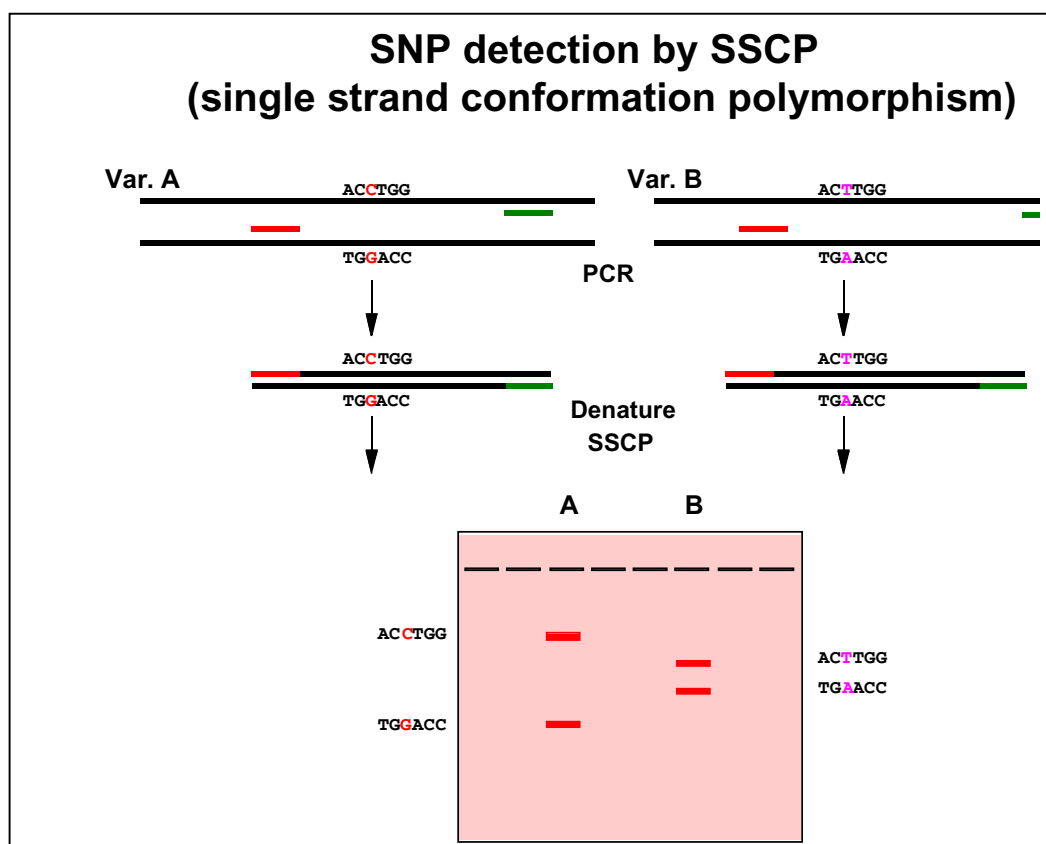


Figure 10.1. The scheme above shows how SNP variation can be detected between varieties A and B (With permission K. Devos).

10.1. REFERENCES

- Cooper, D. N., B. A. Smith, H. J. Cooke, S. Niemann, and J. Schmidtke, 1985. An estimate of unique DNA sequence heterozygosity in the human genome. *Hum.Genet.* **69**(3): 201-205
- Day, I. N., E. Spanakis, D Palamand, G. P. Weavind, and S. D. O'Dell, 1998. Microplate-arrays diagonal-gel electrophoresis (DADGE) and melt-MADGE: tool for molecular genetic epidemiology. *Trends in Biotech.* **16**: 187-290
- Griffin, T. J. and L. M. Smith, 2000. Single-nucleotide polymorphism analysis by MALDI-TOF mass spectrometry. *Trends in Biotech.* **18**: 77
- Hacia, J. G. and F. S. Collins, 1999. Mutational analysis using oligonucleotide microarrays. *J.Med.Genet.* **36**: 730-736
- Kahl, G., 2001. *The Dictionary of Gene Technology*. Wiley-VCH, Weinheim.
- Ren, J., 2001. High-throughput single-strand conformation polymorphism analysis by capillary electrophoresis. *J.Chromatography B.Biomed.Science Appl.* **74**: 115-128
- Underhill, P. A., L. Jin, R Zemans, P. J. Oefner, and L. L. Cavalli-Sforza, 1996. A pre-Columbian Y chromosome-specific transition and its implications for human evolutionary history. *Proc.Natl.Acad.Sci.USA.* **93**: 196-200.

**FAO/IAEA Interregional Training Course on Mutant
Germplasm Characterization**
APPENDICES 1

APPENDICES

A.1. GENERAL DNA EXTRACTION TECHNIQUES

A.1.1. Phenol/Chloroform Extraction

NOTE: Wear gloves, goggles, and lab coat at all times for safety and to prevent contamination.

Removes protein from DNA preparations. Advisable for example if $A_{260\text{nm}}: A_{280\text{nm}}$ (from the spectrophotometer readings) of the DNA are below 1.6. Phenol extraction requires subsequent ethanol precipitation of the DNA.

Phenol: freshly distilled and equilibrated with 20 % 0.5 M Tris-Base. Prepare a mixture of phenol/chloroform/isoamylalcohol (PCI) (25:24:1).

NOTE: Use caution as phenol is toxic.

- S*.1. The DNA sample is mixed with an equal volume of PCI, vortexed, and centrifuged for about 5 minutes. Remove the upper aqueous phase avoiding contamination with protein from interphase and transfer it to a fresh reaction tube.
- S.2. Remaining traces of phenol in the aqueous phase are extracted with 1 volume of chloroform/isoamylalcohol (24:1). Vortex and centrifuge for 5 minutes. Transfer the upper phase carefully to a fresh reaction tube.

A.1.2. Ethanol Precipitation

NOTE: Wear glasses at all time for safety.

- S.1. Determine volume of the sample, add 0.1 volume 3 M sodium acetate and 2.5 volumes cold ethanol (96%). Mix well and leave at -20°C for 2 hours.
- S.2. Centrifuge for 15 minutes (in microcentrifuge at $>12,000$ rpm), preferably at 4°C .
- S.3. Carefully remove ethanol and wash pellet with cold 70% ethanol to remove salt from the sample – centrifuge for 5 minutes.
- S.4. Dry DNA pellet in vacuum centrifuge or air dry in flow bench.
- S.5. Dissolve DNA in TE buffer or sterile double distilled H_2O (dd H_2O).

* Step

A.1.3. Solutions

- 1.5 x CTAB extraction buffer (1 liter):

CTAB	15.0 g
1 M Tris (pH 8.0)	75 ml
0.5 M EDTA	30 ml
NaCl	61.425 g
ddH ₂ O	to 1 litre

- 10% CTAB (1 litre)

CTAB	100 g
NaCl (0.7M)	40.95 g
ddH ₂ O	to 1 litre

- β -mercaptoethanol,
- Chloroform:isoamylalcohol (24:1),
- Isopropanol,
- Ethanol 96% and 70%
- sodium acetate (3 M)
- TE buffer
 - 10 mM Tris HCl
 - 1 mM EDTA (pH 8.0)

A.2. POLYMERASE CHAIN REACTION PROTOCOL

The polymerase chain reaction (PCR) is basically a technique for *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary DNA strands. The principle of primer extension is illustrated in Figure A.2.1 for one DNA strand. The primer binds to its complementary sequence of the single stranded target DNA and the polymerase extends the primer in 5' - 3' direction by using the complementary DNA as a template. For a PCR reaction, two primers are used, one binding to the “lower” strand (forward primer) and one binding to the “upper” strand (reverse primer). Thus, the requirements for the reaction are: template DNA, oligonucleotide primers, DNA polymerase, deoxynucleotides (to provide both energy and nucleosides for DNA synthesis), and a buffer containing magnesium ions. In general the DNA sequence of both ends of the region to be amplified must be known to be able to synthesize proper primer oligonucleotides. The PCR reaction is a cyclic process, which is repeated 25 to 35 times. One cycle consists of three basic steps with characteristic reaction temperatures:

- S*.1. Denaturation of the double stranded DNA to make the template accessible for the primers and the DNA polymerase (94°C, 30 seconds).
- S.2. Annealing of primers to complementary sequence on template (between 45 and 60°C, depending on the primer sequences, 30 seconds).
- S.3. Extension of primers by DNA-polymerase (72°C - the optimum temperature of *Taq* DNA-polymerase -, 1 minute per kilobase of template to be amplified).

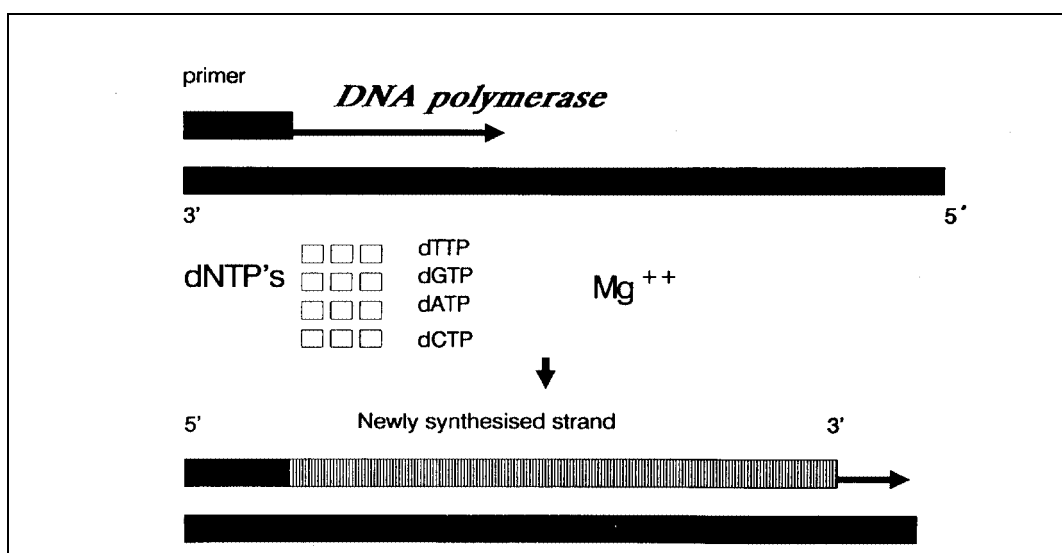


Figure A.2.1. Primer extension. DNA polymerase extends a primer by using a complementary strand as a template (McPherson *et al.*, 1991).

* Step

By multiple repetition of this cycle the number of template molecules increases. This results in exponential amplification of the DNA sequence that is bordered by the two primers used (Figure A.2.2).

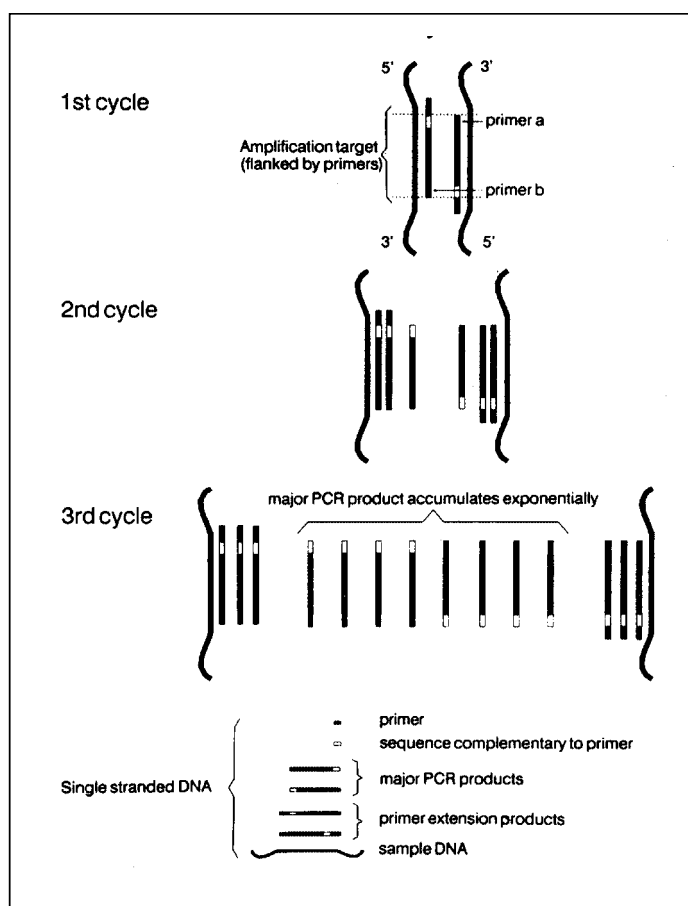


Figure A.2.2. Schematic diagram of PCR. By using primer pairs 'a' and 'b' (short black lines) annealed to complementary strands of DNA (long black lines), two new strands (shaded lines) are synthesized by primer extension. If the process is repeated, both the sample DNA and the newly synthesized strands can serve as templates, leading to an exponential increase of product which has its ends defined by the position of the primers (McPherson *et al.*, 1991).

Successful performance of a PCR experiment is dependent on a number of different factors; some of them have to be determined empirically.

- The selection of the primers is a very important step. They should be long enough to be specific, not anneal against themselves by folding (avoid palindromic sequences), nor should the forward primer anneal with the reverse primer. Furthermore the G/C content of the primers should be similar and they should have similar melting temperatures (T_m). Several computer programs are available on the Internet to help to find the best primer

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APPENDIX 2 3

pairs for a given sequence. Try the addresses below- submit the DNA sequence and some required parameters and you will get a list of possible primers:

<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3> www.cgi
<http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer>
<http://www.nwfsc.noaa.gov/protocols/oligoTMcalc.html>

- The annealing temperature must be determined empirically and is dependent from the T_m 's of the primers. A rule of thumb (Wallace rule) provides a first order approximation for T_m of oligonucleotides that have 20 bases or less:

$$T_m = 2^{\circ}\text{C} (\text{A} + \text{T}) + 4^{\circ}\text{C} (\text{C} + \text{G})$$

The annealing temperature is a few degrees lower than T_m .

- PCR is extremely sensitive! Thus contamination of samples and solutions with minimal amounts of foreign DNA, or the wrong PCR programme can result in unspecific PCR products. Always include controls without template DNA in order to check if there is any contamination in your nucleotides, primers, etc.

A typical PCR experiment is given in the table below. In the FAO/IAEA course, PCR was demonstrated by amplifying a 1050 bp sequence of the rice retrotransposon Tos 17 accession number D88394:

Forward Primer 1 (100 pmol/ μl):

Reverse Primer 2 (100 pmol/ μl):

Reaction volume: 50 μl

Stock solutions	μl	Final conc./amount
10 x PCR buffer (15 mM MgCl_2)	5.0 μl	1 x PCR buffer (1.5 mM MgCl_2)
Primer 1 (100 pmol/ μl)	0.5 μl	1 pmol
Primer 2 (100 pmol/ μl)	0.5 μl	1 pmol
dNTP mix (10 mM)	1 μl	0.2 mM
DNA template (100 ng/ μl)	1 μl	100 ng
<i>Taq</i> DNA Polymerase (5 U/ μl)	0.5 μl	2.5 U
H_2O	41.5 μl	-

NOTE: It is very important to prepare a master mix corresponding to the number of desired samples that contains all the reagents except for the template DNA. Mix well and add the appropriate amount of the master solution to single reaction vials containing the individual template DNA samples you wish analyzed. This procedure significantly reduces the number of pipetting steps, avoids errors derived from pipetting small amounts of liquid, and finally ensures that every tube contains the same concentrations of reagents.

For amplification of the Tos17 sequence the PCR machine was programmed as follows:

<i>Step 1</i>	Initial denaturation	94°C	(4:00 minutes)
<i>Step 2</i>	Denaturation	94°C	(0:30 minute)
<i>Step 3</i>	Primer annealing	56°C	(0:30 minute)
<i>Step 4</i>	Primer extension	72°C	(1:10 minutes)
<i>Step 5</i>	Cycling	Repeat steps 2-4	29 times
<i>Step 6</i>	Final extension	72°C	(6:00 minutes)
<i>Step 7</i>	Hold	4°C	(hold)

NOTE: The PCR programme can vary from primer to primer set and species to species with the annealing temperature being the most variable step.

A.2.1. REFERENCES

McPherson, M., P. Quirke, and G Taylor, 1991. PCR: A Practical Approach. Oxford University Press, New York.

A.3. PLANT GENOME DATABASE CONTACT INFORMATION

A.3. PLANT GENOME DATABASE CONTACT INFORMATION (Taken from an IAEA-TECDOC on “Radioactively Labelled DNA Probes For Crop Improvement” VIENNA SEPTEMBER 6-8, 1999).

DATABASE	CROPS	CURATOR	E-MAIL ADDRESS	DATABASE ADDRESS
AAtdB	<i>Arabidopsis</i>	David Flanders	flanders@genome.stanford.edu	http://genome-www.stanford.edu/Arabidopsis/
Alfagenes	Alfalfa (<i>Medicago sativa</i>)	Daniel Z. Skinner	Dzolek@ksu.ksu.edu	http://naaic.org/
Bean Genes	<i>Phaseolus</i> and <i>Vigna</i>	Phil McClean	mcclean@beangenes.cws.ndsu.nodak.edu	http://probe.nalusda.gov:8300/cgi- bin/browse/beangenes
ChlamyDB	<i>Chlamydomonas reinhardtii</i>	Elizabeth H. Harris	chlamy@acpub.duke.edu	http://probe.nalusda.gov:8300/cgi- bin/browse/chlamydb
CoolGenes	Cool season food legumes	Fred Muehlbauer	muehlbau@wsu.edu	http://probe.nalusda.gov:8300/cgi- bin/browse/coolgenes
CottonDB	<i>Gossypium</i> species	Sridhar Madhavan	msridhar@tamu.edu	http://probe.nalusda.gov:8300/cgi- bin/browse/cottondb
GrainGenes	Wheat, barley, rye and relatives	Olin Anderson	oandersn@pw.usda.gov	http://probe.nalusda.gov:8300/cgi- bin/browse/graingenes
MaizeDB	Maize	Mary Polacco	maryp@teosinte.agron.missouri.edu	http://www.agron.missouri.edu/
MilletGenes	Pearl millet	Matthew Couchman	Matthew.Couchman@bbsrc.ac.uk	http://jiiio5.jic.bbsrc.ac.uk:8000/cgi- bin/ace/search/millet
PathoGenes	Fungal pathogens of small-grain cereals	Henriette Giese	h.giese@risoe.dk	http://probe.nalusda.gov:8300/cgi- bin/browse/pathogenes
RiceGenes	Rice	Susan McCouch	srm4@cornell.edu	http://genome.cornell.edu/rice/
RiceGenome Project	Rice			http://www.staff.or.jp
SolGenes	<i>Solanaceae</i>	Molly Kyle	mmk9@cornell.edu	http://genome.cornell.edu/solgenes/welcome.html
SorghumDB	<i>Sorghum bicolor</i>	Russel Kohel/Bob Klein	nus6389@tam2000.tamu.edu	http://probe.nalusda.gov:8300/cgi- bin/browse/sorghumdb
Soybase	Soybeans	David Grant	dgrant@iastate.edu	http://129.186.26.94/
TreeGenes	Forest trees	Kim Marshall	kam@s27w007.pswfs.gov	http://dendrome.ucdavis.edu/index.html
National Center for Genome Resources	Various			http://www.ncgr.org/

A.4. ACRONYMS OF CHEMICALS & BUFFERS

AMPPD	4-Methoxy-4-(3-phosphatephenyl)spiro(1,2-dioxetan-3,2'-adamantan)
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
CSPD[®]	Chemiluminescence substrate (a registered trademark of Tropix Inc., USA)
CTAB	Hexadecyltrimethylammonium Bromide
ddH₂O	Double distilled Water
DIG	Digoxygenin
N₂ liquid	Liquid Nitrogen
NBT	Nitro Blue Tetrazolium
PCI	Phenol/Chloroform/Isoamylalcohol (25:24:1)
SDS	Sodium Dodecyl Sulfate
SSC	Saline-Sodium Citrate Buffer
TBE	Tris-Borate-EDTA Buffer
TE	Tris-EDTA Buffer
TEMED	N,N,N',N'-Tetramethylenediamine
TRIS	[Tris(hydroxymethyl)aminomethane]