FOREWORD

The following protocols are used routinely for genotyping plants at the molecular biology laboratory (MBL) of the International Potato Center (CIP). We use restriction fragment length polymorphism (RFLP) to anchor genetic linkage groups or analyze specific loci and random amplified polymorphic DNA (RAPD), single sequence repeats (SSR) or microsatellites, and amplified fragment length polymorphism (AFLP) to generate genetic linkage groups and calculate genetic distances. Before using protocols such as RAPD, AFLP, and Taq polymerase commercially, users should confirm the status of these technologies through their national patent registration office. Users should also note that this training publication is for internal use only and all information is intended for research purposes only.

Suggestions for improving the current version are welcome. This document is the result of the collaborative efforts of the following CIP staff and students, who are warmly acknowledged for their contributions: Ana Hurtado, Carmen Herrera, Diego Fajardo, Luis Ñopo, María del Carmen Beltrán, and Nelson Espinoza.

Please do not hesitate to contact us (cip@cgiar.org). For more information about CIP and CIP training materials, please visit our website (www.cipotato.org).

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AMPPD	disodium3-(4,methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3,3,1]decan}-4-yl)phenyl phosphate
BSA	bovine serum albumin
CTAB	hexadecyltrimethylammonium bromide
DIG	digoxigenin
dNTP	deoxynucleoside triphosphate mix
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
Hr	hour(s)
IPTG	isopropylthiol-β-D-galactoside thiogalactoside
LB	luria broth base
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RT	room temperature
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
SSR	single sequence repeats
Sec	second(s)
Taq	enzyme from *Thermus aquaticus*
TBE	tris-borate-EDTA buffer
TE	Tris/EDTA buffer
Tris	N-tris[Hidroxymethyl]methyl-2-aminoethanosulfonic acid

PLANT DNA EXTRACTION

DNA quality is a crucial factor in the successful application of molecular techniques. In most cases the final DNA quality will be determined by the physiological condition of the plant material rather than by the specific protocol used.

Genomic DNA used for RAPDs and SSRs can be obtained by following the small-scale protocol for fresh or lyophilized tissues. Polysaccharide contaminants can be reduced by storing plants in the dark for one or two days prior to DNA extraction.

Genomic DNA used for RFLP or AFLP must be of a higher quality to ensure complete digestion by restriction enzymes. Toward that end, the medium-scale extraction method can be used with either fresh or lyophilized tissues. We recommend using material multiplied in vitro as fresh tissue.

All of these protocols have been used successfully for potatoes, sweetpotatoes, and Andean roots and tubers.

DNA Extraction (Small-Scale)

This protocol is used routinely in our laboratory and has given good results with most root and tuber crops studied so far. A 100-mg sample of fresh leaf tissue should yield about 100 µg of DNA.

Reference: Isolation of DNA from small amounts of plant tissues from Doyle and Doyle, 1990 (*BRL Focus* 12, 13–15) modified at the NCSU Forest Biotechnology Laboratory.

Protocol:

1. Weigh 100 mg of leaf tissue and grind into a pre-chilled mortar in liquid nitrogen to obtain a fine powder.

2. Transfer powder into an Eppendorf tube. Add 700 µl of fresh 2X CTAB buffer and 2 µl of ß-mercaptoethanol and vortex. Place in a 65°C water bath for 45 min, mixing tubes every 15 min. Cool samples to RT for 2 min.

3. Add 700 µl of chloroform:isoamyl alcohol (24:1) to each tube. Vortex briefly and gently to avoid shearing the DNA. Then turn over several times.

4. Spin 5 min at 14,000 rpm in a microcentrifuge. Remove aqueous top layer and transfer to a new, labeled Eppendorf tube. Be careful to avoid transferring

interphase material. Dispose of chloroform:isoamyl alcohol waste properly in a labeled waste container.

5. Add 50 µl 10% CTAB (in 0.7 M NaCl), vortex gently, and mix thoroughly.

6. Repeat Steps 3 and 4.

7. Add an equal volume of cold isopropanol (400–500 µl) to each tube. Turn over several times and let tubes sit at 4°C for 30 min or at –20°C for 15 min.

8. Spin at 14,000 rpm for 20 min. Pour off the supernatant carefully to avoid losing the DNA pellet. Invert tubes and air-dry (1–2 min).

9. Wash the DNA pellet with 1 ml of 70% EtOH (for 3 min) and spin for 30 min at 14,000 rpm. Carefully pour off the EtOH, wash the pellet in 1 ml of 90% EtOH, spin for 30 min at 14,000 rpm, and carefully pour off the EtOH. Invert tubes and air-dry overnight or use a vacuum pump for 15 min.

10. Dissolve the DNA in 150 µl of $T_{10}E_1$ or distilled H_2O per sample, add 1–2 µl of DNAse-free RNAseA (10 mg/ml). Incubate at 37°C for 1 hr.

11. Store at 4°C (or at –20°C, for long-term storage).

2X CTAB Buffer

Stock	Final	100 ml
CTAB	2%	2 g
5 M NaCl	1.4 M	28 ml
0.5 M EDTA	20 mM	4 ml
1 M Tris HCl, pH 8.0	100 mM	10 ml
PVP	1%	1 g
Distilled H_2O		Up to 100 ml

Note: Buffer can be stored at RT indefinitely. CTAB will dissolve after salt is added.

$T_{10}E_1$ Buffer

Stock	Final	1 L
1 M Tris HCl, pH 8.0	10 mM	10 ml
0.5 M EDTA, pH 8.0	1 mM	2 ml
Distilled H_2O		Up to 1000 ml

Note: Sterilize in autoclave and store at RT.

DNA Extraction (Medium-Scale)

Isolation of DNA from medium amounts of plant tissue is used routinely in our laboratory and has given good results with most root and tuber crops studied so far. If any problems develop using this procedure, check the quality of the plant material and the chemicals. If problems persist, try using other protocols.

In the following protocol, we prefer Option A. One 4-g sample of fresh leaf tissue should yield around 400 µg of total DNA.

Reference: Adapted from Murray and Thompson, 1980. Rapid isolation of high molecular weight plant DNA (*Nucl. Acid Res.* 8, 4321–4325).

Protocol:

1. Weigh 4 g of fresh leaf tissue and grind into a pre-chilled mortar containing liquid nitrogen to obtain a fine powder. Transfer the powder into a 50-ml Falcon tube containing 20 ml of extraction buffer plus 200 µl of ß-mercaptoethanol (add just prior to use).

2. Incubate at 65°C for 20 min, mixing tubes every 5 min.

3. Cool samples to RT for 10 min. Add equal volume of chloroform:isoamyl alcohol (24:1). Turn over gently several times for 5 min.

4. Spin at 4000 rpm for 20 min at 4°C.

5. Transfer aqueous top layer to a new 50-ml Falcon tube.

6. Add equal volume of chloroform:isoamyl alcohol (24:1). Turn over gently several times for 5 min.

7. Spin at 4000 rpm for 20 min at 4°C.

8. Transfer aqueous top layer to a new 50-ml tube containing two-thirds volume of cooled isopropanol (about 15 ml). Turn tube over gently.

Option A:

9a. Leave tube at RT for 30 min.

10a. Collect the DNA by "fishing" with a sterile glass rod or with a Pasteur pipette.

11a. Transfer the DNA cloth to a new Eppendorf tube containing 300 µl of Wash 1 buffer. Leave at RT for 20 min.

12a. Transfer to a new Eppendorf tube containing 300 µl of Wash 2 buffer for 5 sec.

13a. Transfer the DNA to a new Eppendorf tube and air-dry at RT (uncovered) for 30 min.

14a. Dissolve the DNA in 500–1000 µl of $T_{10}E_1$.

15a. Add 5–10 µl of DNAse-free–RNAse A (10 mg/ml). Incubate at 37°C for 30–60 min.

16a. Store at 4°C (or at –20°C for long-term storage).

Option B:

9b. Leave at –20°C for at least 1 hr. Spin at 4000 rpm for 30 min.

10b. Pour off the isopropanol and air-dry at RT (or use a vacuum-pump).

11b. Dissolve the DNA pellet in 500–1000 µl of $T_{10}E_1$ and add 5–10 µl of DNAse-free RNAse A (10 mg/ml). Incubate at 37°C for 1 hr.

12b. Store at 4°C (or at –20°C for long-term storage).

Buffer

Stock	Final	1 L
1.0 M Tris HCl, pH 8.0	100 mM	100 ml
5.0 M NaCl	1.4 M	280 ml
0.5 M EDTA	25 mM	50 ml
CTAB	2%	20 g
Distilled H_20		Up to 1000 ml

Note: Sterilize in autoclave, then store at RT. Just before use, add 1% ß-mercaptoethanol.

Wash 1

Stock	Final	100 ml	500 ml
Absolute ethanol	76%	76 ml	380 ml
2.5 M sodium acetate	0.2 M	8 ml	40 ml
Distilled H_2O		16 ml	80 ml

Wash 2

Stock	Final	100 ml	500 ml
Absolute ethanol	76%	76 ml	380 ml
1 M ammonium acetate	10 mM	1 ml	5 ml
Distilled H_2O		23 ml	115 ml

$T_{10}E_1$ Buffer

Stock	Final	1 L
Tris HCl 1 M, pH 8.0	10 mM	10 ml
EDTA 0.5 M, pH 8.0	1 mM	2 ml
Distilled H_2O		Up to 1000 ml

Note: Sterilize in autoclave and store at RT.

MINI-SCALE PLASMID EXTRACTION

***Protocol*:**

1. Transfer a single bacterial colony into 2 ml of bacterial culture containing the appropriate antibiotic. Incubate overnight at 37°C (with agitation).

2. Pour 1.5 ml of the culture into an Eppendorf tube. Centrifuge at 5000 rpm for 15 min.

3. Add 100 µl of TELT buffer. Vortex to re-suspend the cells.

4. Add 10 µl of Lysozyme solution (10 mg/ml, freshly prepared), and incubate for 5 min at RT (no longer!).

5. Boil for 1 min (no longer!).

6. Cool down for 5 min on ice and 8 min at RT.

7. Centrifuge at 12,000 rpm for 10 min to remove cell debris.

8. Transfer supernatant to an Eppendorf tube containing 250 µl of cold ethanol. Mix well and cool for 30–60 min at –20°C.

9. Centrifuge at 12,000 rpm for 15 min.

10. Wash the DNA pellet with 70% ethanol and spin again.

11. Air-dry the pellet (invert the tube on paper towel for 5 min and air-dry the pellet for 10 min or more).

12. Dissolve the pellet in 30–50 µl of $T_{10}E_1$:

TELT Buffer

Tris pH 7.5	50 mM
EDTA	62.5 mM
Triton X100	0.4%
LiCl	2.5 M

T_{10} E_1 Buffer

Stock	Final	1 L
1 M Tris HCl, pH 8.0	10 mM	10 ml
0.5 M EDTA, pH 8.0	1 mM	2 ml
Distilled H_2O		Up to 100 ml

Note: Sterilize in autoclave and store at RT.

PCR AMPLIFICATION OF PLASMID INSERTS

The protocol described below is reprinted with permission from a laboratory protocols booklet published by the International Center for the Improvement of Maize and Wheat (CIMMYT).

Reference: D. Hoisington, 1992 (*Laboratory Protocols*. CIMMYT Applied Molecular Genetics Laboratory. Mexico, D.F.: CIMMYT).

Protocol:

1. Prepare a bulk reaction mixture containing all ingredients except the plasmid DNA:

Stock	Final	Per Reaction
Distilled H_2O		15.9 µl
10X PCR buffer	1X	2.5 µl
dNTPs (10 mM each)	50 µM each	0.5 (0.125 each) µl
Forward primer (2 µM)	0.2 µM	2.5 µl
Reverse primer (2 µM)	0.2 µM	2.5 µl
Taq enzyme (5 U/µl)	0.5 U	0.1 µl

2. Add 24 µl of the bulk mixture to each tube.

3. Add 1 µl of plasmid DNA to each tube (2 ng/µl).

4. Overlay each sample with 25 µl of ultrapure mineral oil.

5. Amplify in a PCR thermocycler, using the following program:

1 cycle	94°C for 1 min
25 cycles	94°C for 1 min
	55°C for 2 min
	72°C for 2 min
1 cycle	72°C for 1 min

6. Check the amplification by loading 5 µl of each sample onto a 1% agarose gel.

NON-RADIOACTIVE RFLP ANALYSIS

Non-radioactive RFLP analysis (ECL direct nucleic acid labeling and detection system) is adapted from Amersham Life Science manufacturer protocols. Restriction of genomic DNA is performed according to manufacturer specifications, and blotting onto nylon membranes is done by capillarity according to Sambrook, Fritsh, and Maniatis, 1989. Probes are labeled and detected using Amersham's ECL kit (catalog #RPN- 3001).

References:

Sambrook, Fritsh, and Maniatis, 1989 (*Molecular Cloning: A Laboratory Manual*, 2nd edition. CSHL Press Editors).

Protocol:

1. Prepare the labeled probe:
    - Dilute the DNA to a concentration of 10 ng/μl. Use 10 ng of probe for each ml of hybridization buffer. If more is required, make sure to maintain the proper concentration when increasing the volume.
    - Denature the DNA sample in boiling water for 5 min.
    - Cool the sample immediately on ice for 5 min. Centrifuge briefly.
    - Add the labeling reagent in a volume equal to that of the DNA. Mix gently.
    - Add the glutaraldehyde solution in a volume equal to that of the labeling reagent. Mix gently and centrifuge briefly.
    - Incubate for 10 min at 37°C. If not used immediately, the probe can be kept on ice for up to 10 min.

      Note: It is important to maintain the proper DNA: labeling reagent: glutaraldehyde ratio to ensure accurate labeling. Altering the ratio can result in high "background" and lower sensitivity.

2. Carry out the hybridization and stringency washes:
    - Using the required volume of hybridization buffer, at RT, add 0.5 M of MaCl and 5% blocking reagent slowly to the buffer and mix for 1 h with a magnetic stirrer. Then heat to 42°C for 1 hr.
    - Wash the membranes in 5X SSC and place them carefully in a hybridization tube to avoid forming bubbles between the membrane and the glass. Add 15 ml of buffer. If several membranes are being hybridized at once, put them in a plastic container and cover them with hybridization buffer.

- Pre-hybridize for at least 15 min at 42°C, agitating gently. Prepare the probes.
- After the pre-hybridization, add the labeled probe to the pre-hybridization buffer. Incubate overnight at 42°C (with agitation).

  Note: Avoid placing the probe directly on the membrane.
- Heat the Wash 1 buffer to 42°C. Use about 2–5 ml/cm^2 of membrane. Transfer the blots carefully to this solution, and wash for 20 min with gentle agitation, ensuring the temperature does not exceed 42°C. Place the films in the cassettes.
- Wash the blots again, using a fresh Wash 1 buffer at 42°C for 20 min.
- Wash in the Wash 2 buffer for 5 min with gentle agitation at RT. Repeat with a fresh batch of Wash 2 buffer.

  Note: Stringency may be increased by using a lower final concentration of SSC.

3. Carry out signal generation and detection:
   - Add equal volumes of Detection Reagent 1 and Detection Reagent 2 to a tray to ensure that there is enough solution to cover the membrane. Place the membrane and incubate for 1 min.
   - Drain the excess Wash 2 buffer from the membrane and wrap in Saran Wrap™. Avoid the formation of bubbles on the DNA side.
   - In a darkroom, place the membrane in a cassette with the DNA toward the film. If the DNA have a high molecular weight, expose them for 2 hr at RT; if they are amplified fragments, expose them for 10 min.
   - Remove the film and develop. If necessary, expose a new film for the appropriate length of time.

Wash 1

Stock	Final	1 L
Urea	6 M	360 g
SDS	0.4%	4 g
20X SSC	0.5X	25 ml
Distilled H_2O		Up to 1000 ml

Wash 2

Stock	Final	1 L
20X SSC	0.2X	100 ml
Distilled H_2O		Up to 1000 ml

TAE 50X Buffer

Stock	1 L
2 M Tris base	242.0 g
0.05 M EDTA	18.6 g
Distilled H_2O	Up to 1000 ml
(Adjust pH to 8.0 with glacial acetic acid)	

SSC20X Solution

Stock	1 L
0.3 M Na_3 citrate	88.2 g
3 M NaCl	175.3 g
Distilled H_2O	Up to 1000 ml
(Adjust pH to 7.0)	

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a popular molecular marker system because it is technically easy to implement, relatively cheap, and produces results quickly. Here we provide CIP protocols that have been modified to reduce operating costs.

It is essential to standardize amplification conditions and run all samples for comparative analysis using the same protocol and the same PCR equipment. When working with a large number of samples, we use 96-well boxes for DNA storage, and repetitive multichannel pipettes (Eppendorf tubes) and 96-well microplates for PCR amplification.

Reference: Adapted from J. Williams, M. Hanafey, A. Rafalski, and S. Tingley, 1993 (Genetic analysis using amplified polymorphic DNA markers, *Meth. Enz.* 218, 704–740).

Protocol:

1. Compose a base mixture:

Stock	Final	10 µl
Distilled H_2O		4.4 µl – X
10X PCR buffer	1X	1.5 µl
25 mM $MgCl_2$	1.5 mM	0.9 µl
2.5 mM dNTP	0.2 mM each	1.2 µl
10 ng/µl RAPD primer	20 ng	2.0 µl
Taq polymerase	0.5 U	X

   Note: $MgCl_2$ is optional; the reaction buffer includes the recommended amount. Prepare 10% extra base mixture to allow for pipetting errors. The volume of Taq polymerase (X) is adjusted according to manufacturer specifications.

2. Add 10 µl of the base mixture to each microplate well.

3. Add 5 µl of DNA (2 ng/µl for potato 2n = 2x; 4 ng/µl for potato 2n = 4x; 5 ng/µl for sweetpotato 2n = 6x) to the respective wells.

4. Overload each sample with 40–50 µl (1 drop) of mineral oil.

5. Place the microplate in the MJ Research model PTC-100 or in the Techne model PHC-3 PCR thermocycler machine.

6. Carry out amplification according to the following conditions:

No. Cycles	MJ Research PTC-100	Techne PHC-3
1 cycle	94°C for 3 min	92°C for 4 min
40 cycles	94°C for 1 min	92°C for 1 min
	35°C for 1 min	32°C for 1 min
	72°C for 2 min	72°C for 2 min
1 cycle	72°C for 7 min	72°C for 7 min

7. Add 2 µl of loading buffer to each sample in the microplate.

8. Reactions are then loaded on agarose gels (1.4% agarose in 1X TBE; 3 µl ethidium bromide 10 mg/ml for 250 ml gels).

9. Gels are run at 100 V for 4 h (250 ml gels, 2 combs per gel).

10. Stain with ethidium bromide (10 mg/ml), photograph, and evaluate.

10X PCR Buffer

Stock	Final	100 ml
1 M Tris HCl, pH 8.3	100 mM	10 ml
1 M KCl	500 mM	50 ml
1 M $MgCl_2$	15 mM	1.5 ml
Triton-X	1%	1g
Distilled H_2O		Up to 100 ml

10X TBE Buffer

Stock	1 L
Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 ml
Distilled H_20	Up to 1000 ml

SINGLE SEQUENCE REPEATS (SSR) OR MICROSATELLITES

Amplification products can be detected using either a radioactive protocol (which is accurate but has a high workload) or a non-radioactive protocol.

Reference: Adapted from J. Provan, W.J. Powel, and R. Waugh, 1996 [Microsatellite analysis of relationships within cultivated potato (*Solanum tuberosum*), *Theor. Appl. Genet.* 92, 1078–1084].

Protocol:

1. Set up the following reactions:

Stock	Final	Radioactive (10 µl)	Non-radioactive (20 µl)
Distilled H_20		4.05 – X µl	11.2 – X µl
10X reaction buffer	1X	1.0 µl	2.0 µl
5 mM dNTP	0.2 mM (each)	0.4 µl	0.8 µl
10 mM primer	5 pmol	0.5 µl	1.0 µl
Taq polymerase		X	X
DNA template 2 ng/µl		4 µl	5 µl
dCTP [α-^{32}P]	1 µCi	0.05 µl	

Note: The volume of Taq polymerase (X) is adjusted according to manufacturer's specifications. Prepare 10% extra base mixture to allow for pipetting errors. Work quickly on ice once Taq is added to the base mixture or to the genomic DNA (to avoid unspecific amplifications).

2. Carry out amplification according to the following conditions.

1 cycle	94°C for 3 min
30 cycles	94°C for 1 min
	$T°_{ann}$ for 2 min
	72°C for 1.5 min
1 cycle	72°C for 5 min

Note: $T°_{ann}$ determined for each primer pair.

3. Carry out gel electrophoresis and detection:

- *Radioactive method*—Add 2 µl of sequencing gel loading buffer to each tube. Denature the DNA, heating at 94°C for 5 min, chill immediately on ice, and analyze on a polyacrylamide gel (6% acrylamide, 8 M urea, 1X TBE).

- *Non-radioactive method*—Add 2 µl of loading buffer to each tube. Analyze on a Metaphor agarose 3.5% gel at 120 V for 4 hr. The cost of using Metaphor gel electrophoresis can be reduced by mixing 1% Metaphor with 3% agarose. Ethidium bromide (10 mg/ml) is added to the gel (3 µl per 250 ml) and to the electrophoresis buffer (20 µl per 1 L TBE 1X).

10X Reaction Buffer

Stock	Final	10 ml
1 M Tris base	900 mM	9.0 ml
5 M $(NH_4)_2SO_4$	200 mM	0.4 ml
1 M $MgCl_2$	25 mM	0.25 ml
Distilled H_2O		Up to 10 ml

Note: Adjust to pH 9.0 with HCl.

10X TBE Buffer

Stock	1 L
Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 ml
Distilled H_20	Up to 1000 ml

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

This method requires the following precautions: (i) genomic DNA should be of good quality to ensure complete restriction in 2 h; (ii) nucleotides and oligonucleotides should be of the highest quality (not older than 1 year); and (iii) all enzymes and solutions should be separated for AFLP only.

This protocol consists of four steps: (I) restriction digestion of the genomic DNA; (II) ligation of specific adapters; (III) amplification of the restricted DNA fragments; (IV) selective amplification of the restricted DNA fragments.

Reference: Adapted from P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. vande Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau, 1995 (AFLP: A New Technique for DNA Fingerprinting. *Nucl. Acid Res.* 23, 4407–4414).

I. Restriction Digestion of the Genomic DNA

5X RL Buffer

Stock	Final	1 ml
1 M Tris HAc, pH 7.5	50 mM	50 µl
1 M Mg Cl_2	50 mM	50 µl
1 M Kac	250 mM	250 µl
250 mM DTT	25 mM	100 µl
10 µg/µl BSA	250 ng/µl	25 µl
Distilled H_2O		Up to 1000 µl

Note: Aliquot in 1 ml and store at –20°C. Ac = acetate.

Protocol:

1. Set up the Restriction Master Mix (RMM):

Reagents	Per Reaction (µl)
Distilled H_2O	10.25
5X RL buffer	8.00
10 U/µl EcoRI	0.50
4 U/µl MseI	1.25

2. Set tubes on amplification plate:

Genomic DNA (1 µg)	In 20 µl distilled H_2O
RMM	20 µl
Total	**40 µl**

3. Incubate for 3 hr at 37°C.

4. Check for complete restriction digestion by 1% agarose gel electrophoresis using 5 µl.

II. Ligation of Specific Adapters

Prepare the EcoA and MseA adapter solutions:

EcoA (5 µM):

	µg	Stock	600 µl
EcoA1	17	9.459 µg/µl	1.8
EcoA2	15	13.268 µg/µl	1.1
Distilled H_2O			Up to 600 µl

MseA (50 µM):

	µg	Stock	140 µl
MseA1	40	9.437µg/µl	4.25
MseA2	35	10.721µg/µl	3.25
Distilled H_2O			Up to 140 µl

***Protocol*:**

1. Set the Ligation Master Mix (LMM):

Reagents	Per Reaction (µl)
Distilled H_2O	9
EcoA	1
MseA	1
10 mM ATP	1
5X RL buffer	2
T_4 DNA ligase (1 U/µl)	1

2. Add 15 µl of LMM to the 35 µl of the restricted DNA.
3. Incubate at 37°C for 3 hr.
4. Dilute with 150 µl of $T_{10}E_{0.1}$ to obtain a final volume of 200 µl.
5. Store at –20°C.

III. Amplification of the Restricted DNA Fragments

Prepare the 10X PCR buffer solution:

Stock	Final	10 ml
1 M Tris HCl, pH 8.3	100 mM	1 ml
1 M $MgCl_2$	15 mM	0.15 ml
1 M KCl	500 mM	5 ml
Distilled H_2O		Up to 10 ml

Note: Aliquot in 1 ml and store at –20°C.

Protocol:

1. Set up the Pre-amplification Master Mix (PMM) reaction:

Stock	Per Reaction (µl)
Distilled H_2O	10.92
EcoA1 50 ng/µl	0.60
MseA1 50 ng/µl	0.60
5 mM dNTPs	0.80
10X PCR buffer	2.00
Taq polymerase (5 U/µl, Perkin-Elmer)	0.08

Note: Dilute the EcoA1 and MseA1 priners to 50 ng/µl.

2. Set up the reactions:

DNA restricted and ligated	5 µl
PMM	15 µl
Total	**20 µl**

3. Perform 20 amplification cycles:

20 cycles	92°C for 60 sec
	60°C for 30 sec
	72°C for 60 sec

4. Dilute the 20 µl in 500 µl $T_{10}E_{0.1}$ and store at –20°C.

IV. Selective Amplification of the Restricted DNA Fragments

Prepare the 10X T_4 kinase buffer solution:

Stock	Reagents	10 ml
1 M Tris HCl, pH 7.5	250 mM	2.5 ml
1 M $MgCl_2$	100 mM	1 ml
250 mM DTT	50 mM	2 ml
100 mM spermidine (3 HCl form)	5 mM	0.5 ml
Distilled H_2O		Up to 10 ml

Note: Aliquot in 1 ml and store at –20°C.

Protocol:

1. Label the EcoRI primer and set the labeling reaction:

Reagents	Per Reaction (µl)
Distilled H_2O	0.24
Eco32 (50 ng/µl)	0.1
10X T_4 buffer	0.05
γP^{33} ATP (10 µCi/µl of 2000 Ci/mmol)	0.1
T_4 kinase (Pharmacia 10 U/µl)	0.01

2. Incubate at 37°C for at least 30 min.

3. Denature the enzyme by incubating at 70°C for 10 min and placing on ice.

4. Set up the Amplification Master Mix (AMM):

Primer/dNTPs	Per Reaction (µl)
Distilled H_2O	11.02
Eco* 50 ng/µl primer	0.50
Mse 50 ng/µl primer	0.60
5 mM dNTPs	0.80
10X PCR Buffer	2.00
Taq polymerase (5 U/µl, Perkin-Elmer)	0.08

5. Set up the reactions:

Pre-amplified DNA	5 µl
AMM	15 µl
Total	**20 µl**

6. Perform the amplification cycle:

Cycle 1:	94°C for	30 sec
	65°C for	30 sec
	72°C for	60 sec
Cycles 2–13:	94°C for 30 sec	
	(65 – 0.7)°C in each cycle for 30 sec	
	72°C for 60 sec	
Cycles 14–36:	94°C for 30 sec	
	56°C for 30 sec	
	72°C for 60 sec	

7. Carry out gel analysis on a 6% denatured polyacrylamide gel with 7 M of urea:

$T_{10}E_{0.1}$ Buffer

Stock	Final	1L
1 M Tris HCl, pH 8.0	10 mM	10 ml
0.5 MEDTA,pH 8.0	0.1 mM	0.2 ml
Distilled H_2O		Up to 1000 ml

Note: Sterilize in autoclave and store at RT.

AFLP Primers and Adapters for EcoRI and MseI

EcoRI adapter

EcoA1: 5-CTC GTA GAC TGC GTA CC-3

EcoA2: 5-AAT TGG TAC GCA GTC-3

Selective primers

Primer +0:	5-GAC TGC GTA CCA ATT C-3	E00
Primers +1:	5-GAC TGC GTA CCA ATT C|A-3	E01
Primers +3:	5-GAC TGC GTA CCA ATT C|AA A-3	E31
	5-GAC TGC GTA CCA ATT C|AA C-3	E32
	5-GAC TGC GTA CCA ATT C|AA G-3	E33
	5-GAC TGC GTA CCA ATT C|AA T-3	E34
	5-GAC TGC GTA CCA ATT C|AC A-3	E35
	5-GAC TGC GTA CCA ATT C|AC C-3	E36
	5-GAC TGC GTA CCA ATT C|AC G-3	E37
	5-GAC TGC GTA CCA ATT C|AC T-3	E38
	5-GAC TGC GTA CCA ATT C|AG A-3	E39
	5-GAC TGC GTA CCA ATT C|AG C-3	E40
	5-GAC TGC GTA CCA ATT C|AG G-3	E41
	5-GAC TGC GTA CCA ATT C|AG T-3	E42
	5-GAC TGC GTA CCA ATT C|AT A-3	E43
	5-GAC TGC GTA CCA ATT C|AT C-3	E44
	5-GAC TGC GTA CCA ATT C|AT G-3	E45
	5-GAC TGC GTA CCA ATT C|AT T-3	E46

MseI adapter

MseA1: 5-GAC GAT GAG TCC TGA G-3

MseA2: 5-TAC TCA GGA CTC AT-3

Selective primers

Primer +0:	5-GAT GAG TCC TGA GTA A	M00
Primers +1:	5-GAT GAG TCC TGA GTA A|A-3	M01
	5-GAT GAG TCC TGA GTA A|C-3	M02

Primers +3:	5-GAT GAG TCC TGA GTA A|AA	A-3	M31
	5-GAT GAG TCC TGA GTA A|AA	C-3	M32
	5-GAT GAG TCC TGA GTA A|AA	G-3	M33
	5-GAT GAG TCC TGA GTA A|AA	T-3	M34
	5-GAT GAG TCC TGA GTA A|AC	A-3	M35
	5-GAT GAG TCC TGA GTA A|AC	C-3	M36
	5-GAT GAG TCC TGA GTA A|AC	G-3	M37
	5-GAT GAG TCC TGA GTA A|AC	T-3	M38
	5-GAT GAG TCC TGA GTA A|AG	A-3	M39
	5-GAT GAG TCC TGA GTA A|AG	C-3	M40
	5-GAT GAG TCC TGA GTA A|AG	G-3	M41
	5-GAT GAG TCC TGA GTA A|AG	T-3	M42
	5-GAT GAG TCC TGA GTA A|AT	A-3	M43
	5-GAT GAG TCC TGA GTA A|AT	C-3	M44
	5-GAT GAG TCC TGA GTA A|AT	G-3	M45
	5-GAT GAG TCC TGA GTA A|AT	T-3	M46
	5-GAT GAG TCC TGA GTA A|CA	A-3	M47
	5-GAT GAG TCC TGA GTA A|CA	C-3	M48
	5-GAT GAG TCC TGA GTA A|CA	G-3	M49
	5-GAT GAG TCC TGA GTA A|CA	T-3	M50
	5-GAT GAG TCC TGA GTA A|CC	A-3	M51
	5-GAT GAG TCC TGA GTA A|CC	C-3	M52
	5-GAT GAG TCC TGA GTA A|CC	G-3	M53
	5-GAT GAG TCC TGA GTA A|CC	T-3	M54
	5-GAT GAG TCC TGA GTA A|CG	A-3	M55
	5-GAT GAG TCC TGA GTA A|CG	C-3	M56
	5-GAT GAG TCC TGA GTA A|CG	G-3	M57
	5-GAT GAG TCC TGA GTA A|CG	T-3	M58
	5-GAT GAG TCC TGA GTA A|CT	A-3	M59
	5-GAT GAG TCC TGA GTA A|CT	C-3	M60
	5-GAT GAG TCC TGA GTA A|CT	G-3	M61
	5-GAT GAG TCC TGA GTA A|CT	T-3	M62

NON-RADIOACTIVE AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

This protocol consists of four steps: (I) restriction digestion of the genomic DNA; (II) ligation of specific adapters; (III) pre-amplification reaction; (IV) selective AFLP amplification.

The AFLP Core Reagent kit and the AFLP Starter Primer Kit protocols have been modified to increase AFLP reactions. Amplification products are detected by silver-staining after denaturing sequencing gel electrophoresis.

Reference: Adapted from Life Technologies (Rockville, MD, USA) protocols.

I. Restriction Digestion of the Genomic DNA

Protocol:

1. Add the following to an Eppendorf tube:

Stock	Per Reaction
5X reaction buffer	5 µl
DNA (250 ng in 18 µl)	18 µl
*Eco*RI/*Mse*I	2 µl

2. Mix gently. Incubate for 2 hr at 37°C.

3. Deactivate restriction endonucleases by incubating 15 min at 70°C. Place tube on ice.

II. Ligation of Specific Adapters

Protocol:

1. Add the following to the digested DNA:

Stock	Per Reaction
Adapter ligation solution	24 µl
T4 DNA ligase	1 µl

2. Mix gently and incubate at 20°C for 2 hr.

3. Perform a 1:10 dilution of the ligation mixture using the TE buffer included in the kit. Dilute a small amount of the reaction mixture (unused portion may be stored at –20°C).

III. Pre-Amplification Reaction

Protocol:

1. Add the following to an amplification tube/plate:

Stock	Per Reaction
Pre-amp primer mix	10 µl
10X PCR buffer for AFLP	1.25 µl
Taq DNA polymerase (1 unit/µl)	0.25 µl
Diluted template DNA	1.25 µl

2. Perform the amplification cycle according to the following conditions:

20 cycles	94°C for 30 sec
	56°C for 60 sec
	72°C for 60 sec

3. Perform a 1:10 dilution with the TE buffer included in the kit. Prepare enough for 30 selective AFLP amplifications (unused portions can be stored at –20°C).

IV. Selective AFLP Amplification

Protocol:

1. For each primer pair, add the following components to an amplification tube/plate:

Stock	Per Reaction
AFLP-grade water	3.41 µl
EcoR I primer	0.09 µl
Mse I primer	2.25 µl
10X PCR buffer for AFLP	1.0 µl
Taq DNA polymerase (1 unit/µl)	0.25 µl
Diluted template DNA	3 µl

2. Perform the amplification cycle according to the following conditions:

Cycle 1	94°C for 30 sec 65°C for 30 sec 72°C for 60 sec
Cycles 2–13	94°C for 30 sec (65 – 0.7)°C, 30 sec each cycle 72°C for 60 sec
Cycles 14–36	94°C for 30 sec 56°C for 30 sec 72°C for 60 sec

3. Carry out gel analysis on a 6% denaturing polyacrylamide gel with 7 M urea.

4. Carry out detection according to the silver-staining protocol.

Stock	Final	1 L
1 M Tris HCl, pH 8.0	10 mM	10 ml
0.5 M EDTA, pH 8.0	1 mM	2 ml
Distilled H_2O		Up to 1000 ml

Note: Sterilize in autoclave and store at RT.

DNA GEL ELECTROPHORESIS

Preparation of Recycled Agarose Gels (1.4%)

This protocol has been devised for re-use of agarose gels. While no recycling limit has been determined as yet, the separation quality should be checked by comparing the standard band separation with the standard DNA ladder.

***Protocol*:**

1. Wash three 250-ml agarose gels (1.4%) overnight in 5 L of distilled water (with agitation).

2. Melt the agarose in a microwave oven.

3. If debris is visible in the liquid agarose solution, filter it with an 0.8-µm Millipore nitrocellulose filter type AA. Maintain the gel in liquid form.

4. Combine 75 ml of 10X TBE and 12 µl of ethidium bromide (10 mg/ml).

5. Add distilled water to the gel solution, increasing the volume to 750 ml.

6. Pour the gels into molds with two combs.

7. Store for 1 hr (with the combs) at RT or for 30 min at 4°C.

Loading Buffer

Stock	100 ml
0.25% bromophenol blue	0.25 g
40% w/v sucrose	40 g
Distilled H_2O	Up to 100 ml

Note: For applications other than RAPD, add 35 mg of Xylene Cyanol, 35 mg of bromphenol blue, and 50 mg of Orange G.

10X TBE Buffer

Stock	1 L
Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8.0	40 ml
Distilled H_2O	Up to 1000 ml

Polyacrylamide Gel for DNA Electrophoresis

In this protocol denatured PAGE gel is used for DNA sequencing and for microsatellite and AFLP amplification product separation on Model S2 sequencing gel electrophoresis apparatus (Life Technologies, Rockville, MD, USA).

Reference: Adapted from Promega (Madison, WI, USA) manufacturer protocols.

Protocol:

1. Prepare the sequencing plates:

   Note: The glass plates must be meticulously clean. Rinse washed plates thoroughly in de-ionized water to remove residue and perform a final ethanol wash of plates.

Short glass plate:

- Prepare fresh binding solution by adding 3 µl of bind silane to 1 ml of 95% ethanol and 3 µl of glacial acetic acid.
- Wipe scrupulously cleaned plate using tissue paper moistened with 1 ml of freshly prepared binding solution. Make sure the plate is completely covered.
- After 4–5 min, apply approximately 2 ml of 95% ethanol to the tissue paper, wiping first in one direction and then perpendicular to the first direction, using gentle pressure. Repeat this wash three times, using fresh paper towel each time to remove excess binding solution (this step is essential to prevent contamination from the binding solution, which could tear the gel).

Long glass plate:

- Change gloves before preparing the long glass plate to prevent cross-contamination with the binding solution.
- Wipe a scrupulously cleaned plate with tissue saturated with repellant solution.
- After 5–10 min, remove the excess repellant solution by wiping the plate with tissue.

2. Prepare the sequencing gel:

   - Assemble the glass plates ("sandwich-style") just prior to pouring the gel. Special care should be taken to prevent leakage and to ensure uniform pressure of the clamps and adequate combs.

- Prepare the "pre-mix" solution using 6% polyacrylamide (20:1 acrylamide:bisacrylamide:

Stock	Final	1 L
Urea	7 M	420 g
Acrylamide-Bisacrylamide	6%	57 g – 3 g
10X TBE	1X	50 ml
Milli-Q H_2O		Up to 1000 ml

- Filter the solution and store it at 4°C in a bottle wrapped in aluminum.
- Prepare the gel with 0.4-mm spacers as follows:

Polyacrylamide pre-mix solution	60 ml
TEMED	33 µl
10% Ammonium persulfate	33 µl

- *Pour the gel solution*—Make sure there are no bubbles. If there are any, position the glass plate sandwich vertically, and tap the plates gently. Place the combs between the plates carefully. Let them polymerize horizontally for at least 2 hr. Pre-run the gel at 500 V for about 15–30 min to equilibrate.

- *Load the samples*—Run the gel at 400 V overnight, or run a rapid electrophoresis at 1200 V for 5–6 hr.

- *Fix the DNA*—Put the short glass plate in a 10% acetic acid solution (make sure that the gel is on top). Shake it homogeneously for 20 min (this step is critical for DNA precipitation and urea removal). The DNA can be detected by autoradiography, when labeled with radioactive compound, or by silver-staining.

- *Perform autoradiography*—Assemble the "sandwich-style" glass-plate system again, this time with the film between the plates. Be sure to store it in dark place for two days. Develop the film. If the film sticks to the gel there is either too much urea (creating visible urea crystals) or the gel was not completely dry before use.

- *Silver-stain the sequencing gel*—Use the following protocol:

10X TBE

Stock	1 L
Tris base	108 g
Boric acid	55 g
0.5 M EDTA, pH 8,0	40 ml
Distilled H_2O	Up to 1000 ml

Loading Dye

Stock	10 ml
Formamide	9.4 ml
0.2 M EDTA	500 µl
Dye	100 µl

Note: Dye = 50 mg xylene cyanol + 50 mg bromophenol dissolved in 1 ml of distilled H_20.

Silver-Staining the Sequencing Gel

Silver-staining is used to detect microsatellites and AFLP amplification products. Water and chemical quality has a great influence on the staining process.

Reference: Adapted from Promega (Madison, WI, USA) manufacturer protocols.

Protocol:

1. Prepare the solutions:

   - *Fixing/stop solution (10% glacial acetic acid)*—Add 100 ml of glacial acetic acid to 900 ml of ultrapure (Milli-Q) or double-distilled water. Prepare two separated solutions and save for re-use.
   - *Staining solution*—Combine 2 g of silver nitrate ($AgNO_3$) and 3 ml of 37% formaldehyde in 2 L of ultrapure water.
   - *Developing solution*—Dissolve 60 g of sodium carbonate (Na_2CO_3) in 2 L of ultrapure water. Chill to 10°C in an ice bath. ***Immediately before use*** add 3 ml of 37% formaldehyde and 400 µl of sodium thiosulfate (10 mg/ml).

2. Separate the plates:

   - After electrophoresis, carefully separate the plates using a plastic wedge. The gel should be attached securely to the short glass plate.

3. Fix the gel:

   - Place the gel (the plate) in a shallow plastic tray, cover with fix/stop solution, and agitate well for 20 min or until the tracking dye is no longer visible.

4. Wash the gel:

   - Rinse the gel twice (2 min each time) with ultrapure water using agitation. Take gel (plate) out of the wash and let it drain 10–20 sec before moving it to the next wash.

5. Stain the gel:

   - Transfer the gel to the staining solution and agitate well for 30 min.
   - Complete the preparation of the developing solution. Pour half of the pre-chilled solution into a tray, and set it aside. Keep the remaining solution on ice.

     Note: The timing of the next [rinse] step is very important. Rinsing for too long can result in weak or no signals.

6. Rinse the gel:

   - Dip the gel briefly into the tray containing ultrapure water, drain, and place the gel immediately into the tray of chilled developing solution.

     Note: The time taken to dip the gel in the water and transfer it to developing solution should not exceed 5–10 sec.

7. Develop the gel:

   - Agitate the gel well until the template band starts to develop.
   - Transfer the gel to the remaining chilled solution, and continue developing for an additional 2–3 min or until all bands are visible.

8. Fix the gel:

   - To terminate the developing reaction and fix the gel, pour the gel into the tray containing the stop solution, and incubate (with agitation) for 2–3 min.

9. Rinse the gel twice in ultrapure water (2 min each time).

10. Dry the gel by storing it at RT or by using convection heating.

11. Make permanent records of the results. APC film, a convenient format for keeping permanent records of silver-stained gels, produces a direct-positive, mirror image of the original.

    Note: The gel must be completely dry before APC film development. Handle all plates with gloves to avoid fingerprints.

    - In a darkroom (using a safelight) place the dry, stained gel attached to the plate (gel side up) on a white fluorescent light box.
    - Place the APC film, emulsion-side down, over the gel to be copied. Place a clean glass plate on top of the film to maintain contact between the gel and the film. Turn on the lightbox and expose the film for 1–2 min. The length of the exposure time may vary with different light sources. Find the optimum exposure time by exposing strips of the APC film for various time intervals.
    - Develop the film as follows: submerge in developer solution for 1–5 min, wash in de-ionized water for 1 min, submerge in fixer solution for 3 min, and wash in de-ionized water for 1 min.

PURIFICATION OF TAQ POLYMERASE (SHORT PROTOCOL)

The preparation used for this protocol will also be used in PCR amplifications, so be sure to use molecular biology–grade reagents and to avoid contamination with biological material or metal ions.

Reference: Adapted from F.G. Pluthero, 1993 (Rapid purification of high-activity Taq DNA polymerase. *Nucleic Acids Research* 21, 4050–4051).

Protocol:

1. Add 500 µl of an *E. coli* culture (cultivated overnight and containing the pTaq plasmid) to 1 L of LB with ampicillin (100 mg/L).

2. Incubate at 37°C to OD_{600} = 0.8 (approximately 3–6 hr). Add IPTG to a final concentration of 125 mg/L (using 625 µl from a filter-sterile solution of 5 g/ml).

3. Continue incubation for 12 hr.

4. Harvest the cells by centrifugation at 6000 rpm in a GSA rotor for 10 min.

5. Wash with 100 ml of Buffer A, re-suspend, and centrifuge at 5000 rpm for 10 min.

6. Re-suspend cells in 50 ml of pre-lysis buffer (Buffer A + 4 mg/ml lysozyme) and incubate at RT for 15 min.

7. Add 50 ml of lysis buffer and incubate at 75°C in pyrex flask for 1 hr.

8. Transfer the mixture to plastic bottles and centrifuge at 15,000 rpm for 10 min at 4°C in a SA-600 rotor.

9. Transfer the clarified lysate to a clean pyrex flask.

10. Add 30 g of $(NH_4)_2SO_4$ per 100 ml of lysate while stirring rapidly at RT.

11. Centrifuge at 15,000 rpm for 10 min. Discard the supernatant.

12. Dissolve the protein pellet with 20 ml of Buffer A per 100 ml of original clear lysate.

13. Dialyze the proteins with storage buffer at 4°C (changing solution after 12 hr).

14. Dilute the resulting solution 1:1 with sterilized storage buffer and store in aliquots at –70°C.

15. Estimate the polymerase activity according to commercial Taq polymerase standards.

Buffer A

Stock	Final	500 ml
1 M Tris HCl, pH 7.9	50 mM	25 ml
Dextrose	50 mM	4.5 g
0.5 M EDTA	1 mM	1 ml
Distilled H_2O		Up to 500 ml

Lysis Buffer

Stock	Final	100 ml
1 M Tris HCl, pH 7.9	10 mM	1 ml
1 M KCl	50 mM	5 ml
0.5 M EDTA	1 mM	0.2 ml
PMSF	1 mM	17.4 mg
Tween 20	0.5%	0.5 ml
Nonidet P40	0.5%	0.5 ml
Distilled H_2O		Up to 100 ml

Note: Make sure to dissolve PMSF in dimethylsulfoxide (DMS) before use.

Storage Buffer

Stock	Final	2 L
1 M Tris HCl, pH 7.9	50 mM	100 ml
KCl	50 mM	7.5 g
0.5 M EDTA	0.1 mM	0.4 ml
DTT	1 mM	0.3 g
PMSF	0.5 mM	0.2 g
Glycerol	50%	1000 ml
Distilled H_2O		Up to 2000 ml