

# Standard Operating Procedures For Accredited Test Laboratory

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(Name & Address of Accredited Test Laboratory)

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सत्यमेव जयते

**National Certification System for Tissue Culture Raised Plants (NCS-TCP)**  
**Department of Biotechnology, Government of India**  
**New Delhi**

**May 2008**

<b>SOPs for Accredited Test Laboratory</b>		
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**1. Document Issue & Revision:**

This document issue and revision is controlled by the Document Approval Authority (Department of Biotechnology). As and when a section of this document is revised, the revised section is issued in its entirety together with a revision number, identifying the new issue status and the issue date of each section. The revised sections are automatically issued to each of this document copy holders listed in Section 2 of 'Control of Document':

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### 1.1. Scope:

This document provides guidance and describes the standard operating procedures (SOPs) for Accredited Test Laboratory involved in virus/quality (genetic fidelity) testing and certification of tissue culture raised plants.

### 1.2. Purpose

The purpose of this document is to facilitate adoption of standard operating procedures by all the accredited test laboratories under National Certification System for Tissue Culture Plants established by the Department of Biotechnology, Ministry of Science & Technology for accreditation of test laboratory in accordance with the guidelines established by the Department of Biotechnology, in exercise of the powers conferred under Section 8 of the Seeds Act, 1966.

### 1.3. Definitions & Terms:

<b>Accredited Test Laboratory</b>	A test laboratory accredited by the Department of Biotechnology for virus/quality (genetic fidelity) testing and certification of tissue culture raised plants.
<b>Acclimatization</b>	It is a physiological adaptation of plants to climate or environment such as, light, humidity, temperature, etc.
<b>Recognized tissue culture production facility</b>	A tissue culture production facility recognized by the Department of Biotechnology for quality production of tissue culture plants.
<b>Clone</b>	A progeny of plant derived through vegetative propagation having identical genetic make-up with that of parent plant.
<b>Controlled Document:</b>	Documents formally identified. These documents are registered, maintained and their change, as well as, their implementation is regulated.
<b>Controlled Record:</b>	A record that requires to be kept and maintained under safeguard for future reference
<b>Culture Medium</b>	It is a liquid or gelatinous substance containing nutrients for the growth of explants.
<b>Corrective Action</b>	Action to eliminate the cause of a detected non-conformity.
<b>Data</b>	Quantified information in documents.

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<b>Document:</b>	Procedures, work instructions, references, specifications or regulatory material for the administration of the system.
<b>Explant</b>	An explant is any portion of the plant that will be used to initiate the culture it can be a portion of the shoot or of the leaves or even just some cells.
<b>Hardened Plant</b>	In-vitro derived plants which have developed good root and stem system to grow in the field conditions and ready for field plantation.
<b>Inoculation</b>	Transferring of explants under aseptic condition onto the media in a culture tube/bottle.
<b>Incubation</b>	Maintenance of inoculated plants in a bottle/tube under controlled environment conditions of temperature, light, humidity and nutrients to provide optimal conditions for growth.
<b>Internal Audit</b>	Independent activity to verify, through an exam and evaluation of objective evidence, if the processes and elements applicable to the quality system have been developed, documented and implemented.
<b>Internal Document</b>	Document generated outside the limits of the administrative system for example: a regulatory document that is referred to a procedure or work instruction.
<b>Micropropagation</b>	It is the practice of rapidly multiplying the stock material to produce a large number of progeny plants using modern plant tissue culture methods.
<b>Mother Plant</b>	A plant which acts as a source of material for plant propagation by micropropagation
<b>NCS-TCP</b>	National Certification System for Tissue Culture Raised Plants established by the Department of Biotechnology, Ministry of Science & Technology.
<b>Non-Conformity</b>	Any situation that differs from standard procedures, guidelines or regulations

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<b>Objective Evidence</b>	Data supporting the existence or verify something
<b>Plantlet</b>	A baby plant produced in vitro on an auxenic culture medium from a meristematic plant tissue.
<b>Plant Tissue Culture</b>	Plant tissue culture is a technique of culturing plant cell, tissue or organ in artificial, controlled and aseptic conditions. It mainly covers micropropagation, organogenesis and somatic embryogenesis.
<b>Pest</b>	Any species, strain or biotype of plant, or pathogenic agent, injurious to plants or plant products.
<b>Procedure</b>	Document that describes, "Who does the job", "when", "where", and "why".
<b>Protocol or Work Instruction</b>	A written instruction to carry out a specific task or activity or job
<b>Record:</b>	Document (electronic or print), product or sample statement, which will confirm that a procedure (or part of the procedure) has been carried out.
<b>Somaclonal variation</b>	It is the term used to describe the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are a major source of this variation.
<b>Stock Culture</b>	Tissue culture derived from mother plant
<b>Standard Operating Procedures (SOPs)</b>	Standard operating procedures (SOPs) are sets of written instructions that document the routine or repetitive activity followed by an organization. The development and use of SOPs are an integral part of a successful quality system as it provides individuals with the information to perform a job properly.

#### **1.4. References:**

*Guidelines for accreditation of test laboratory, 2006, Department of Biotechnology, New Delhi.*

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## **1.5. Resource Requirements:**

### **1.5.1. Staff/Training:**

The Accredited Test Laboratory will have a scientist each with a postgraduate/doctorate qualification specialized in Virology/Molecular Biology with substantial experience in heading the Virology/Molecular Biology laboratory, as the case may be, and supported by at least a minimum of one or two laboratory technicians each with postgraduate qualification preferably in Virology/Molecular Biology and or/ related fields and trained in serological (ELISA/DIBA)/molecular testing (RT-PCR/NASH) for viruses, viroids, phytoplasmas and bacteria affecting plants and or/ genetic fidelity tests (AFLP, ISSR, RFLP, RAPD, SSR), as the case may be.

### **1.5.2. Equipments & Reagents:**

#### **1.5.2.1. General Laboratory Equipments:**

- Digital Top Pan/Analytical Balance
- Deep Freezer (-80 °C & -20 °C)
- Distilled/RO/Millipore Unit
- Hot Plate/Magnetic Stirrer
- Autoclave
- Microwave Oven
- Vertex Mixer
- pH/EC Meter
- Refrigerator (4 °C)
- Tissue Grinder/Comodril/Pestle & Mortar
- Incubator Shaker
- Ice Maker
- Microcentrifuge/Minicentrifuge
- Laminar Air Flow Cabinet
- Fume Hood
- Water Bath with Thermostat (30 to 100 °C)
- Laboratory Chemicals/Glassware
- Disposable Gloves

#### **1.5.2.2. Virus Testing Equipments & Supplies:**

##### **Sero-diagnosis**

- ELISA Reader with Printer
- Microplates (96 wells/strips)
- Multichannel Pipette
- Micropipettes (10µl, 50 µl, 100 µl)

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- Enzyme Conjugate & Substrate
- Specific Antisera/Buffers/Reagents
- Nitrocellulose Membranes (for DIBA)

Molecular diagnosis:

- Thermal Cycler
- Horizontal Gel Electrophoresis Unit & Power pack
- Gel Documentation Unit with Computer & Printer
- Hybridization Oven
- Specific Primers
- PCR Tubes (200 µl, 500 µl)
- RNeasy Kit
- Taq DNA Polymerase
- Nucleic Acid Buffers/Reagents
- C-DNA Probes

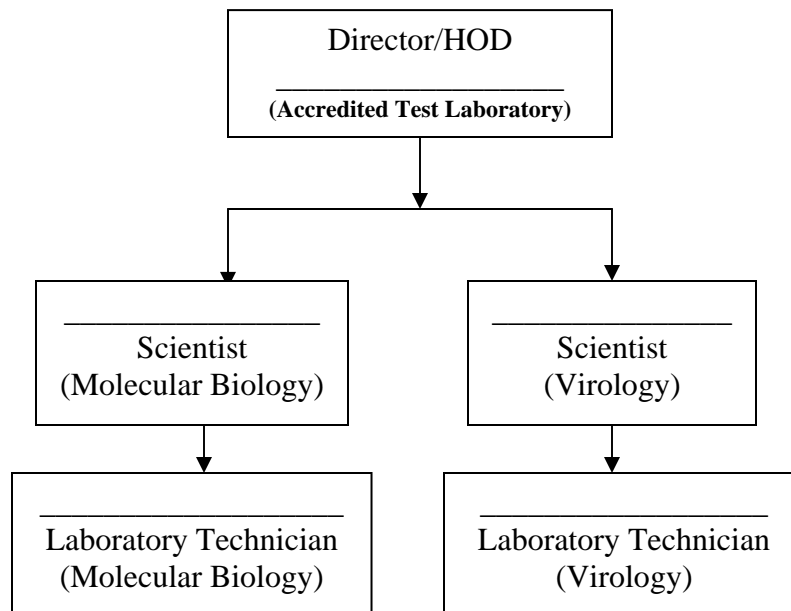
1.5.2.3. Genetic fidelity testing equipments & supplies:

- Thermal Cycler
- DNA Extraction Equipment
- Dry Heating Block
- DNeasy Kit
- Hybridization Oven
- UV Cross Linker
- Horizontal Gel Electrophoresis Unit & Power pack
- Gel Documentation Unit with Computer & Printer
- PCR Tubes (200 µl, 500 µl)
- DNA Sequencer
- Specific Primers
- Specific Markers (RAPD/ISSR/AFLP/SCAR)
- Nucleic Acid Buffers/Reagents

## **2.1. Organization Structure:**

The organization structure of Accredited Test Laboratory is described as under:

### **Organizational Chart of Accredited Test Laboratory**



## **2.2. Responsibilities:**

### **2.2.1. Director/HOD:**

The Director/HOD of Accredited Test Laboratory will be responsible for overall management of Accredited Test Laboratory including both virus & quality (genetic fidelity) testing of tissue culture plants. He will be specifically responsible for:

- recruitment of technical/administrative personnel
- approval of purchase of equipments and quality chemicals
- approval of standard operating procedures (SOPs) for Accredited Test Laboratory
- planning resources for activities of tissue culture testing in consultation with Scientists (Virology/Molecular Biology) respectively
- Approval & certification of tissue culture plants and or/ disapproval based on test reports provided by the Scientists (Virology/Molecular Biology) respectively

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### **2.2.2. Scientist (Virology)**

The Scientist (Virology) is responsible for:

- research and development/standardization/validation of virus testing protocols for tissue culture plants
- organizing training of laboratory technicians in virus testing
- maintenance of appropriate records related to virus testing of tissue culture plants
- organizing virus testing of tissue culture plants
- calibration of measuring/monitoring equipments used for virus testing
- issue of test report for virus testing

### **2.2.3. Scientist (Molecular Biology)**

The Scientist (Molecular Biology) is responsible for:

- research and development/standardization/validation of quality (genetic fidelity) testing protocols for tissue culture plants
- organizing training of laboratory technicians in quality (genetic fidelity) testing
- maintenance of appropriate records related to quality testing of tissue culture plants
- organizing quality (genetic fidelity) testing of tissue culture plants
- calibration of measuring/monitoring equipments used for quality testing
- issue of test report for quality (genetic fidelity) testing

### **2.2.4. Laboratory Technicians (Molecular Biology/Virology):**

The technicians attached to each of virus and quality testing laboratory will be responsible to assist the Scientist (Virology/Molecular Biology) respectively in sampling, preparation of sample for testing, carrying out actual tests as per established protocols under the supervision of Scientist (Virology/Molecular Biology) and preparation of test reports and maintenance of records of testing/calibration of equipments etc.

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### **3.1. Receipt/Registration of Application:**

- 3.1.1. An Accredited Test Laboratory will receive an application for virus/quality (genetic fidelity) testing of tissue culture plants (Annexure-3A) from a certified tissue culture production facility sufficiently in advance of shipment along with fees prescribed by the Department of Biotechnology from time to time.
- 3.1.2. The application received at the office of Director/HOD of Accredited Test Laboratory will be verified and complete application will be entered in a certification register (Annexure-3D). The registered application will be forwarded to the Scientist (Virology/Molecular Biology), as the case may be, for organizing sampling of tissue culture plants for virus/quality testing.
- 3.1.3. The Scientist (Virology/Molecular Biology), as the case may be, will intimate the concerned tissue culture production facility (within a maximum of two working days) regarding the requirements for forwardal of sample for testing or else will intimate the site visit of technical personnel from Accredited Test Laboratory for drawl of samples for testing.

### **3.2. Sampling of tissue culture plants:**

- 3.2.1. In case of mother culture every mother plant (clone) will be sampled for virus testing prior to initiation into tissue culture production.
- 3.2.2. In case of tissue culture raised plants (ex-agar plants/hardened plants), the following scale of sampling for virus and or/quality (genetic fidelity) testing, as the case may be, depending on the lot (batch) size of tissue culture plants produced, just prior to dispatch/shipment of consignment.

Lot size	Number of tissue culture plants to be sampled
Up to 1000 Nos	1% plants subject to a minimum of 10 Nos
1001 to 10000 Nos	0.5% of plants subject to a minimum of 10 Nos
10001 to 100000 Nos	0.1% of plants subject to a minimum of 50 Nos

- 3.2.3. Finally a random sample of 10 plants will be collected out of the composite sample drawn from each batch of tissue culture production separately for virus/quality testing without contamination.
- 3.2.4. In case of sampling for quality (genetic fidelity) testing, sample from mother plant (clone) from which tissue culture plants produced will also be collected and forwarded for testing.

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**3.3. Packing/Labelling/Forwardal of samples for testing:**

- 3.3.1. The sample collected will be blotted dry to remove excess moisture before packing.
- 3.3.2. The sample will be placed in between paper towels, packed in self sealing/zip-lock polythene bags of appropriate size. The sample will be affixed with a label (Annexure-3C) and kept in a ventilated card board box and /or thermocool box for forwardal to Accredited Test Laboratory within specified time under cool conditions.
- 3.3.2. The packing box will be marked on top of the box with the address of Accredited Test Laboratory with appropriate instructions such as "*Handle with care/Tissue Culture Plants/Rush Delivery*" and either couriered or delivered in person to the concerned Accredited Test Laboratory within 24 hrs period under cool conditions.

**3.4. Receipt /storage of sample at Accredited Test Laboratory:**

- 3.4.1. The samples on receipt at the Accredited Test Laboratory will be entered in a laboratory test register (Annexure-3D).
- 3.4.2. Each sample will be allotted a unique 12 digit code number (2 digits for ATL/3 digits for tissue culture production facility/6 digits for date of sampling/single digit for the sample (s) drawn) to facilitate proper identification of the sample through out the testing process and divided into three sub-samples. One of the sub-samples will be maintained as reference sample and other two sub-samples will be utilized for testing, as the case may be, along with a job card (Annexure-3E). The replicate samples will be either stored in vacuum desiccator with anhydrous calcium chloride (Ca Cl<sub>2</sub>) or freeze dry or store at -80 °C in a deep freezer at the Accredited Test Laboratory.

## Annexure-3 A

**Application for Virus/Quality (genetic fidelity) Testing & Certification of Tissue Culture Raised Plants**

1. Name/Location Address of the recognised tissue culture production facility:						
2. Certificate No./date of issue/validity:						
3. Name of authorised person & his contact details (Telephone/ Fax/Mobile/E-Mail):						
4. Details of tissue culture plants required to be sampled:						
Plant species	Variety	Accession/ Batch No	Batch size	No of packages	Tests required for	Category of tissue culture material*
						<input type="checkbox"/> Mother plant <input type="checkbox"/> Invitro stock-culture <input type="checkbox"/> Ex-agar washed plants <input type="checkbox"/> Primary hardening plants <input type="checkbox"/> Secondary hardening plants *Tick out in appropriate box
5. Purpose of testing/certification*:					<input type="checkbox"/> Import Quarantine requirements <input type="checkbox"/> Mother plant/stock-culture for initiation <input type="checkbox"/> Domestic sale & distribution <input type="checkbox"/> Phytosanitary certification * Tick out in appropriate box	
6. Particulars of payment of testing fees:						
Amount in Rs:						
Demand Draft/Banker's Cheque No./Date of Issue:						
Bank Name/Branch:						
7. Date by which sampling:						
8. Date by which certification is requested:						
9. Any additional information:						
<b>Declaration</b>						
I/we hereby declare that the information furnished above is complete and correct to the best of my/our knowledge and belief. I/we will meet the TA/DA of technical personnel deputed for sampling in addition to the testing fees as indicated above and provide necessary facilities for sampling.						
Date: _____						
Place: _____						
_____ (Signature/Name/Stamp of Applicant/Date)						
<b>For Office (Testing Facility) Use</b>				<b>Application Reg. No. _____/Date _____</b>		
Check list	Status		Scrutinized by	Action by TCPF	Applicant Response	
Application complete	Yes	No				
Payment of Fees	Yes	No				
Final Action Taken:						
_____ (Signature/Name of Director or HOD of Accredited Test laboratory/Date)						



Annexure-3 C

**Sampling Label**

Sample No*:	<table border="1"> <tr> <td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td> </tr> </table> <p>(12 Digit Code)</p>															
Application Reg. No/Date:																
Plant Species/Variety:																
Lot/Accession No:																
Lot size:																
Tissue Culture Production Facility:																
Date of Sampling:																
Sample size/No of samples drawn:																
Samples drawn by:	<p>_____</p> <p>(Signature/Name/Designation Stamp/Date of Authorised person of/by Accredited Test Laboratory)</p>															
In the presence of:	<p>_____</p> <p>(Signature/Name of authorized person from Tissue Culture Production Facility)</p>															
<p>* Sample Number will be entered by Accredited test laboratory and will be maintained through out certification.</p>																



Annexure-3 E

**Job Card**

1. Plant Species/Variety:	
2. Sample code No:	
3. Sample size:	
4. Sampled by:	
5. Testing For:	
6. Testing Protocol:	
7. Date on which TC samples received:	
8. Date on which test results required:	
9. Sample issued for testing by:	
10. Remarks (Condition of sample/packing):	

\_\_\_\_\_  
Signature of Scientist-incharge  
(Virology//Molecular Biology)

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#### **4. Preparation/handling of samples for testing:**

- 4.1. The laboratory technician will always wear clean laboratory overcoat while working in the laboratory.
- 4.2. He/she will wear fresh disposable gloves while handling nitrocellulose membranes and while carrying out molecular testing viz., RNA/DNA isolation, handling agarose gels containing ethidium dibromide.
- 4.3. He/she will as far as possible handle only one sample at a time during preparation/testing to avoid contamination. If multiple samples are handled at a time, he will ensure to follow appropriate precautions to avoid cross-contamination while pipetting out the sample (antigen), antisera, PCR mix etc.
- 4.4. He/she will thoroughly clean/wash and sterilize the tissue grinder/pestle & mortar/Como drill used for tissue grinding between samples.
- 4.5. He/she will use clean, separate beakers for preparing, antigen, antisera, enzyme conjugate, substrate, buffer solutions and reagents.
- 4.6. He/she will always use clean sterile microtips, while pipetting out samples (antigen), antisera, enzyme conjugates, buffers, nucleic acid reagents etc and change tips at the end of each pipettings in order to avoid cross-contamination.
- 4.7. He/she will prepare stock solutions of buffers/reagents and store them in a refrigerator until use, but however substrate buffer will be prepared fresh.
- 4.8. He/she will not wash or rinse the microplates before use or reuse the used microplates.
- 4.9. He/she will cross-absorb the antisera with a healthy plant sap to remove cross reacting antibodies to false positive reactions if required as per the instructions of manufacturer/provider and both antisera and enzyme conjugates will be diluted according to their titre values as per the instructions of manufacturer/provider.
- 4.10. He/she will use clean, blunt forceps, while handling the nitrocellulose/nylon membranes during testing.
- 4.11. He/she will wear protective clothing against radiation with cassette provided by BARC to measure exposure of radiation, while handling radioactive nucleotides for preparing C-DNA probes.
- 4.12. He/she will always include negative (healthy) and known positive controls to validate the assay.

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- 5.1. The scientist/laboratory technician (plant virology) will use established protocols specified in Annexure-5A for virus testing as approved by the national referral laboratory (i.e.), Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi.
- 5.2. The list of tissue culture plant species and viruses covered under the national tissue culture certification system for tissue culture plants as approved by the Department of Biotechnology are given at Annexure-5B, which may be revised from time to time. However, in respect of export consignments, the virus testing will be carried as per the phytosanitary requirements specified by the importing country.
- 5.3. If any new protocols used or any deviations from established protocols will require validation by the national referral laboratory (i.e.), Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi before their adoption and use.
- 5.4. Where preliminary testing by ELISA revealed is distinctly high readings (say more than 3 times the healthy control), the sample will be rejected for batch certification.. However, if the reading is just near the double of healthy (i.e. threshold value), the results will have to be confirmed with RT-PCR. If ELISA results are clearly negative (i.e. nearby the healthy control), the sample should be cleared for batch certification. However in case of clonal certification, the negative/doubtful results of ELISA required to be confirmed by RT-PCR.
- 5.5. Where preliminary testing by RT-PCR proved to be positive the sample will be rejected for certification. If the results of RT-PCR are negative for virus, the test results will be repeated by NASH to confirm the test results before approving the sample for certification.

## **Virus Testing Protocols**

### **Enzyme-Linked Immunosorbent Assay (ELISA)**

#### **1. Scope/Purpose:**

This protocol describes the testing procedure of Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of plant viruses using microtitre plates. This test employs antisera (virus antibody); universal conjugate (enzyme (alkaline phosphatase) labeled goat anti-rabbit Ig G); and the substrate (p-nitrophenyl phosphate-PNPP) for the detection of viruses.

Two types of testing protocol viz., (a) direct antigen coated enzyme-linked immunosorbent assay (DAC-ELISA); and (b) double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). DAC-ELISA usually employed for screening large number of viruses but in case of plant viruses, where specific strain of virus to be detected, the DAS-ELISA will be employed.

Also at least two negative controls (healthy plant sap) besides buffer controls will be used in order to have more confident background values. It is also advised to cross-adsorb antisera with healthy plant sap to prevent false positives.

#### **2. Requirements of Test**

##### **2.1 Equipments:**

- ELISA Reader with Printer
- Balance
- Magnetic Stirrer
- Microcentrifuge
- Deep Freezers (-20 °C, -80 °C)
- Incubator
- pH Meter
- Refrigerator
- Micropipettes (10µl, 50 µl, 100 µl)
- Multichannel Adjustable micropipette (4-well type)
- Tissue Grinder/Pestle and Mortar
- Polystyrene Microtips

##### **2.2. Supplies:**

- Microplates (polysterene, 96 wells)/strips
- Antisera (polyclonal/monoclonal)
- Antigen (virus affected plant material)
- Enzyme (alkaline phosphatase) labeled goat anti-rabbit Ig G
- Goat Anti-rabbit Enzyme Conjugate (universal conjugate)
- Substrate (p-nitrophenyl phosphate-PNPP)

### 2.3. Buffers/Reagents

- **Stock buffer (phosphate buffer- saline, pH 7.4)**

NaCl	8.0 g
Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	1.44 g or
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	2.90 g or
Na <sub>2</sub> HPO <sub>4</sub>	1.50 g
KH <sub>2</sub> PO <sub>4</sub>	0.20 g
KCl	0.20 g
Distilled water	to make 1 litre

- **Wash buffer (PBS-Tween, PBS-T)**

Add 0.5 ml Tween – 20 to 1 litre PBS.

- **Coating buffer (Carbonate buffer, pH 9.6)**

Na <sub>2</sub> CO <sub>3</sub>	1.59 g
NaHCO <sub>3</sub>	2.93 g
Distilled water	To make 1 litre

- **Enzyme conjugate diluent/buffer (PBS-T polyvinyl-pyrrolidone and ovalbumin, PBS-TPO)**

Add 20.0 g polyvinyl-pyrrolidone (PVP, MW 40,000) and 2.0 g egg ovalbumin to 1 litre PBS-T.

- **Antibody diluent/buffer**

Same as PBS-TPO

- **Substrate buffer (diethanolamine buffer, pH 9.8)**

Diethanolamine	97 ml
Distilled water	800 ml

Adjust the pH to 9.8 with 1N HCl, add about 67 ml and make up the volume to 1 litre with distilled water.

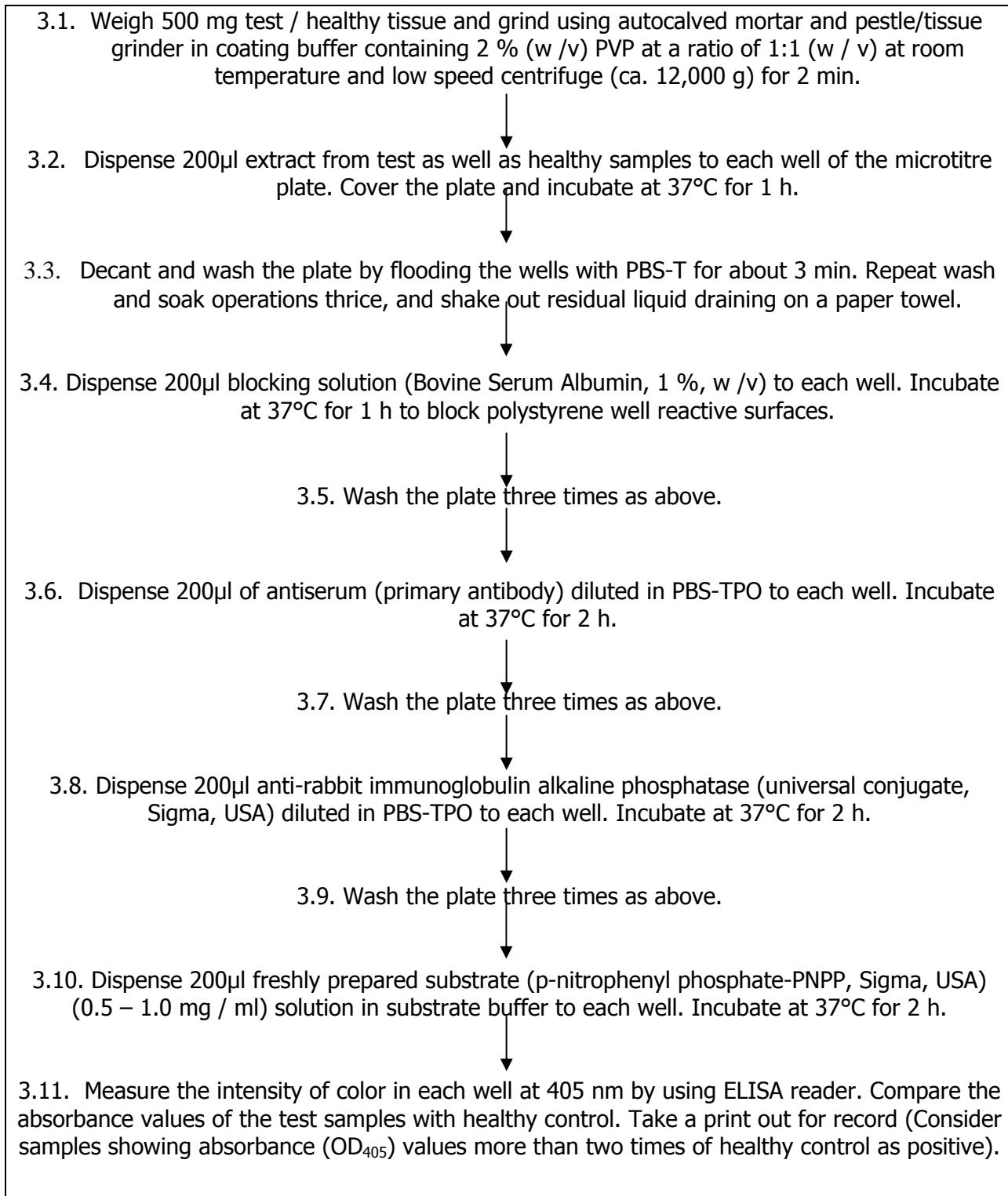
- **Blocking solution**

Add 5.0 g bovine serum albumin (BSA / spray dried milk (SDM) to 1 litre PBS-T.

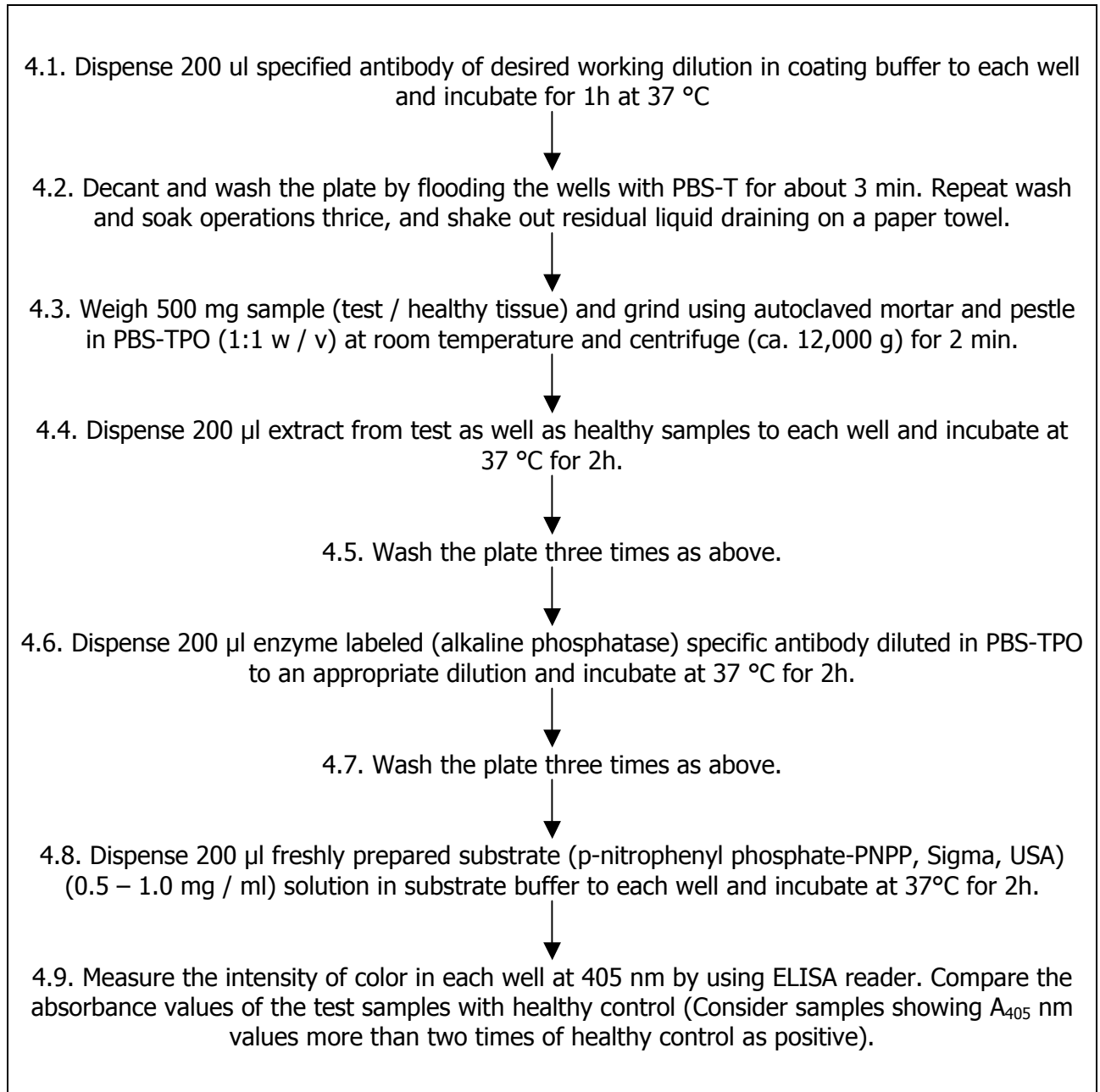
- **Fixing solution**

Na OH 120.0 g  
Distilled water to make 1 litre  
(All buffers contain 0.02 % sodium azide as a preservative)

### 3. Test Protocol: DAC-ELISA



#### 4. Testing Protocol: DAS-ELISA



#### References:

*Clark, M. F. and A. N. Adams. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483.*

*Hampton, R., E. Ball., and S. De Boer. (1990). Serological methods for detection and identification of viral and bacterial plant pathogens- a laboratory manual. APS Press, Minneosta, USA, 389 pp.*

### Helpful Hints:

- Draw test plan in checkerboard format and include minimum of two replications for each treatment.
- Include negative (healthy) control to validate the assay.
- Determine the optimum dilutions of various reagents to be used.
- Do not wash or rinse microtitre plates prior to use. Once used plates should not be reused.
- Use separate beakers for preparing antigen, antiserum, conjugate and substrate dilutions.
- Dilute antisera according to their titre value and commercial conjugates as per the manufacturer's directions.
- Antisera used should be free from cross-reacting antibodies against host proteins to avoid false positive reactions. Otherwise cross-absorb the antiserum with sap extracted from healthy plant by incubating required quantity of antiserum in about 1:50 dilution of healthy plant sap extracted in PBS. Incubate at 37°C for 1 h and centrifuge at 10,000 rpm for 5 min. The supernatant is then directly used.
- Always use freshly prepared substrate buffer.
- PNPP is photo-degradable. Hence cover with aluminium foil to avoid direct contact with light. Use as quickly as possible, once PNPP solution is prepared.

## Dot Immuno-binding Assay (DIBA)

### **1. Scope/Purpose:**

This protocol describes the testing procedure of Dot Immuno-binding Assay (DIBA) for the detection of plant viruses by using nitrocellulose or nylon membranes. This test employs antisera, enzyme labeled Ig G and substrate for the detection of viruses.

### **2. Requirements of Test**

#### **2.1 Equipments:**

- Micropipettes (10 $\mu$ l, 50  $\mu$ l, 100  $\mu$ l)
- Multichannel Adjustable micropipette (4-well type)
- Tissue Grinder/Pestle and Mortar
- Polystyrene microtips

#### **2.2. Supplies:**

- Nitrocellulose/Nylon membranes
- Antisera
- Antigen (virus affected plant material)
- Enzyme (alkaline phosphatase) labeled anti-Ig G
- Substrate (p-nitrophenyl phosphate-PNPP)

#### **2.3. Buffers/reagents:**

- **Stock buffer (tris buffer saline, TBS, pH 7.5)**

0.02 M Tris	4.84 g
0.5 M Na Cl	58.48 g

Adjust the pH to 7.5 with H Cl and make up to 2 litres.

- **Antigen extraction buffer (TBS + 50mM DIECA)**

Add 11.25 g diethyl dithiocarbamate (DIECA) to 1 litre TBS

- **Blocking solution (TBS + SDM)**

Add 5.0 g spray dried milk (SDM) to 100 ml TBS

- **Antibody and enzyme conjugate diluent / buffer**

Same as TBS-SDM

- **Substrate buffer (pH 9.5)**

Bromo chloro indolyl phosphate (BCIP)	50 mg
Dimethyl formamide (DMFA)	1 ml

Adjust the pH 9.5 with 1 N H Cl and make up to 1 litre

- **Substrate solution**

Solution A

0.1 M Tris	12.11 g
0.1 M Na Cl	5.85 g
5 m M MgCl <sub>2</sub> 6H <sub>2</sub> O	1.01 g

Solution B

Nitroblue tetrazolium (NBT)	75 mg
Dimethyl formamide (DMFA)	1 ml

Store solutions A and B refrigerated in amber bottles. Add NBT to 0.33 mg/ml and BCIP to 0.17 mg/ml to the substrate buffer just before use

- **Fixing solution (pH 7.5)**

10 m M Tris	1.21 g
1 m M EDTA	0.29 g

Adjust the pH to 7.5 with 1 N H Cl and make up to 1 litre  
(All buffers contain 0.02 % sodium azide as preservative)

### 3. Testing Protocol: DIBA:

- 3.1. Weigh test and healthy tissue (100 mg) and grind using autoclaved mortar and pestle in antigen extraction buffer at a ratio of 1:10 (w /v) at room temperature and express through cheese cloth.
- 3.2. Pipette out 0.8 ml expressed sap into 1.5 ml eppendorf tube. Add 0.4 ml  $\text{CHCl}_3$ , vortex and centrifuge (ca. 12,000 g) for 2 min.
- 3.3. Pipette out 200 $\mu\text{l}$  clarified sap (upper aqueous layer) into 800 $\mu\text{l}$  antigen extraction buffer and vortex.
- 3.4. Put on the gloves, cut desired size piece of NCM and draw a lattice of squares of 1 x 1cm each with a soft lead pencil. Always use forceps for handling the membrane.
- 3.5. Wet the NCM by floating it in TBS and then air dry. Spot the test / control samples (5-10  $\mu\text{l}$ ) by hand.
- 3.6. Allow NCM to air dry and immerse in blocking solution with gentle oscillation for 1 h at room temperature or overnight at 4°C.
- 3.7. Rinse once in TBS for ca. 10 min.
- 3.8. Incubate for 1 h at room temperature or overnight at 4°C in crude antiserum (primary antibody) diluted in TBS-SDM.
- 3.9. Rinse thrice in TBS for ca. 10 min. each.
- 3.10. Incubate for 1 h at room temperature or overnight at 4°C in secondary antibody (anti-rabbit Ig G alkaline phosphatase) diluted in TBS-SDM.
- 3.11. Rinse thrice in TBS for ca. 10 min. each.
- 3.12. Incubate in substrate solution at room temperature in the dark and watch for color development (good purple color development take 5-10 min.).
- 3.13. Rinse thrice in the fixing solution for ca.10 min. each and then air dry NCM between Whatman filter paper sheets.
- 3.14. Assess the result by visual observation by comparing the intensity of purple color or by using densitometer.
- 3.15. Photograph NCM (dry or wet) and store protected from light.

## References

*Banttari, E.E. and P.H. Goodwin, (1985). Detection of potato viruses S, X and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes. Plant Dis. 69: 202-205.*

*Hawkes, R., E. Niday., and J. Gordon. (1982). A dot immunobinding assay for monoclonal and other antibodies. Anal. Biochem. 119: 142-147.*

## Helpful Hints

- Nylon or PVDF membranes in place of nitrocellulose may also be used.
- Wear gloves while handling membranes.
- NCM, being fragile, should be handled with blunt tipped forceps.
- Always use buffer and negative (healthy) controls.
- The antibody and conjugate solution can be re-used for 3-5 times.
- If the antigen concentration is good, colour development starts immediately after addition of substrate solution.
- Antisera used should be free from cross-reacting antibodies against host proteins to avoid false positive reactions. Otherwise cross-absorb the antiserum with sap extracted from healthy plant by incubating required quantity of antiserum in about 1:50 dilution of healthy plant sap extracted in PBS. Incubate at 37°C for 1 h and centrifuge at 10,000 rpm for 5 min. The supernatant is then directly used.
- The membrane once developed may be photographed or preserved between folds of filter paper for several years.
- Extra care should be taken while spotting the samples. See that sample diameter is less as much as possible to ensure concentration of antigen at the same area. Spotting can be done manually or also through the use of dot - blot apparatus. If done manually, do not use more than 2.5 µl for dotting each time. If planning to dot 10 µl of sample, take 2.5 µl of sample and slowly release it on the centre of the grid area without touching the membrane. Allow it air dry. Repeat this process for another three times.
- If one does not have antiserum or other facilities for processing the membrane, the dotted membrane can be sent any where in the world where such facility exists. The membrane can also be sent back after processing.

# Reverse Transcription-Polymerase Chain Reaction (RT-PCR)/PCR

## **1. Scope/Purpose:**

This protocol describes the testing procedure of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for the detection of plant viruses (RNA Viruses) by using primer-mediated *in vitro* reaction involving amplification of target nucleic acid sequences. Since majority of the plant viruses are RNA viruses, one step reverse transcription-polymerase chain reaction (RT-PCR) is used to amplify a segment of RNA that lies between two regions of known sequences. A one step RT-PCR will have four phases in the process viz., (i) C-DNA synthesis using reverse transcription at 42°C; (ii) denaturation at a high temperature (90°-95°C); (iii) annealing of target specific primers, and (iv) primer extension by a thermostable DNA polymerase. Two-step RT-PCR, which is also known as quantitative real-time PCR is used for detection of low abundance transcripts. DNA Plant viruses are directly detected by PCR techniques.

## **2. Requirements of Test**

### **2.1 Equipments:**

- Thermal Cycler
- Horizontal Gel Electrophoresis Unit with Power Pack
- Gel Documentation Unit with Computer and Printer
- Refrigerated Micro-centrifuge
- Deep Freezers (-20 °C, -80 °C)
- Micropipettes (1µ, 10µl, 50 µl, 100 µl, 1000 µ, 5000 µ)
- Multichannel Adjustable micropipette (4-well type)
- Tissue Grinder/Pestle and Mortar
- Polystyrene microtips

### **2.2. Supplies:**

- PCR Tubes (500µl/200 µl)
- Virus affected plant tissue/healthy tissue
- Specific primers
- RNeasy Kit
- Taq DNA Polymerase

### **2.3. Chemicals and Solutions:**

- **Ethidium bromide (10 mg/ml):**

Dissolve 1 g ethidium bromide in 100 ml H<sub>2</sub>O and transfer to a dark bottle and store at 4°C.

- **0.5 M EDTA (pH 8.0):**

EDTA: 186.1 g

Dissolve in 800 ml H<sub>2</sub>O. Adjust the pH to 8.0 with 10 N Na OH. Make the volume to 1 litre. Dispense into aliquots and sterilize.

- **Running buffer (50 X TAE (Tris Acetate EDTA, pH 8.0))**

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

Adjust pH 8.0 with 1 N Na OH. Make up volume to 1 litre. Dilute 50 X TAE buffer to 1 X before use.

### **3. Testing Protocol- RT-PCR:**

#### **3.1. Isolation of total nucleic acid:**

3.1.1. Weigh test / healthy sample (~100 mg) and cut into strips. Extract total nucleic acid using RNeasy kit according to the manufacturer's instructions. Inactivate the RNA at 70°C for 2 min and then snap chill on ice for 10 min and use this RNA as template for reverse transcription – polymerase chain reaction (RT-PCR).

#### **3.2. cDNA amplification:**

3.2.1. Assemble RT-PCR reaction components on wet ice and prepare amplification mix by dispensing into ~ 200µl microfuge tube in the order listed below (see box)

<b>Composition of PCR mix</b>		
<b>Reagents</b>	<b>Volume required (µl)</b>	
	<b>Test sample</b>	<b>Negative control</b>
10 X PCR buffer	10.0	10.0
5 X Q solution	20.0	20.0
10 mM Dithiothreitol	10.0	10.0
100mM dNTPs	2.0	2.0
Forward primer, 100 pM (100 pM/µl)	1.0	1.0
Reverse primer, 100 pM (100 pM/µl)	1.0	1.0
RNase inhibitor 2 units (4units /µl)	0.5	0.5
Omniscript reverse transcriptase 2.5 units (5units /µl)	0.5	0.5
Taq DNA polymerase 2.5 units (5 units /µl)	0.5	0.5
Template	44.5	44.5
RNase free water	10.0	10.0
<b>Total</b>	<b>100</b>	<b>100</b>

3.2.2. Mix RT-PCR reaction assembly by inversion and place the tubes in a thermal cycler and proceed with thermal cycling profile chosen for reaction (see box).

3.2.3. Analyze the amplified product by electrophoresing 10µl from the total reaction on 1 % agarose gel in Tris – acetate EDTA (TAE) containing ethidium bromide.

Temperature profile for RT-PCR			
Steps	Temperature	Time	Cycle
Reverse transcription	42°C	45 min	1
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	
Annealing	45-50°C	1 min	30
Extension	72°C	1 min	
Final extension	72°C	10 min	1

### 3.3. Analysis of amplicons:

3.3.1. Melt 0.5 g agarose in 50 ml 1 X TAE running buffer and add ethidium bromide (0.5 mg / ml) to the agarose after cooling to around 50°C.



3.3.2. Pour the melted agarose into the casting tray for polymerization and fill the buffer tank with running buffer (1 X TAE) and remove the comb.



3.3.3. Load 10 µl RT-PCR products with 2 µl 6X loading dye and run the gel at 60 volts for 2 h along with marker.



3.3.4. Examine the gel under ultraviolet transilluminator and photograph.

### References:

*Dijkstra, J. and C.P. de Jager, (1998). Practical Plant Virology: protocols and exercises. Springer, New York, 459 pp.*

*Pappu, S.S., A. Brand, H.R. Pappu, E.P. Rybicki, K.H. Gough, M.J. Frankel, C.L. Nzblett. (1993). A polymerase chain reaction method adopted for selective amplification and cloning of 3' sequences of potyviral genomes: application to Dasheen mosaic virus. J. Virol. Methods. 41: 9-20*

### Helpful Hints:

- Always perform a healthy control reaction.
- Use fresh gloves for RNA isolation and each reaction set-up.
- Wear gloves while handling agarose gels containing ethidium bromide.
- Ensure that the RNA used is inactivated at 70°C for 5 min and then snap chill on ice.
- Thaw and vortex all the reagents before use (except DNA polymerase and reverse transcriptase).
- Optimize the annealing temperature for the primer pair before setting the reaction.
- Keep all the reagents at -20°C after use for long storage.
- Ensure 50X TAE buffer is diluted to 1X buffer before running the gel.

# Nucleic Acid Spot Hybridization (NASH)

## **1. Scope/Purpose:**

This protocol describes the testing procedure of Nucleic Acid Spot Hybridization (NASH) for detecting very small amount of virus in the plant material. The detection is based on mobilization of target (virus) NA on to a solid matrix followed by hybridization with NA probes under appropriate conditions. Nitrocellulose or charged nylon membranes are the commonly used solid matrix for hybridization. Both radioactive probes ( $^{32}\text{P}$  DNA probes) as well as non-radioactive probes (chemiluminescent, digoxigenin-labelled cRNA probes) are available for nucleic acid spot hybridization.

## **2. Requirements:**

### **2.1. Equipments:**

- Hybridization oven
- Hybridization flask
- Lucite screen, a separate room for handling radioactivity, X-ray cassette, X-ray film developing facility, radioactivity, monitor vacuum oven, shaking water bath, bag sealer, etc.

### **2.2. Supplies:**

- Nitrocellulose or nylon membranes
- Pestle and mortar/tissue grinder
- Virus infected and healthy plant samples

### **2.3. Buffers and Reagents:**

- **Standard saline citrate (SSC) buffer 20X, pH 7.0:**

Na Cl (3.0 M)	175.3 g
Na Citrate (0.30 M)	88.2 g

Dissolve in 800 ml  $\text{H}_2\text{O}$ . Adjust the pH to 7.0 and make up the volume to 1 litre. Dispense into aliquots and sterilize before storing at room temperature.

- **Tris H Cl- EDTA (TE) buffer (pH 8.0):**

Tris 10m M: 121.1 g

Dissolve in 800 ml  $\text{H}_2\text{O}$ . Adjust the pH to 8.0 with concentrated H Cl. Make the volume to 1 litre. Dispense into aliquots and sterilize.

EDTA 0.5 M: 186.1 g

Dissolve in 800 ml H<sub>2</sub>O. Adjust the pH to 8.0 with 10 N Na OH. Make the volume to 1 litre. Dispense into aliquots and sterilize. Make appropriate dilutions to prepare required amount of 1X TE.

- **Oligolabelling buffer (5X):**

Tris Cl (pH 8.0)	250 mM
MgCl <sub>2</sub>	25 mM
β- mercaptoethanol	5 mM
ATP, GTP, TTP	2 mM each
HEPES (adjusted to pH 6.6 with 4N Na OH)	1 M
Oligonucleotides	1 mg / ml

- **SSPE 20X (pH 7.4):**

Na Cl	175.3 g
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	27.6 g
EDTA	7.4 g

Dissolve in 800 ml H<sub>2</sub>O. Adjust the pH to 7.4 with Na OH and make up the volume to 1 litre. Dispense into aliquots and sterilize by autoclaving.

- **10 % SDS:**

Dissolve 100 g analar grade SDS in 900 ml H<sub>2</sub>O. Heat at 68°C to dissolve. Adjust the pH to 7.2 by adding few drops of concentrated HCl. Adjust the volume to 1 litre. Dispense into aliquots.

- **0.5 N Na OH:**

Dissolve 20 g Na OH in 800 ml H<sub>2</sub>O and make up the volume to 1 litre (no need to sterilize).

- **3 M sodium acetate, pH 5.0:**

Dissolve 408.1 g of sodium acetate 3 H<sub>2</sub>O in 800 ml H<sub>2</sub>O. Adjust the pH to 5.0 with glacial acetic acid. Adjust the volume to 1 litre and autoclave.

- **Bovine serum albumin (1 %):**

Dissolve 10 mg of BSA in 1 ml H<sub>2</sub>O. Filter sterilize and aliquot them. Store at -20°C.

- **Stop buffer:**

Na Cl	20 mM
Tris H Cl	20 mM
EDTA	2 mM
SDS	0.25 %
dCTP	1 mM

- **Denhardt's reagent 50X:**

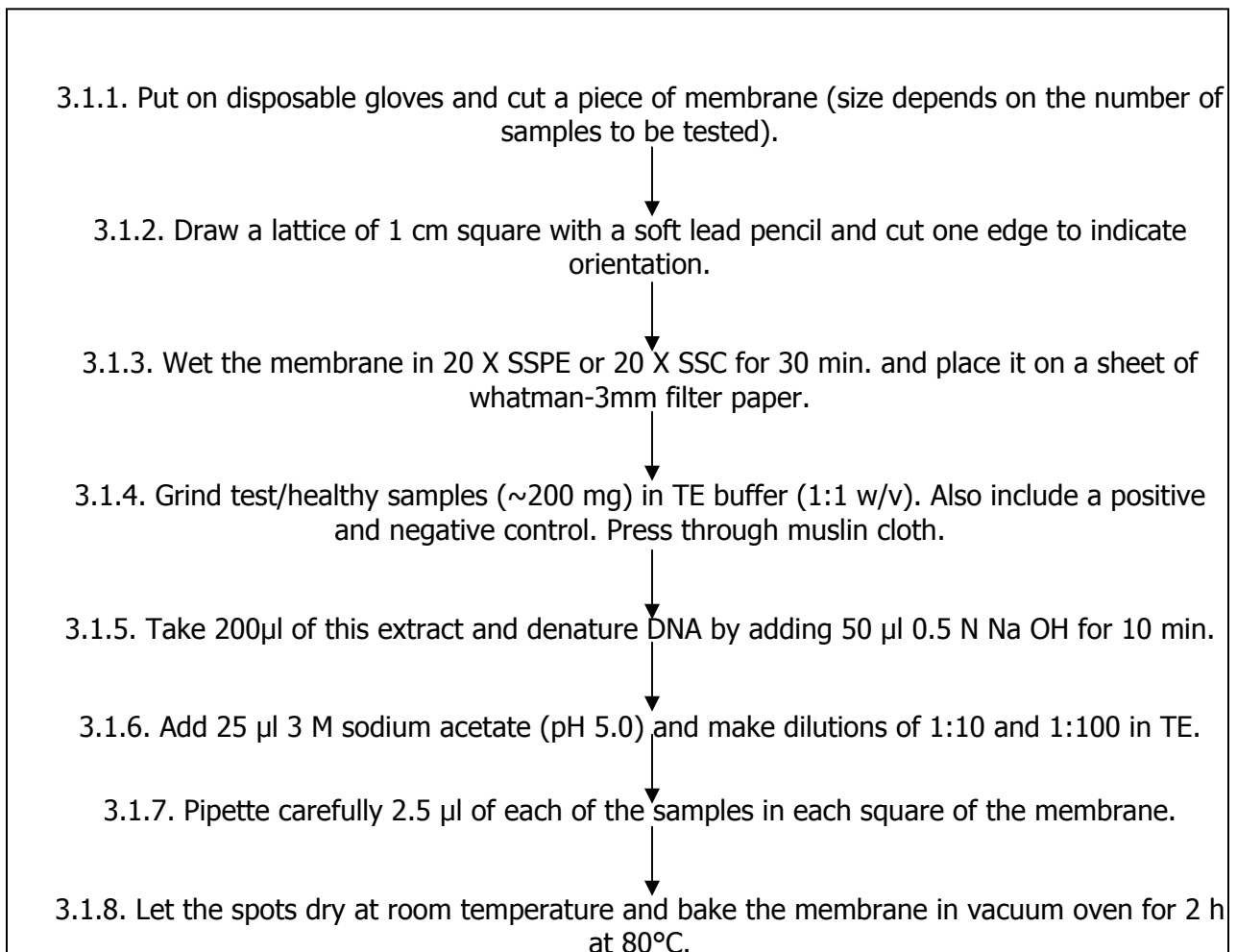
Ficoll (type 400)	5 g
PVP	5 g
BSA	5 g
H <sub>2</sub> O	500 ml

- **Salmon sperm DNA (1 %):**

SS DNA is dissolved in water at a concentration of 10 mg/ ml. The solution is stirred for 2-4 h at room temperature. The concentration of Na Cl is adjusted to 0.1 M and the solution is extracted with phenol once and then with phenol chloroform once. DNA is sheared by passing 12 times through a 17 guage hypodermic needle. DNA is precipitated by ice-cold ethanol and then recovered by centrifugation, redissolved at a conc. Of 10 mg / ml. Boil the solution for 10 min., aliquot and freeze at -20°C.

### **3. Testing Protocol-NASH:**

#### **3.1.Dot blotting:**



### **3.2. Probe production (Random primer method):**

3.2.1. Mix the following in the given order in eppendorf tube. Protect yourself from radiation while preparing probe.

H <sub>2</sub> O	33 µl
Oligolabelling buffer	10 µl
BSA	2 µl
Template	1 µl (20 ng DNA / µl)
32 <sub>P</sub> -dCTP	3 µl (10 µCi)
Klenow enzyme	1 µl

3.2.2. Incubate the mix behind Lucite green at room temperature overnight. Add 2 µl of 1 m M EDTA to stop the reaction.

### **3.3. Pre-hybridization:**

3.3.1. Pre-hybridize the membrane by treating in 100 ml pre-hybridization solution in a sealable plastic bag by incubating with constant agitation for 15 min. at 60°C.

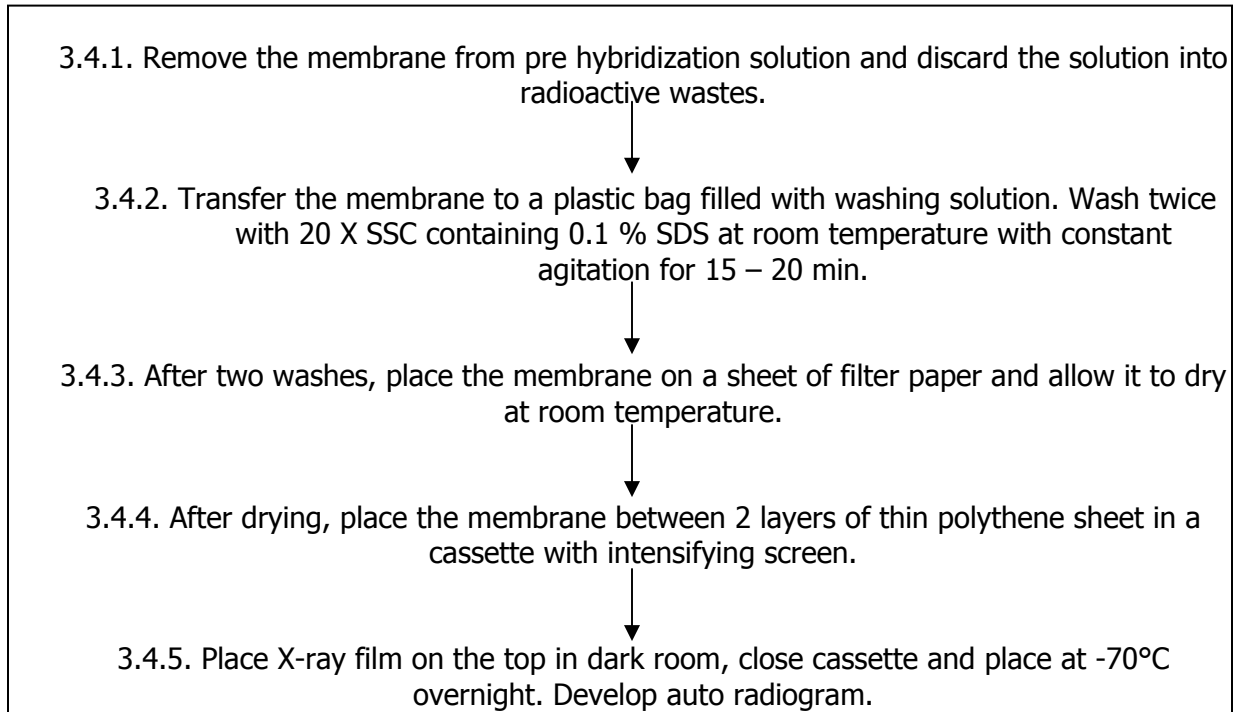
3.3.2. Pre- hybridization solution (10 ml)

H <sub>2</sub> O	6.3 ml
50 X Denhardt's solution	0.8 ml
Denatured Salmon sperm DNA (10 mg/ml)	0.1 ml
20 X SSC	2.5 ml
10 % SDS	0.3 ml
Heat in boiling water bath for 90 sec. and chill	

### **3.4. Hybridization:**

3.4.1. Add probe at the rate of  $1 \times 10^6$  cpm/ml of solution after denaturing by boiling for 5 min in a water bath. Re-seal plastic bag and incubate with constant agitation for overnight at 65°C.

### **3.5. Washing Procedure:**



### **Reference:**

*Dijkstra, J. and C.P. de Jager, (1998). Practical Plant Virology: protocols and exercises. Springer, New York, 459 pp.*

### **Helpful Hints:**

- Use gloves at all times and frequently wash or change them.
- Cross- contamination during DNA isolation should be avoided.
- Always use clean, blunt forceps and avoid contacting dirty surfaces as binding of probes to contaminants on the membrane may lead to high background.
- Blot can be re-used after stripping the probe. Nitrocellulose blots have been re-used 6-7 times with limited loss in sensitivity.

## Annexure-5 B

## List of Plant Species &amp; Viruses Covered under National Certification System for Tissue Culture Plants.

S. No	Plant Species		Viruses/Viroids/phytoplasmas/bacteria covered under NCS-TCP	Test protocol*			
	Common Name	Scientific Name		ELISA	DIBA	RT-PCR /PCR	NASH
<b>Fruit Plant Species</b>							
1	Apple*	<i>Malus domestica</i>	Apple chlorotic leaf spot virus (ACLSV); Trichovirus; Flexiviridae.	+		+	
			Apple mosaic virus (ApMV); Ilarvirus; Bromoviridae.	+		+	
			Apple stem grooving virus (ASGV); Capillovirus; Flexiviridae.	+	+	+	
			Apple stem pitting virus (ASPV); Foveavirus; Flexiviridae.	+		+ <sup>1C</sup>	
2	Banana*	<i>Musa spp</i>	Banana bract mosaic virus (BBrMV); Potyvirus; Potyviridae.	+		+	
			Banana bunchy top virus (BBTV); Babuvirus; Nanoviridae.	+		+	+
			Banana streak virus-Mysore strain (BSMyV); Badnavirus; Caulimoviridae.	+		+	+
			Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
3.	Citrus*	<i>Citrus spp</i>	Citrus greening ( <i>Liberobacter asiaticum</i> )	+		+	
			<sup>1</sup> Citrus exocortis viroid (CEVd); Pospiviroid; Pospiviroidae.			+	
			Citrus tristeza virus (CTV); Closterovirus; Closteroviridae.	+	+	+	
			Citrus mosaic virus (CMBV); Badnavirus; Caulimoviridae.			+	+
			Indian citrus ringspot virus (ICRSV); Mandarivirus; Flexiviridae.	+		+	
4.	Strawberry	<i>Fragaria spp</i>	<sup>§</sup> Raspberry ringspot virus (RpRSV); Nepovirus; Comoviridae.	+		+	+
			<sup>§</sup> Strawberry crinkle virus (SCV); Cytorhabdovirus; Rhabdoviridae.	+			
			<sup>§</sup> Strawberry latent ringspot virus (SLRSV); Sadwavirus; unassigned.	+		+	
			<sup>§</sup> Strawberry mild yellow edge virus (SMYEV); Potexvirus; Flexiviridae.	+			

			Strawberry mottle virus (SMoV); Sadwavirus; unassigned.	+			
			Strawberry pseudo mild yellow edge virus (SPMYEV); Carlavirus; Flexiviridae.	+		+	
			<sup>§</sup> Strawberry vein banding virus (SVBV); Caulimovirus; Caulimoviridae.	+		+	
			Tomato black ring virus (ToBRV); Nepovirus; Comoviridae.	+		+	+
<b>Spices</b>							
6	Black Pepper*	<i>Piper nigrum</i>	Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			Piper yellow mottle virus (PYMOV); Badnavirus; Caulimoviridae.	+		+	
			Piper little leaf (phytoplasmas).	+		+	
7.	Cardamom, large	<i>Amomum subulatum</i>	Cardamom mosaic virus (CdMV); Macluravirus; Potyviridae.	+		+	
8.	Cardamom, small	<i>Elettaria cardamomum</i>	Cardamom mosaic virus (CdMV); Macluravirus; Potyviridae.	+		+	
9.	Vanilla*	<i>Vanilla</i> spp.	Vanilla mosaic virus (VanMV); Potyvirus; Potyviridae.	+		+	
			Vanilla necrosis virus (Watermelon mosaic virus (WMV)); Potyvirus; Potyviridae.	+		+	
			Cymbidium mosaic virus (CYMMV); Potexvirus; Flexiviridae.	+	+	+	+
			Odontoglossum ring spot virus (ORSV); Tobamovirus; unassigned.	+	+	+	+
<b>Commercial Crops</b>							
10.	Potato*	<i>Solanum tuberosum</i>	Potato leaf roll virus (PLRV); Polorovirus; Luteoviridae.	+ <sup>DAS</sup>	+	+	+
			Potato virus M (PVM); Carlavirus; Flexiviridae.	+ <sup>DAS</sup>	+	+	
			Potato virus S (PVS); Carlavirus; Flexiviridae.	+	+	+	+
			Potato virus X (PVX); Potexvirus; Flexiviridae.	+ <sup>DAS</sup>		+	+
			Potato virus Y (PVY); Potyvirus; Potyviridae.	+	+	+ <sup>IC</sup>	+
			Potato apical leaf curl virus (Tomato leaf curl virus (TICV)); Begomovirus; Geminiviridae	+		+	
11.	Sugarcane*	<i>Saccharum officinarum</i>	Sugarcane mosaic virus (SCMV); Potyvirus; Potyviridae.	+	+	+	
			Sugarcane yellow leaf virus (ScYLV); Polorovirus; Luteoviridae			+	
			Sugarcane bacilliform Mor virus (SCBMV); Badnavirus; Caulimoviridae.			+	

Ornamental Plant Species							
12.	Alocassia	<i>Alocassia spp</i>	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
13.	Alstroemeria	<i>(Alstroemeria spp)</i>	Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			Alstroemeria mosaic virus (AIMV); Potyvirus; Potyviridae.	+		+	
			Alstroemeria streak virus (Alstroemeria mosaic virus); Potyvirus; Potyviridae.	+		+	
			§Alstroemeria carlavirus (Lily symptomless virus); Carlavirus; Flexiviridae.	+	+	+	
			§Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae.	+		+	+
14.	Anthurium	<i>Anthurium spp</i>	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
15.	Asparagus	<i>Asparagus spp</i>	Asparagus virus-1 (AV-1); Potyvirus; Potyviridae.	+		+	
			Asparagus virus-2 (AV-2); Ilarvirus; Bromoviridae	+		+	
			Asparagus virus-3 (AV-3); Potexvirus; Flexiviridae	+		+	
16.	Caladium	<i>Caladium spp</i>	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
17.	Calla Lily	<i>Zantedeschia aethiopica</i>	Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
			§Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae	+		+	+
18.	Carnation	<i>Dianthus caryophyllus</i>	Carnation etched ring virus (CERV); Caulimovirus; Caulimoviridae.	+		+	
			§Carnation Italian ringspot virus (CIRV); Tombusvirus; Tombusviridae.	+		+	
			Carnation latent virus (CLV); Carlavirus; Flexiviridae.	+		+	
			Carnation mottle virus (CarMV); Carmovirus; Tombusviridae.	+		+	
			Carnation necrotic fleck virus (CNFV); Closterovirus; Closteroviridae.	+		+	
			Carnation ringspot virus (CRSV); Dianthovirus; Tombusviridae.	+		+	
			Carnation vein mottle virus (CVMV); Potyvirus; Potyviridae.	+		+	
19.	Chrysanthemum	<i>Dendranthema morifolium</i>	Chrysanthemum virus B (CVB); Carlavirus; Flexiviridae.	+ <sup>DAS</sup>		+	
			Chrysanthemum aspermy virus (Tomato aspermy virus); Cucumovirus; Bromoviridae.	+		+	
			Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			§Chrysanthemum stem necrosis virus (CSNV); Tospovirus; Bunyaviridae.	+		+	+

			<sup>2</sup> Chrysanthemum stunt viroid (CSVd); Pospiviroid; Pospiviroidae.			+	+
20.	Colocasia	<i>Colocasia</i> spp.	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
21.	Cordyline	<i>Cordyline</i> spp	Impatiens necrotic spot virus (INSV); Tospovirus; Bunyaviridae	+		+	
			<sup>§</sup> Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae.	+		+	+
22.	Geranium	<i>Geranium</i> spp	<sup>§</sup> Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae.	+		+	+
			<sup>§</sup> Pelargonium flower break virus (PFBV); Carmovirus; Tombusviridae.	+		+	
			<sup>§</sup> Pelargonium vein clearing virus (PVCV); Eggplant mottled dwarf virus; Nucleorhabdovirus; Rhabdoviridae.	+		+	
			<sup>§</sup> Pelargonium leaf curl virus (PLCV); Tombusvirus; Tombusviridae	+		+	
			<sup>§</sup> Arabis mosaic virus (ArMV); Nepovirus; Comoviridae.	+		+ <sup>1C</sup>	
			<sup>§</sup> Tomato ring spot virus (ToRSV); Nepovirus; Comoviridae.	+		+	
			Tomato black ring virus (ToBRV); Nepovirus; Comoviridae.	+		+	+
23.	Gerbera	<i>Gerbera jamesonii</i> .	Tobacco rattle virus (TRV); Tobravirus; un assigned.	+		+	
			Gerbera mosaic virus (Tobacco mosaic virus (TMV)); Tobamovirus; unassigned.	+	+	+	+
			Gerbera symptomless virus (GeSLV); unassigned; Rhabdoviridae.	+		+	
			Gerbera phyllody (Phytoplasma).	+		+	
24.	Ginger	<i>Zinziber</i> spp	Cucumber mosaic virus (CMV); <u>Cucumovirus</u> ; Bromoviridae.	+		+	
			Ginger chlorotic fleckvirus (GCFV); Sobemovirus; unassigned	+		+	
25.	Lily (Asiatic/ Oriental lily)	<i>Lilium longifolium</i>	Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			Lily symptomless virus (LSV); Carlavirus; Flexiviridae.	+	+	+	
			Lily mottle virus (LMoV); Potyvirus; Potyviridae.	+		+	
			<sup>§</sup> Lily virus X (LVX); Potexvirus; Flexiviridae.	+		+	
			<sup>§</sup> Strawberry latent ring spot virus (SLRSV); Sadwavirus; unassigned.	+			
			<sup>§</sup> Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae.	+		+	+
			Tulip breaking virus (TuBV); Potyvirus; Potyviridae.	+		+	
26	Limonium	<i>Limonium</i> spp	Impatiens necrotic spot virus (INSV); Tospovirus; Bunyaviridae.	+		+	

			Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae.	+		+	
			<sup>§</sup> Clover yellow vein virus (CIYVV); Potyvirus; Potyviridae	+ <sup>DAS</sup>		+	
			Tobacco rattle virus (TRV); Tobravirus; un assigned.	+		+	
27.	Orchids	<i>Cymbidium spp;</i> <i>Dendrobium spp;</i> <i>Cattelyia spp;</i> <i>Phalenopsis spp. Etc.</i>	Cymbidium mosaic virus (CYMMV); Potexvirus; Flexiviridae.	+	+	+	+
			Cymbidium ringspot virus (CymRSV); Tombusvirus; Tombusviridae.	+		+	
			Impatiens necrotic spot virus (INSV); Tospovirus; Bunyaviridae.	+		+	
			Odontoglossum ring spot virus (ORSV); Tobamovirus; unassigned.	+	+	+	+
			<sup>§</sup> Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae	+		+	+
27.	Philodendron	<i>Philodendron spp</i>	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae	+	+	+	
28.	Spathyphyllum	<i>Spathyphyllum spp</i>	Dasheen mosaic virus (DsMV); Potyvirus; Potyviridae.	+	+	+	
29.	Syngonium	<i>Syngonium spp</i>	Impatiens necrotic spot virus (INSV); Tospovirus; Bunyaviridae	+		+	
			<sup>§</sup> Tomato spotted wilt (TSWV); Tospovirus; Bunyaviridae.	+		+	+
30.	Weeping fig	<i>Ficus benjamina</i>	Ficus chlorosis virus (Fig leaf chlorosis virus) (FigLCV); Potyvirus; Potyviridae.	+		+	
			Cucumber mosaic virus (CMV); Cucumovirus; Bromoviridae	+		+	
<b>Forest plant species</b>							
31.	Bamboo	<i>Bambusa vulgaris</i>	Bamboo mosaic virus (BaMV); Potexvirus; Flexiviridae.	+		+	

<sup>§</sup> not known to occur in India and or/ restricted distribution in India;

<sup>1</sup>Citrus exocortis also can be detected by **sPAGE** (sequential polyacrylamide gel electrophoresis);

<sup>2</sup>Chrysanthemum stunt viroid, Potato spindle tuber viroid also can be detected by **R-PAGE** (return gel PAGE);

+: Detection of virus by specified test; +<sup>DAS</sup>: Detection of virus by double antibody sandwich-ELISA; +<sup>IC</sup>: Detection of virus by Immunocapture-RT-PCR); +<sup>dsRNA</sup>:

Detection by dsRNA;

\***ELISA**: Enzyme-linked Immunosorbent Assay; **DIBA**: Dot-immunobinding Assay; **RT-PCR**: Reverse transcription polymerase chain reaction; **NASH**: Nucleic Acid Spot Hybridization;

@Viruses/viroids nomenclature is as per the International Taxonomy Committee for Viruses.

\* Covered under certification standards established by the Department of Biotechnology in accordance with provisions of Seeds Act, 1966.

<b>SOPs for Accredited Test Laboratory</b>		
<b>Section-6</b>	<b>Quality (genetic fidelity) Testing</b>	<b>Page 1 of 1</b>
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- 6.1. The scientist/laboratory technician (molecular biology) will use established protocols specified in Anexure-6 for genetic fidelity testing by the Centre for DNA Fingerprinting & Diagnostics, Hyderabad.
- 6.2. If any new protocols used or any deviations from existing protocols will require validation by the national referral laboratory (i.e., The Centre for DNA Fingerprinting & Diagnostics, Hyderabad) before their adoption and use.
- 6.3. The test results will be recorded by counting the presence/absence of each distinct band across all the samples tested in replicates and then subjected either to a parsimony or other phylogenetic analysis, cluster analysis using a simple matching coefficient as per established software programmes recommended by the Centre for DNA Fingerprinting & Diagnostics, Hyderabad.
- 6.4. The Inter-Simple Sequence Repeats' (ISSR) protocols will be followed for quality certification until such time the Simple Sequence Repeats' (SSR) or Microsatellites' protocols are established and validated by the Centre for DNA Fingerprinting & Diagnostics, Hyderabad.

## **Quality (Genetic Fidelity) Testing Protocols**

One of the approaches for estimating genetic diversity is to measure the genetic variation among individuals of a population(s) using molecular genetic markers. Many of the genetic markers belong to so-called anonymous DNA marker type since the positions from which they come is usually unknown. These types of markers include microsatellites or simple sequence repeats (SSRs), Inter-SSR (ISSR), restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), and amplified fragment length polymorphisms (AFLPs). These marker types generally measure apparently neutral DNA variation, and are very useful with varying efficiency in the analysis of phylogenetic relationships, population structure, mating system, gene flow, parental assignment, introgressive hybridization, marker-aided selection and genetic linkage studies. In contrast, gene specific markers (termed genic markers) like isozymes or mitochondrial gene sequencing are also extensively used in population genetics. The choice of the marker to be used depends on the type of utility it provides including ease to obtain, economy and other factors. The use of co-dominant markers, particularly those with a high degree of polymorphism such as SSRs, is known to improve the efficiency and accuracy of linkage analysis.

### **Microsatellites as Molecular Markers:**

Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem. They are widespread in both eukaryotic and prokaryotic genomes and have higher rates of mutation than the rest of the genome. Microsatellites are classified according to the type of repeat sequence as perfect, imperfect, interrupted or composite. Perfect microsatellite is the repeat sequence, which is not interrupted by any base not belonging to that motif (*e.g.* TATATATATATATA- represented as (TA)<sub>8</sub>). An imperfect microsatellite is a repeat motif that is interrupted by base(s) that does not match the motif sequence (*e.g.* TATATACTATATA). In case of an interrupted microsatellite, there is a small sequence within the repeated sequence that does not match the motif sequence (*e.g.* TATATACGTGTATATATATA) while in a composite microsatellite the sequence contains two adjacent distinctive sequence-repeats (*e.g.* TATATATAGTGTGTGTGT).

Microsatellites find extensive use in (1) the construction of genetic maps, (2) association studies between repeat number instability and human genetic diseases, (3) population genetics studies, and (4) genotyping and paternity analysis.

### **Inter-Simple Sequence Repeats (ISSRs):**

Inter-Simple Sequence Repeats (ISSRs) is marker system that has recently been developed as an anonymous, RAPDs-like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes (particularly the nuclear genome) and circumvents the challenge of characterizing individual loci that other molecular approaches require. Microsatellites are very short stretches of DNA that are "hypervariable", expressed as different variants within populations and among different species. They are characterized by mono-, di-, tri- or tetra-nucleotide repeats, *e.g.*, AA..., or AG..., CAG....., GATA..., that have several repeat units side-by-side. In ISSRs, one specifically targets the di- and trinucleotide repeat types of microsatellite,

because these are characteristic of the nuclear genome (mononucleotide types are found in the chloroplast genome and therefore might not be very specific and informative).

The variation comes in two "forms" in the ISSR markers: one to several loci (band groups), with each of these having one to several immediately adjacent bands. These latter might represent "allelic" variants for different numbers of tandem repeats in either of the microsatellites flanking the amplified fragment, in both of them, or perhaps more likely variants differing in indels of the intervening region. If the PCR products are separated well in the final agarose gel, one can actually discriminate the allelic variants pretty clearly.

The Quality testing protocols generally involve the following steps:

### **1. DNA Extraction:**

There are various methods practiced to isolate genomic DNA, the conventional method for the isolation of genomic DNA is by organic phase extraction i.e. Phenol: Chloroform: Isoamyl alcohol, followed by an ethanol precipitation. Many people also have very good success with readymade kits such as DNeasy kits (Qiagen, Valencia, CA), Wizard Preps (Promega Corp. Madison WI), etc.

#### **1.1 Isolation of Nuclear/ Genomic DNA:**

Total genomic DNA is generally isolated employing the CTAB based method. This is considered as standard protocol for DNA fingerprinting, which requires good quality high molecular weight DNA. The method is based on Doyle & Doyle (1990, Focus, Volume 12: pp. 13-15) is described below.

##### **1.1.1. Standard Procedure:**

- (a) Lyophilize 200-300 mg of fresh leaf material.
- (b) Grind 20 mg of lyophilized leaf material to a fine powder using quartz sand using pestle and mortar.
- (c) Transfer the powdered material to 700  $\mu$ l of pre-warmed Extraction buffer and 700  $\mu$ l of 2X CTAB buffer and incubate for 60 min at 60°C with occasional stirring.
- (d) Extract with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1).
- (e) Centrifuge at 10,000 rpm for 15 min at room temperature (20°C).
- (f) Separate the aqueous phase and transfer to a fresh tube.
- (g) Add 2  $\mu$ l of RNase A (10 mg/ml) to final concentration of 50 mg/ml and incubate for 30 min at 37°C.
- (h) Extract with an equal volume of chloroform: isoamyl alcohol (24:1) at 10,000 rpm for 10 min.
- (i) To the aqueous phase add 0.6 volumes of ice-cold isopropanol and incubate at -20°C

for 30-60 min.

- (j) Centrifuge at 10,000 rpm for 10 min at 4°C. Wash the DNA pellet obtained with 70% ethanol and 10 mM ammonium acetate.
- (k) Dry the DNA pellet and dissolve in 100 µl of water or low concentration TE buffer.

### **1.1.2. Optional:**

- (a) Add 0.5ml of 7.5M ammonium acetate to each tube, mix and chill on ice for 15 min.
- (b) Centrifuge for 30 min at 10000 g, at 4 °C. To the supernatant add two volumes of chilled ethanol and keep at -20°C to precipitate DNA.
- (c) Centrifuge at 10000 rpm for 10 min at 4°C .
- (d) Wash the pellet with 70% ethanol, air dry and dissolve in water or TE buffer.

### **1.1.3. Alternate Method:**

- (a) 0.5-1.0 g of leaf tissue is grinded with liquid nitrogen in mortar and pestle and transferred to oak ridge tube.
- (b) 4 ml of extraction buffer is added to each tube and is incubated at 37°C for 1 hour.
- (c) 4 ml of CTAB buffer is added to each tube and mixed well.
- (d) Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) is added and mixed.
- (e) It is then centrifuged at 12000 rpm for 10 minutes at 4°C.
- (f) Supernatant is transferred to fresh tubes.
- (g) Then equal volume of chloroform: isoamyl alcohol (24:1) is added and centrifuged at 12000 rpm for 10 minutes at 4°C.
- (h) Again supernatant is transferred to fresh tube. 2/3<sup>rd</sup> volume of isopropanol is added and incubated at -20°C for a minimum of 30 minutes.
- (i) Then it is centrifuged at 12000 rpm for 8 minutes.
- (j) The supernatant is discarded and the pellet is washed twice with 70% ethanol.
- (k) The pellet is now air dried and then dissolved in appropriate volume of TE buffer.
- (l) Add 2 µl of RNase A (10 mg/ml) to final concentration of 50 mg/ml and incubate for 30 min at 37° C.

## 2. Quantification of DNA :

### 2.1 By Agarose Gel Electrophoresis Method:

5 µl of DNA was mixed with 1 µl of 6X loading dye and loaded on to a 0.8 to 1.0 % agarose gel along with 500 ng of lambda *Hind* III digest marker and electrophoresed at 90V for 30 min. The quantity of extracted DNA was estimated based on the intensity of lambda *Hind* III digest marker bands as the top band accounts half amount (250 ng) of total loaded amount. The quality of genomic DNA was confirmed for its integrity.

#### 2.1.1. Agarose Gel Preparation (MiniGel - 40 ml (Medium Gel – 70 ml):

You will need 40 ml of buffer for the minigel apparatus. You will need an additional 400 ml buffer to run the gel.

- (a) Tape ends of gel holder and insert comb. Make sure that there is a paper width of space between the comb and the bottom of the gel tray and that the comb is level. Make sure the gel holder is also level.
- (b) Prepare 0.8% agarose by mixing 0.32 g (0.56 g for medium gel) Agarose in 40 ml (70 ml for medium gel) of 1X TAE in a 125 ml conical flask.
- (c) Heat in the microwave, 30 seconds/time until dissolved. Swirl after each heating. If almost dissolved, heat for shorter periods of time.  
**(Caution:** Agarose can develop superheated spots and can explode when swirled. Use hot gloves and heat just to boiling).
- (d) Allow it to cool to ~55 °C; add Ethidium bromide to a final concentration of 0.5 µg/ml and mix the contents well.
- (e) Pour into gel and cool for 30 minutes before use.
- (f) Remove the tape on each side and put the gel in the electrophoresis apparatus. Cover the gel with 0.5X TAE.
- (g) Prepare the DNA sample. DNA 5 µl plus 6x loading dye 1 µl.
- (h) Load your samples and the lambda DNA in separate wells of the gel. Load the samples in the wells using a P20 micropipette. Change tips between samples. Place the tip of the pipette containing the sample to be loaded under the buffer, just at the opening of the well. The loading buffer contains glycerol which increases the density of the sample to be loaded. The sample will settle in the well because it is denser than the buffer. Be careful not to poke a hole in the bottom of the well with the pipette tip.
- (i) Place the cover on the gel electrophoresis unit and plug the leads into the power pack. Make sure the positive lead runs from the bottom of the gel (DNA is negatively charged)

and will run to the positive pole).

- (j) Turn on the power and run the gel at 95-105 V for 30-45 minutes or until the tracking dye is 2/3 to 3/4 of the distance to the end of the gel.
- (k) Turn off the power.
- (l) Unplug the leads by pulling on the plastic clips. Do not pull on the wires.
- (m) Remove the top of the unit and take out the gel and gel holder (caution, the gel can slide off the holder).  
**Caution:** Wear gloves while handling gel with Ethidium bromide (a potent mutagen).
- (n) Photograph the gel under UV light in Gel documentation system.

## **2.2. Spectrophotometer Method:**

- (a) Take 1ml of TE buffer in a cuvette and calibrate the spectrophotometer at 260 nm and 280 nm wavelength.
- (b) Add 2 to 5µl of DNA mix properly and record the optical density (OD) at both 260 and 280 nm.
- (c) Estimate the DNA concentration employing the following formula:  
Amount of DNA (µg / µl) = 
$$\frac{(OD)_{260} * 50 * \text{dilution factor}}{1000}$$
- (d) Judge the quality of DNA from the ratio of OD values recorded at 260 and 280 nm.

The ratio between the readings ( $OD_{260}/OD_{280}$ ) provides an estimate of sample purity. Pure preparations have values close to 1.8 and protein contaminated samples have significantly lower.  $OD_{260}$  also allowed the calculation of nucleic acid concentrations of the samples, i.e. an OD of 1 corresponded to approximately 50µg /ml for double stranded DNA.

## **3. Polymerase Chain Reaction (PCR):**

PCR is an in vitro enzymatic process by which a specific region of DNA is synthesized into many copies, and this was discovered by Kary Mullis. This molecular photocopying process involves heating and cooling of samples in a machine called thermal cycler in the presence of oligonucleotide primers, dNTPs and heat stable enzyme called Taq Polymerase in a cycle pattern over about 30 cycles. During each cycle, a copy of target DNA sequence is generated for every molecule containing the target sequence. After about 30 cycles, a billion copies of the target region on the DNA template have been generated. This PCR product, also called as amplicon.

The PCR process consists of the following steps:

- (a) Initial denaturation: Each double-stranded segment is separated into two strands by heating at 95-98 °C.

- (b) Denaturation during cycling: Denaturation at 94-95 °C for 30-45 sec is usually sufficient.
- (c) Primer annealing: The reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified, annealing of primers to the denatured DNA strands when the temperature comes down to 45-55 °C.
- (d) Primer extension: The elongation of primers is catalyzed by a heat stable DNA polymerase and the four nucleotide building blocks, dNTPs. The primers serve as the starting point for the replication of the target sequence and a copy of the complement of each of the separated strands is made, so that there are two double stranded DNA segments at the end of the first cycle. Taq polymerase will extend the annealed primers at about 60 bases per sec at 75 °C for 45 sec.
- (e) Cycle number: Generally 25-35 cycles.
- (f) Final extension: Usually, after last cycle, the reaction kept at 72 °C for 5-15 min which promote completion of partial extension products and annealing of single stranded complementary products.

### **3.1. PCR Reaction Components:**

**Template DNA:** about 1-10ng of genomic DNA is required as a starting template. The quality of the template influences the outcome of the PCR. Impure template and other inhibitors decrease the efficiency of the reaction.

**Primers:** the oligonucleotide sequences have the following general Characteristics 18-24 bases long contains no internal secondary structure contain 40-60% GC content have a balanced distribution of GC and AT rich domains should not have complementary to each other have a melting temperature  $T_M$  that allows an annealing temperature of 55-65°C the concentration of the primer concentrations are between 0.1 and 0.6. Higher primer concentration may promote mispriming and accumulation of non specific product.

**DNA polymerase:** the heat stable Taq polymerase of 0.5-5 units.

*MgCl<sub>2</sub> concentration:* the optimal MgCl<sub>2</sub> concentration is 1.5mM. Mg<sup>2+</sup> influences enzyme activity and increases the  $T_M$  (melting temperature) of double stranded DNA. Excess Mg<sup>2+</sup> in the reaction can increase non-specific primer binding and increase non-specific background.

*dNTPs:* all four dNTPs each of 200mM. Always use balanced mix of all four dNTPs to minimize polymerase error rate.

*pH:* the pH of the reaction medium should be about 8.3-9.0 for optimal results.

The most commonly used thermo-stable polymerase is Taq polymerase, which comes from a bacterium named *Thermus aquaticus* that inhabits hot springs.

The PCR reaction contained the following components in 25 µl reaction volume.

<b>Components</b>	<b>Final concentration</b>
10X Buffer	1X
25 mM MgCl <sub>2</sub>	1.5 mM
dNTPs (each)	200 µM
Primers	0.5- 1 µM
Taq Polymerase	0.5-2.0 units
Template DNA	~5 to 15 ng

### **3.1.1. Additional components (optional):**

<b>Components</b>	<b>Final concentration</b>
Spermidine	0.4 mM
Triton X-100	0.1%

The PCR amplification conditions for ISSR assay are as follows: an initial denaturation at 94 °C for 5 minutes, followed by 35-45 cycles of denaturation (at 94 °C for 45 seconds), annealing\* (at 45-55 °C for 45 seconds) and extension (at 72 °C for 2 min), and a final extension step at 72 °C for 7 min. The amplification products are visualized on 1.2- 1.5% agarose gels under UV light. (\*: the annealing temperature would vary depending on the melting temperature of the primer used and needs to be worked out separately for each primer)

### **3.2. PCR Master Mix:**

Make a standard PCR reaction Master Mix with one of the primers, aliquot 25 microliters of the Master Mix into individual tubes as usual, put 1-1.5 microliter of undiluted DNA sample into each tube (one should do a series of DNA amounts and MgCl<sub>2</sub> amounts for a couple of your samples, to determine the optimum concentrations to give good bands in the initial phase), and amplify them.

Test the quantity of DNA in at least a few sets of extracts (corresponding to many individuals in a population) to determine whether the concentration is roughly comparable across extracts. If different extracts within and among populations show high variability in DNA concentration, all the extracts should be routinely measured for concentration, and then DNA must be standardized approximately (by dilution, or drying and rehydration in less water) across samples and populations.

### **3.3. Tips for Setting up PCR:**

- (a) The components of the reaction can be added in any order, provided that water is added first. Pipetting should be on ice, and the vials were placed from ice directly into the preheated metal block or water bath of the thermal cycler.
- (b) Results of the PCR were the same when 100 or 50 or 25 or 6.2ul reaction volumes were used. With smaller volumes, pipetting is critical, especially for dNTPs. The PCR products were separated by electrophoresis on 3% agarose gels.

- (c) The Tris buffer in the PCR reaction is pH sensitive with temperature variation, and higher temperatures cause the solution pH to go down by about 0.02 with every 1°C. A Tris buffer with pH 8.3 at 25°C will go down to pH about 6.9 at 95°C. Thus, not only is the template DNA well denatured but the polymerase is activated just when it is needed, and not in a situation where primers and mispriming can occur as easily.

### **3.4. PCR Inhibition:**

The plant samples that are sent to a DNA Laboratory may have been exposed to a harsh environment for few days during transportation. Environmental exposure degrades DNA molecules by randomly breaking them into smaller pieces. Another important challenge to amplifying DNA from the plant sample is the fact that PCR amplification process can be affected by inhibitors present in the samples themselves. . The result of amplifying a DNA sample containing an inhibitor is a loss of the alleles from the larger sized ISSR/SSR loci. Thus, the failure to amplify the larger ISSR/SSR loci for a sample can be either due to degraded DNA, where there are not enough intact copies of the DNA template, or due to the presence of a sufficient level of PCR inhibitor to reduce the activity of the polymerase.

PCR inhibitors may be removed or their effects reduced by one or more of the following:

- (a) The genomic DNA template may be diluted, which also dilutes the PCR inhibitor, thus DNA may be amplified in the presence of less inhibitor.
- (b) Alternatively, more DNA polymerase can be added to overcome the inhibitor. With this approach some fraction of the Taq polymerase binds to the inhibiting molecules and removes them from the reaction so that the rest of the Taq can do its job and amplify the DNA template.
- (c) In addition, additives as bovine serum albumin (BSA) have been shown to prevent or minimize the inhibition of PCR (Comey et al.1994).
- (d) More recently, sodium hydroxide treatment of DNA has been shown to neutralize inhibitors of Taq polymerase (Bourke et al.1999)

### **4. Testing Protocol-ISSR:**

Fifteen to twenty ISSR primers were screened for each species under investigation to select 6 to 10 best primers for each of the species (Table 1 & 2). The selected primers were used to assess the clonal fidelity of the micropropagated plants taken out at different passages of subculturing. The PCR reaction contained the following components in 25 µl reaction volume.

<b>Components</b>	<b>Final concentration</b>
10X Buffer	1X
25 mM MgCl <sub>2</sub>	1.5 mM
dNTPs (each)	200 µM

Primers	0.5- 1 $\mu$ M
Taq Polymerase	0.5-2.0 units
Template DNA	~5 to 15 ng

Additional components (optional):

<b>Components</b>	<b>Final concentration</b>
Spermidine	0.4 mM
Triton X-100	0.1%

The PCR amplification conditions are as follows: an initial denaturation at 94 °C for 5 minutes, followed by 35-45 cycles of denaturation (at 94 °C for 45 seconds), annealing\* (at 45-55 °C for 45 seconds) and extension (at 72 °C for 2 min), and a final extension step at 72 °C for 7 min. The amplification products are visualized on 1.2- 1.5% agarose gels under UV light. (\*: the annealing temperature would vary depending on the melting temperature of the primer used and needs to be worked out separately for each primer)

#### **4.1. PCR Master Mix:**

Make a standard PCR reaction Master Mix with one of the primers, aliquot 25 microlitres of the Master Mix into individual tubes as usual, put 1-1.5 microlitre of undiluted DNA sample into each tube (one should do a series of DNA amounts and MgCl<sub>2</sub> amounts for a couple of your samples, to determine the optimum concentrations to give good bands in the initial phase), and amplify them.

Test the quantity of DNA in at least a few sets of extracts (corresponding to many individuals in a population) to determine whether the concentration is roughly comparable across extracts. If different extracts within and among populations show high variability in DNA concentration, all the extracts should be routinely measured for concentration, and then DNA must be standardized approximately (by dilution, or drying and rehydration in less water) across samples and populations.

It is important to amplify each set of samples and a particular primer twice--giving two amplification replicates. A few bands appear and disappear at random, depending on conditions and the probabilistic nature of PCR. Bands are scored as "**present**" for a sample and a given primer only where they occur in both replicates, and "**absent**" where they occur in only one replicate or neither of them. Each fragment scored as "present" is treated as a "dominant" (amplified) band for that locus, while one scored as "absent" is treated as a "recessive" (null) band; note that homozygous dominant and heterozygous genotypes can't be distinguished in diploid individuals. This must be accommodated in statistical formulas in arriving at F-equivalent "phi" statistics.

**4.2. Primer sequences:** See Tables 1, 2

**4.3. Scoring the data:**

Data points are the presence/absence of each distinct (not "ghost") band across all samples for the same primer, in both replicate sets of amplifications.

#### **4.4. Analysis:**

These are then subjected either to a parsimony or other phylogenetic analysis, cluster analysis using a simple matching coefficient such as Jaccard's or an estimate of genetic distance (e.g., Nei's distance) modified to accommodate dominant ( e.g., RAPD-like) markers. Arlequin, NTSYS, PopGene, etc softwares are used to accomplish the cluster algorithms. Once the data of all primers and individuals are obtained as a binary matrix, it could be used for obtaining distance matrix and phylogenetic analyses during genetic fidelity testing.

- (a) Arlequin (<http://lgb.unige.ch/arlequin/>)
- (b) NTSYS (<http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html>)
- (c) PopGene (<http://www.ualberta.ca/~fyeh/index.htm>)

**Table 1. Sequence of ISSR primers commonly used for genetic fidelity testing:**

<b>ISSR Primer</b>	<b>Nucleotide sequence</b>
UBC 807	5'-AGA GAG AGA GAG AGA GT-3'
UBC 808	5'-AGA GAG AGA GAG AGA GC-3'
UBC 810	5'-GAG AGA GAG AGA GAG AT-3'
UBC 811	5'-GAG AGA GAG AGA GAG AC-3'
UBC 812	5'-GAG AGA GAG AGA GAG AA -3'
UBC 815	5'-CTC TCT CTC TCT CTC TG-3'
UBC 818	5'-CAC ACA CAC ACA CAC AG-3'
UBC 830	5'-TGT GTG TGT GTG TGT GG-3'
UBC 834	5'-AGA GAG AGA GAG AGA GYT-3'
UBC 835	5'-AGA GAG AGA GAG AGA G C-3'
UBC 836	5'-AGA GAG AGA GAG AGA GYA-3'
UBC 838	5'-TAT ATA TAT ATA TAT ARC-3'
UBC 840	5'-GAG AGA GAG AGA GAG AYT-3'
UBC 841	5'-GAG AGA GAG AGA GAG AYC-3'
UBC 842	5'-GAG AGA GAG AGA GAG AYG-3'
UBC 843	5'-CTC TCT CTC TCT CTC TRA-3'
UBC 844	5'-CTC TCT CTC TCT CTC TRC-3'
UBC 848	5'-CAC ACA CAC ACA CAC ARG-3'
UBC 850	5'-GTG TGT GTG TGT GTG TYC-3'
UBC 852	5'-TCT CTC TCT CTC TCT CRA-3'
UBC 857	5'-ACA CAC ACA CAC ACA CYG-3'
UBC 860	5'-TGT GTG TGT GTG TGT GRA-3'
UBC 868	5'-GAA GAA GAA GAA GAA GAA-3'
UBC 873	5'-GAC AGA CAG ACA GAC A-3'
UBC 888	5'-BDB CAC ACA CAC ACA CA-3'
UBC 889	5'-DBD ACA CAC ACA CAC AC-3'
UBC 891	5'-HVH TGT GTG TGT GTG TG-3'

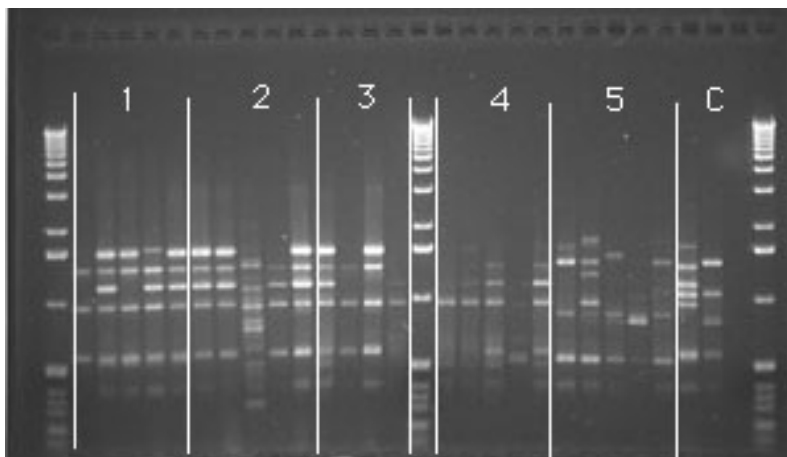
**Single letter abbreviations for mixed base positions:**

N = (A, G, C, T) ; R = (A, G) ; Y = (C, T) ; B = (C, G, T) (i.e. not A); D = (A, G, T) (i.e. not C);  
H = (A, C, T) (i.e. not G); V = (A, C, G) (i.e. not T); K = (G, T) (Keto in large groove); M = (A, C)  
(amino in large groove); S = (G, C) (Strong [3 H-bonds]); W = (A, T) (Weak [2 H-bonds])

**Table 2. ISSR primers for different crop plant species:**

Species	Technique	Suitable Primers
Apple	ISSR	UBC 812, 814, 818, 830, 836, 840, 841, 842, 848, 850, 857, 860
Citrus	ISSR	UBC 809, 812, 815, 816, 840, 841, 842, 848, 850, 857, 860, 868
Potato	ISSR	UBC 812, 818, 830, 836, 841, 842, 848, 850, 857, 860
Vanilla	ISSR	UBC 812, 814, 818, 830, 836, 841, 842, 857, 860
Banana	ISSR	UBC 807, 808, 811, 812, 818, 830, 834, 836, 840, 841, 842, 850, 868
Sugarcane	ISSR	UBC 807, 835, 836, 840, 841, 844

**Fig.1. Prototype gel picture of an ISSR assay:**



## **5. Reagents:**

This section shows a list of reagents used in the Testing Laboratory.

### **Buffers and Solutions Preparation**

#### **Extraction Buffer:**

<b>Final Concentration</b>	<b>For 100ml</b>	<b>Stock</b>
200 mM Tris-HCl (pH 8.0)	20 ml	1 M Tris-HCl
200 mM NaCl	6.66 ml	3 M NaCl
25 mM EDTA	5 ml	0.5 M EDTA
0.5% SDS	2.5 ml	20% SDS
H <sub>2</sub> O	65.9 ml	--

#### **2X CTAB Solution:**

<b>Final Concentration</b>	<b>For 100ml</b>	<b>Stock</b>
2% CTAB	20 ml	10% CTAB
200 mM/ 100 mM Tris-HCl (pH 8.0)	10 ml	1 M Tris-HCl
20 mM EDTA (pH 8.0)	4 ml	0.5 EDTA
1.4 M NaCl	46.6 ml	3 M NaCl
1% PVP	1g	--
H <sub>2</sub> O	Make upto 100 ml	--

#### **1M Tris- HCl, pH 8.0**

Dissolve 121.1g Tris base in 800 ml distilled water. Adjust the pH 8.0 by adding concentrated HCl. Adjust the final volume to 1 liter with distilled water. Autoclave the solution and store at room temperature.

[**Caution:** Hydrochloric acid (HCl) causes severe burns and is irritating to the eyes. When handling with this chemical use a fume hood and avoid inhalation and contact with the skin. Wear a lab coat, gloves, mask, and protective eyewear.

#### **0.5 M EDTA, pH 8.0**

Add 186.1 gms of Ethylene diamine tetra acetate (EDTA) to 800 ml of distilled water, stir vigorously on a magnetic stirrer. Adjust to pH 8.0 by adding NaOH pellets. Adjust the final volume to 1 liter. Autoclave the solution and store at room temperature.

[**Note:** EDTA will not go into solution without pH adjustment]

#### **5M NaCl**

Dissolve 292.2 g NaCl in 800 ml of distilled water. Adjust final volume to 1 liter. Autoclave the solution and store at room temperature.

#### **20% SDS**

Dissolve 200g of sodium dodecyl sulfate in 800 ml of distilled water. Solution may be heated for dissolution. Adjust volume to 1 liter and store at room temperature.

[**Caution:** SDS is an irritant and a strong sensitizer. Avoid skin contact and inhalation. Wear a lab coat, gloves and protective eyewear when handling SDS. Prepare SDS solution in a well ventilated area]

### **20 mg/ml Proteinase K**

Dissolve 200 mg Proteinase K in 10ml of distilled water. Aliquot solution in 0.5 ml tubes store at -20°C. Don't autoclave.

### **3M Sodium acetate, pH 5.2**

Dissolve 40.8 g of Sodium acetate tri hydrate in 80 ml of distilled water. Adjust the pH to 5.2 by adding glacial acetic acid. Adjust the final volume to 100ml. Autoclave the solution and store at room temperature.

### **Preparation of Tris Equilibrated Phenol**

- (a) Take 100g of phenol melt the phenol at 65 °C and pour into a clean glass bottle. Add 200 mg of 8-hydroxyquinoline and mix the solution thoroughly.
- (b) Add half of the volume of 1M Tris-HCl (pH 8.0), mix thoroughly by using magnetic stirrer.
- (c) Allow the solution to settle. (Until the phases separate).
- (d) Discard the upper aqueous phase into waste container.
- (e) Repeat the extraction procedure until the pH of aqueous phase is 7.5.
- (f) After the final extraction, add 10 ml of 0.1M Tris Buffer. Store phenol for up to 3 months at 4°C

[**Caution:** Phenol can cause severe burns. Safety glasses and gloves should be worn when working with phenol. If phenol solution comes in contact with skin or eyes, wash immediately with large volumes of water.]

### **Phenol: Chloroform: Isoamyl alcohol (25:24:1)**

Mix equilibrated Phenol: Chloroform: Isoamyl alcohol in the ratio of 25:24:1, store at 4°C.

### **70% Alcohol**

Mix 70 ml of absolute alcohol and 30 ml distilled water.

### **Ethidium Bromide Solution (10mg/ml)**

100 mg of Ethidium bromide dissolve in 10 ml of deionized water. Wrap in aluminum foil or transfer to a dark bottle and store at room temperature.

[**Caution:** Ethidium bromide is a powerful mutagen. Wear gloves at all times and use a mask when weighing out ethidium bromide powder.]

### 50X TAE Buffer (pH 7.2)

242 g Tris base  
57.1 ml glacial acetic acid  
100 ml 0.5M EDTA Stock

Add Tris and EDTA stock to 500 ml of distilled water. Add glacial acetic acid. Make up the volume to 1L with distilled water.

### 10X TBE Buffer

107.8g Tris base  
7.44 EDTA (Na<sub>2</sub> EDTA.2H<sub>2</sub>O)

### 55.0 g boric acid

Dissolve Tris base and EDTA in 800 ml distilled water. Slowly add boric acid and monitor the pH until the desired pH of 8.3 is obtained. Bring the volume to 1 liter with distilled water.

### TE

10mM Tris-HCl,  
0.1mM EDTA, pH 7.5  
1.21 g Tris base  
0.037 g EDTA (Na<sub>2</sub>EDTA.2H<sub>2</sub>O)

Dissolve Tris base and EDTA in 900 ml distilled water. Adjust to pH 7.5 with HCl. Make up the volume to 1 L with distilled water.

### 6X Glycerol Gel loading solution

0.15 % Bromophenol blue	:	15 mg
0.15% Xylene Cyanol FF	:	15 mg
5mM EDTA	:	100 µl of 0.5 M EDTA (pH- 8.0)
30% Glycerol	:	3 ml

Dissolve all the above components in distilled water and make the volume to 10 ml. Store at 4° C.

### TE Buffer

10mM Tris-HCl, pH 8.0  
Mix together 10 ml of 1 M Tris-HCl, pH 8.0, 0.2 ml of 0.5 M EDTA stock and 990 ml of distilled water. Aliquot in 100 ml volumes and autoclave the solution. Store at 4°C.

**RNase A: (10 mg/ ml)**

- Dissolve Pancreatic RNase (RNase A) at a concentration of 10 mg/ml in 0.01 M Sodium acetate (pH 5.2)
- Heat to 100 °C for 15 minutes
- Allow it to cool slowly to room temperature (do not snap cool).

SOPs for Accredited Test Laboratory		
Section-7	Reporting Results of Testing & Action taken	Page 1 of 6
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### **7.1. Reporting Results of Testing:**

- 7.1.1. The laboratory technician (Virology/Molecular Biology), as the case may be, will complete the testing and enter the results of laboratory testing in the laboratory testing register within a maximum period of 3-4 days from the date of receipt of the sample.
- 7.1.2. He will prepare the test report as per the format prescribed at Annexure-7A and submit to the Scientist (Virology/Molecular Biology), as the case may be, who will verify the results and sign the test report for issue within a day.

### **7.2. Certification of tissue culture plants:**

- 7.2.1. If the test results for the viruses are negative and or the results of quality (genetic fidelity) are not exceeding the tolerance limits set by the crop specific tissue culture standards established by the Department of Biotechnology in consultation with ICAR Institute, the Director/HOD of Accredited Test Laboratory will approve the sample and issue a certificate of approval in the format prescribed in Annexure-7B to the concerned tissue culture production facility/hardening unit in the same day. The certificate of approval is issued for the specific tested clone to facilitate initiation or for specific batch of tissue culture production from which sample drawn and tested to facilitate dispatch for sale and distribution. In case of export consignments, a phytosanitary certificate will be issued by an authorized agency based on the certificate of approval issued by the accredited testing facility.
- 7.2.2. In addition, the concerned accredited test laboratory will issue required number of certification labels (Annexure-7C) duly signed and stamped for affixing on the packages of consignment of tissue culture plants as prescribed under certification standards established by the Department of Biotechnology in accordance with provisions of Seeds Act, 1966.

### **7.3. Action taken in the case of failed samples:**

- 7.3.1. If the test results for the viruses are positive and or the results of quality (genetic fidelity) testing exceeded the tolerance limits set by the crop specific standards established by the Department of Biotechnology in consultation with crop specific ICAR Institute, the Director/HOD of Accredited Test Laboratory will issue a memorandum of disapproval of tissue culture plants for certification in the format prescribed in Annexure-7D to the tissue culture production facility/hardening unit, as the case may be, within the same day of completion of testing, under intimation to concerned National Referral Laboratory and Accreditation Unit (BCIL) of Department of Biotechnology. In case of export consignments, a copy of the same will also be forwarded to phytosanitary certificate issuing authority.

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<b>Section-7</b>	<b>Reporting Results of Testing &amp; Action taken</b>	<b>Page 2-6 of 6</b>
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- 7.3.2. On receipt of memorandum of disapproval, the concerned tissue culture production facility will take appropriate action as specified in the memorandum of disapproval and intimate the action taken to concerned accredited testing facility.

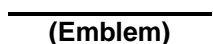
Annexure-7 A

(Logo/Emblem)	_____		Regd. No:
	(Name & Address of Accredited Test Laboratory)		Dated:
<b>TEST REPORT</b>			
1. Plant Species/Variety tested:			
2. Acc. /Batch No. & Batch size:			
3 No. of plants sampled/tested:			
4. Category of tissue culture plants tested:			
5. Name & Address of Tissue Culture Production Facility:			
6. Details of testing:			
Sample No.	Tested for	Testing protocol	Results of Testing
8. Remarks, if any:			
_____			
(Sign/Name of Scientist (Virus/Molecular lab)/date)			

Annexure-7 B

(Logo)	_____	Certificate No.
	_____ <b>(Name of Accredited Test Laboratory)</b>	Date of issue:
<b>CERTIFICATION OF TISSUE CULTURE PLANTS</b>		
<p>This is to certify that the tissue culture plants as described hereunder have been tested and found virus-free* and or/ true to type*, in accordance with certification standards and/or guidelines* established under the National Certification System for Tissue Culture Plants established by the Department of Biotechnology, Ministry of Science &amp; Technology in exercise of the powers conferred under section 8 of the Seeds Act, 1966.</p> <p>Place: _____ Date: _____</p> <p style="text-align: right;">_____ Signature/Name &amp; Designation Stamp of Certifying Authority</p> <p><b>*strikeout, whichever is not applicable.</b></p>		
1. Plant Species/variety:		
2. Accession/Batch No:		
3. Batch size (No. of tissue culture plants):		
4. No of packages		
5. Class of TCP(* strikeout, whichever is not applicable):	Clone/Ex-agar plantlets/Hardened tissue culture plants*	
6. Tissue Culture Production Facility:		

Annexure-7C

<b>Certification Label for Tissue Culture Plants</b>	
 <b>(Emblem)</b>	_____ _____ (Name & Address of Accredited Test Laboratory)
	Certificate No _____ Date of issue _____
Plant species (Common/Botanical Name):	_____
Variety:	_____
Acc./Lot/Batch No:	_____
Batch size:	_____
No of Packages:	_____
Class of TCP(* strikeout, whichever not applicable):	Clone/Ex-agar plantlets/Hardened tissue culture plants*
Tissue Culture Production Facility:	_____
Name/Sign/Stamp of Certifying Authority (ATL) with date	_____ _____

Annexure-7D

Ref. No: \_\_\_\_\_

Dated: \_\_\_\_\_

To:

\_\_\_\_\_  
\_\_\_\_\_

(Name & Address of Tissue culture production Facility)

**Subject: Memorandum of Disapproval for Certification of Tissue Culture Plants**

Sir,

I am to state that the tissue culture plants as described hereunder have been tested in accordance with approved protocols and the same could not be certified due to reasons given below, as per the guidelines/procedures established under the National Certification System for Tissue Culture Plants established by the Department of Biotechnology, Ministry of Science & Technology in exercise of the powers conferred under section 8 of the Seeds Act, 1966.

1. Plant Species/variety:	
2. Accession/Batch No:	
3. Batch size (No. of tissue culture plants):	
4. Category of tissue culture plants:	
5. Reasons for Disapproval for certification:	
6. Action to be taken by the tissue culture production facility:	

Place: \_\_\_\_\_

Date: \_\_\_\_\_

\_\_\_\_\_  
Signature/Name & Designation Stamp  
of Certifying Authority

Copy to:

1. \_\_\_\_\_  
(Name & Address of National Referral Laboratory)

2. \_\_\_\_\_  
(Name & Address of Accreditation Unit (BCIL))

<b>SOPs for Accredited Test Laboratory</b>		
<b>Section-8</b>	<b>Referral Testing</b>	<b>Page 1-2 of 2</b>
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## **8. Referral Testing**

- 8.1. The tissue culture production facility, if aggrieved of the decision of Accredited Test Laboratory, may appeal to the appellate authority under the Chairpersonship of Secretary, Department of Biotechnology for referral testing within a day or two of receipt of memorandum of disapproval. Pending the decision of Appellate Authority the disputed shipment of tissue culture plants will be held in the cold storage room of tissue culture production facility under the sealed custody of certifying authority.
- 8.2. If the Appellate Authority considered the appeal may order with in a maximum of 7 days for referral testing of disputed sample by the national referral laboratory viz., Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi and /or Centre for DNA Finger printing & Diagnostics, Hyderabad, as the case may be, under intimation to the concerned accredited testing facility directing to forward the second sub-sample of disputed sample for referral testing.
- 8.3. The concerned accredited testing facility will forward the second sub-sample of disputed sample for referral testing by the above designated national referral laboratory along with a sample forwardal slip for referral testing (Annexure-8 A).
- 8.4. The concerned national referral laboratory will undertake a referral testing of the second sub-sample of disputed sample maintained by the accredited testing facility involved in the dispute and report the results of referral testing to Appellate Authority with in a maximum period of 3-5 working days.
- 8.5. The Appellate Authority may review the results of referral test report and take a decision and communicate it to the aggrieved tissue culture production facility under intimation to concerned Accredited Test Laboratory within a maximum period of 7 days.
- 8.6. If the decision of Appellate Authority is in favour of the aggrieved tissue culture production facility, the concerned accredited tissue culture testing laboratory will review its decision.

Annexure-8 A

**Sample Forwardal for Referral Laboratory**

1. Ref. No./Date:	
2. Sample forwarded by:	
3. Sample forwarded to:	
4. Date on which sample forwarded:	
5. Sample sent by:	
6. Particulars of sub-sample forwarded for referral testing:	
a. Plant species/variety:	
b. Sample Code Number:	
c. Sub-Sample Size:	
d. Tissue culture Production Unit:	
e. Accession No/Batch No (Which ever applicable):	
f. Batch Size:	
g. Category of tissue culture plants:	
h. Date of Sampling:	
i. Sampled by:	
7. Condition of the sample prior to forwarding (storage, packing etc.):	
8. Testing for:	
9. Testing by:	
10. Reasons for referral testing:	
11. Advise, if any:	
12. Sign/Name/Designation of Sender:	

<b>SOPs for Accredited Test Laboratory</b>		
<b>Section-9</b>	<b>Online Reporting/Monitoring of Testing Activities</b>	<b>Page 1-2 of 2</b>
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- 9.1. The accreditation unit (BCIL) will establish an on-line reporting & monitoring system as a part of website development for National Certification System for Tissue Culture Plants to monitor the virus/quality (genetic fidelity) testing activities of accredited tissue culture testing facilities to ensure that the tissue culture plants produced by the certified tissue culture production facilities are free from viruses, viroids, phytoplasmas, and bacterial pathogens and are true to the type.
- 9.2. The online reporting & monitoring system will be developed using ASP.NET 2.0 with backend is the SQL server 2000.
- 9.3. The accreditation unit (BCIL) will pre-install the software at each of the Accredited Test Laboratory to facilitate on-line reporting of testing activities with user name/password protection authorizing the Scientists (Virus/Molecular Biology) for inputting the data. Until such time, the accredited tissue culture facility will submit a hard copy of the master report of activities of testing facility in the format prescribed at Annexure-9A by e-mail at monthly intervals.
- 9.4. The scientist (Virology/Molecular Biology) will enter the particulars of sampling/testing/certification in internet-enabled database hosted in BCIL website for on-line reporting immediately upon sampling/testing/certification.
- 9.5. The pre-installed soft ware will generate a master report of testing/certification of tissue culture plants in the format prescribed at Annexure-9 A or generate a status report of testing/certification of tissue culture plants by any given date or generate report tissue culture production facility wise or testing facility wise as the case may be, for the access by the accreditation unit (BCIL) of Department of Biotechnology.



<b>SOPs for Accredited Test Laboratory</b>		
<b>Section-10</b>	<b>Calibration of Measuring &amp; Monitoring Equipments</b>	<b>Page 1-3 of 3</b>
<b>May 2008</b>		

**10.1. Identification of Equipment:**

- 10.1.1. The laboratory technician (Virology/Molecular Biology), as the case may be, will identify and documented testing equipment to provide the necessary accuracy of the measurement.
- 10.1.2. He will identify and record each critical equipment using a serial number, lab number/code and or model number and register in a logbook.

**10.2. Calibration of Equipment:**

- 10.2.1. The laboratory technician (Virology/Molecular Biology), as the case may be, will calibrate equipments in accordance with written instructions and tolerances.
- 10.2.2. He will maintain records of calibrated equipment with the information of frequency, conditions, tolerances, method and current status (Annexure-10 A).
- 10.2.3. He will label calibrated equipment with a sticker indicating the status and maintains a calibration record for equipment (Annexure-10 B).
- 10.2.4. He will maintain a certificate of proof of calibration is keeping in the file, when equipment requires calibration from outside the lab.
- 10.2.5. He will label the equipment that does not require calibration is labeled with a "not calibrated" sticker.

**10.3. Maintenance & Utilization of Equipment:**

- 10.3.1. The scientist (Virology/Molecular Biology) will maintain a list of all equipments that includes location, item name, manufacturer, model number, serial number, calibration, frequency, calibration standard and tolerance if applicable and the list is verified and updated annually.
- 10.3.2. He will ensure that the equipments are maintained, stored and handled to preserve their accuracy and protect from damage and deterioration.



Annex-10 B

**Calibration Report Format**

<b>1. Name of equipment:</b>					
<b>2. Identification number:</b>					
<b>3. Tolerance (if applicable):</b>					
<b>4. Manufacturer:</b>					
<b>5. Frequency of calibration:</b>					
<b>6. Method of Calibration:</b>					
<b>7. Remarks:</b>					
<b>8. Details of Calibration</b>					
<b>S. No.</b>	<b>Date</b>	<b>Time</b>	<b>Condition</b>	<b>Adjust</b>	<b>Initial</b>
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
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15					
16					
17					
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19					
20					

SOPs for Accredited Test Laboratory		
Section-11	Documentation Management & Record Control	Page 1 of 3
May 2008		

### **11.1. Document Management:**

- 11.1.1. The accredited testing facility will adopt the standard formats for documentation of information related to sampling, testing and certification of tissue culture plants, as prescribed in the Standard Operating Procedures for Accredited Test Laboratory established herewith by the Accreditation Unit (BCIL), Department of Biotechnology or will establish their own SOPs in line with these established by the Accreditation Unit (BCIL). If the SOPs established by accredited testing facility deviate from the one established by the Accreditation Unit (BCIL) of DBT, they will be required to be technically justified by the accredited testing facility and a copy of the same will be made available to accreditation unit for record.
- 11.1.2. The accredited testing facility will maintain a technical folder to receive and file all the technical information received from the Accreditation Unit (BCIL), Department of Biotechnology related to sampling, testing and certification of tissue culture plants and crop specific standards, testing protocols, phytosanitary requirements etc.
- 11.1.3. If any changes to the Standard Operating Procedures for testing facility established by the Accreditation Unit (BCIL), Department of Biotechnology or revision of document considered necessary, the required changes will be communicated by the testing facility to the document issuing authority (BCIL) in the prescribed format (Annexure-11 A) for necessary approval of change and adoption of revision/modification by the document approving authority (DBT). The accredited testing facility, however, will not make any changes to the SOPs for testing facility established by the Accredited Unit (BCIL) or introduce new document to the SOPs established by the Accreditation Unit (BCIL) without any written approval of Document Approving Authority.
- 11.1.4. As and when any modifications/amendments/revision of documents is brought out, the Accreditation Unit (BCIL), Department of Biotechnology will promptly communicate to all the concerned holders of this document and ensure their replacement. The copy holders should ensure that the obsolete documents are promptly replaced by the revised documents together with revision number to keep it up-to-date. The obsolete documents will be cancelled and filed separately in "obsolete document" folder to prevent confusion or misuse of the document.
- 11.1.5. The accredited testing facility will ensure that photocopies of this document is easily accessible to laboratory technicians/scientists (Virology/Molecular Biology) to facilitate compliance with the Standard Operating Procedures for Accredited Test Laboratory established by the Accreditation Unit (BCIL) of Department of Biotechnology.

SOPs for Accredited Test Laboratory		
Section-11	Documentation Management & Record Control	Page 2-3 of 3
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### **11.2. Record Control:**

- 11.2.1. The accredited testing facility will maintain the record of all activities related to sampling, testing, approval and certification of tissue culture plants and or/disapproval in each folder application-wise maintained separately for each tissue culture production facility-wise.
- 11.2.2. The records will also contain copy of test report including referral testing, certificate of approval of tissue culture plants and or/disapproval certificate issued in respect of failed consignments, records of ELISA/DIBA, documentation records of PCR and autoradiography films of NASH for future reference.
- 11.2.3. The records related to sampling, testing and certification of tissue culture plants will be maintained for a period of at least one year and the records of purchase/calibration of equipment will be maintained as long as the equipment is in use.
- 11.2.4. The accredited testing facility will maintain an up-to-date calico-bound laboratory test register, serially numbered and duly certified by the Scientist (Virology/Molecular Biology), as the case may be.
- 11.2.5. The laboratory test register (Virology/Microbiology) will be retained until such time it is closed and will be available for verification during each audit.

Annexure-11 A

**Document Change Application**

1. Change Requested By:		2. Application Date:	
3. Document Title:			
4. Change Requested:			
5. Reason for change:			
6. Recommendation (Select One):			
<input type="checkbox"/> Reject the change (Reason)			
<input type="checkbox"/> Accept the change with revision (Explain)			
<input type="checkbox"/> Accept the change			
7. If Accepted:	Suggested Date:	Validated change:	
8. Training, if any required:			
9. Received By Coordinator Of Document Control (AU, BCIL): _____ (Signature/Name/Designation)		Date	
10. Approval by DBT:  _____ (Sign/Name/Designation)		Date	
11. Communication of Revision for adoption (AU, BCIL):  _____ (Signature/Name/Designation)		Date	

SOPs for Accredited Test Laboratory		
Section-12	Training/Proficiency Testing	Page 1 of 2
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### **12.1. Training:**

- 12.1.1. Accreditation Unit (BCIL) of Department of Biotechnology, will periodically review with tissue culture testing facilities, identify and record the training needs of the technical personnel in undertaking testing and certification of tissue culture plants in accordance with guidelines established under the National Certification System for Tissue Culture Plants implemented by the Department of Biotechnology, Ministry of Science & Technology.
- 12.1.2. Accreditation Unit (BCIL) of Department of Biotechnology will identify internal/external training needs after taking into account resources available and prepare training programme and request the concerned national referral laboratories for organizing the training. The national referral laboratories viz., Advanced Centre for Virus Research, Division of Plant Pathology, IARI, New Delhi or Centre for DNA Finger Printing & Diagnostics, Hyderabad will develop appropriate training modules in consultation with Accreditation Unit (BCIL) of Department of Biotechnology.
- 12.1.3. Accreditation Unit (BCIL) of Department of Biotechnology will identify human resources (trainers/training coordinator) and prepare training schedule (Title of Training Workshop, Place, Dates (From/To, Trainers & Contact Address of Training Coordinator) for conducting training and budget plan for organizing training workshops.
- 12.1.4. The training-workshops will be organized by respective national referral laboratories viz., Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi or Centre for DNA Finger Printing & Diagnostics, Hyderabad, as the case may be, as per the training schedule finalized by accreditation unit (BCIL) in consultation with concerned national referral laboratory and will be communicated to accredited testing facilities sufficiently in advance to facilitate deputation of personnel for training.
- 12.1.5. The training place will have a comfortable room with sitting chairs with tables/desks for 10-15 trainees and the trainers, LCD Projector and screen for power point presentations and computer facility and printer and white board with marker pens and laboratory facilities for providing hands-on training in virus testing protocols and quality (genetic fidelity) testing protocols, as the case may be.
- 12.1.6. The nominated experts (trainers) will organize training workshop on scheduled dates at specified venue, as per the training modules, approved by the Accreditation Unit (BCIL), Department of Biotechnology.

SOPs for Accredited Test Laboratory		
Section-11	Training/Proficiency Testing	Page 2 of 2
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- 12.1.7. The training workshop will involve one day orientation programme on the National Certification System for Tissue Culture Plants and the Standard Operating Procedures for Accredited Test Laboratory followed by specialised training programme for 5-6 days in virus testing protocols (such as ELISA, DIBA, NASH, RT-PCR) at the Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi or the specialized training programme in genetic fidelity testing (AFLP/ISSR/RFLP/RAPD/ SSR) at Centre for DNA Finger Printing & Diagnostics, Hyderabad.
- 12.1.8. At the end of training the skill competency of trainees will be evaluated in actual conducting of tests as per established protocols and the qualified trainees will be issued a training certificate by the national referral laboratories viz., Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi or Centre for DNA Finger Printing & Diagnostics, as the case may be and provide the Accreditation Unit with a list of qualified trainees for record.
- 12.1.9. The accredited testing facility will maintain a record of technical personnel, who have undergone such training giving information about Name/Designation of Technical Personnel; type of training; period of training (from/to); Training Institute; and Remarks, if any/Sign. of Director/HOD of testing facility.

## **12.2. Proficiency Testing:**

- 12.2.1. The national referral laboratories viz., Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi or Centre for DNA Fingerprinting & Diagnostics, Hyderabad, respectively, will organize proficiency testing of laboratory technicians/scientific experts once in every year to evaluate their competency and consistency in performance of various testing protocols.
- 12.2.2. The national referral laboratories viz., Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi or Centre for DNA Fingerprinting & Diagnostics, Hyderabad, respectively, will submit to the Accreditation Unit (BCIL), Department of Biotechnology, a report of such proficiency testing giving date & venue of proficiency testing; tests carried out; name/designation of participant/accredited testing facility; performance reporting; representing laboratory, for record.

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### **13.1. Communication:**

- 13.1.1. The Accreditation Unit (BCIL) of Department of Biotechnology will timely communicate to the accredited tissue culture testing facilities regarding information on:
- crop specific tissue culture standards
  - country specific phytosanitary requirements for export of tissue culture plants, if any
  - changes to guidelines for accreditation of tissue culture testing facilities
  - validated and approved testing protocols
  - list of regulated pests associated with import of tissue culture plants
  - changes to standard operational procedures for Accredited Test Laboratory
  - list of certified tissue culture production facilities/hardening units & accredited testing facilities
  - any other relevant information
- 13.1.2. The accredited tissue culture testing laboratories will ensure timely reporting of activities related to testing and certification through on-line reporting and monitoring system.
- 13.1.3. The Accredited Test Laboratory will provide periodically any changes to the contact address of Scientists (Virology/Molecular Biology) to Accreditation unit (BCIL) for updating the website.

### **13.2. Auditing:**

- 13.2.1. The Accreditation Unit (BCIL) of Department of Biotechnology will establish a panel of technical experts for auditing of activities performed by the various accredited tissue culture testing facilities to ensure that the standard operating procedures for Accredited Test Laboratory established by the Accreditation Unit are followed for virus/quality (genetic fidelity) testing and certification of tissue culture plants.
- 13.2.2. The Accreditation Unit (BCIL) of Department of Biotechnology, will establish a schedule of audit and nominate at least two experts from the auditing panel for carrying out the technical audit of testing and certification of tissue culture plants and intimate the concerned experts one month in advance, to facilitate making travel arrangements under intimation to concerned Accredited Test Laboratory. The scheduled audits will be carried out once in every year.
- 13.2.3. Besides the above, unscheduled audits will be organized at least once in a year at a short notice without intimating the concerned Accredited Test Laboratory to ensure compliance with the standard operating procedures for Accredited Test Laboratory established by Accreditation Unit (BCIL) of Department of Biotechnology.

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- 13.2.4. Surveillance audits will be carried out at least once in six months or at such intervals, as may be decided by the auditors to ensure corrective actions are taken and preventive measures are implemented subsequent to scheduled auditing.
- 13.2.5. Such audit inspections will involve the verification of records related to testing and certification of tissue culture plants, records of calibration of equipments maintained by the testing facility and records of training; verification of testing protocols being actually practiced; verification of action taken on previous audits; and testing skill competency of technical personnel actually involved in testing.
- 13.2.6. At the end of each audit, an audit report in prescribed format (Annxure-13 A) will be prepared by the auditors in consultation with concerned Accredited Test Laboratory and submit to the Accreditation Unit (BCIL) of Department of Biotechnolgy. The audit report will indicate the non-conformities observed and corrective/preventive action to be taken and time schedules by which the measures required to be implemented to improve the functioning.
- 13.2.7. The concerned Accredited Test Laboratory will communicate to the Accreditation Unit (BCIL). The corrective action/preventive measures taken by the testing facility will be reviewed by the auditor through surveillance audit and reported in a prescribed format (Annexure-13 B) to the Accreditation Unit (BCIL), Department of Biotechnology.
- 13.2.8. Besides the above, the accredited testing facility will have it's own internal auditing inline with the procedures stated above, which will precede external auditing by the Accreditation Unit (BCIL), Department of Biotechnology.

**13.2. Review:**

- 13.2.1. The Accreditation Unit (BCIL), Department of Biotechnology will periodically review the effectiveness of all aspects of National Certification System for Tissue Culture Plants in consultation with all the Accredited Test Laboratory and implement changes to the system if required. Such review meetings will be held annually to discuss the problems/issues related to testing/certification of tissue culture plants and implement corrective action plans/preventive measures for their recurrence.
- 13.2.2. The Accreditation Unit (BCIL), Department of Biotechnolgy will establish a procedure for investigating into non-performing accredited tissue culture testing facilities and recommend to the Department of Biotechnology for appropriate action.

## Annexture-13 A

**Audit (Scheduled) Report.**

<b>1.</b>	<b>Name &amp; Address of Accredited Test Laboratory audited:</b>	
<b>2.</b>	<b>Director/HOD of Accredited Test Laboratory:</b>	
<b>3.</b>	<b>3.1. Name/Designation of Scientist (virology):</b>	
	<b>3.2. Name/Designation of Scientist (Molecular Biology):</b>	
	<b>3.3. Name of laboratory Technicians:</b>	
<b>3.</b>	<b>Auditing related to the period of:</b>	<b>From: _____ To: _____</b>
<b>4.</b>	<b>Date (s) of Auditing:</b>	<b>From: _____ To: _____</b>
<b>5.</b>	<b>List of Records Audited/Documents verified:</b>	
<b>6.</b>	<b>Audited by (Name &amp; Designation):</b>	1. _____ 2. _____
<b>7.</b>	<b>Details of Auditing reported:</b>	
<b>7.1</b>	<b>General Comments:</b>	
<b>7.2</b>	<b>Specific non-conformities observed:</b>	





**Document developed on behalf of Department of Biotechnology (DBT) by  
Accreditation Unit (NCS-TCP) at Biotech Consortium India Limited (BCIL),  
New Delhi**