Standard Operating Procedures For Accredited Test Laboratory

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(Name & Address of Accredited Test Laboratory)

COPY No:____

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

November, 2013

1. Document Issue & Revision:

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Copy 3	Referral Center for Genetic Fidelity testig	National Research Center on Plant Biotechnology (NRCPB), New Delhi-110012
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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:

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

Document:	Procedures, work instructions, references, specifications or regulatory material for the administration of the system.
Explant	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.
Hardened Plant	In-vitro derived plants which have developed good root and stem system to grow in the field conditions and ready for field plantation.
Inoculation	Transferring of explants under aseptic condition onto the media in a culture tube/bottle.
Incubation	Maintenance of inoculated plants in a bottle/tube under controlled environment conditions of temperature, light, humidity and nutrients to provide optimal conditions for growth.
Internal Audit	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.
Internal Document	Document generated outside the limits of the administrative system for example: a regulatory document that is referred to a procedure or work instruction.
Micropropagation	It is the practice of rapidly multiplying the stock material to produce a large number of progeny plants using modern plant tissue culture methods.
Mother Plant	A plant which acts as a source of material for plant propagation by micropropagation
NCS-TCP	National Certification System for Tissue Culture Raised Plants established by the Department of Biotechnology, Ministry of Science & Technology.
Non-Conformity	Any situation that differs from standard procedures, guidelines or regulations

Objective Evidence	Data supporting the existence or verify something
Plantlet	A baby plant produced in vitro on an auxenic culture medium from a meristematic plant tissue.
Plant Tissue Culture	Plant tissue culture is a technique of culturing plant cell, tissue or organ in artificial, controlled and aseptic conditions. It mainly covers micropropogation, organogenesis and somatic embryogenesis.
Pest	Any species, strain or biotype of plant, or pathogenic agent, injurious to plants or plant products.
Procedure	Document that describes, "Who does the job", "when", "where", and "why".
Protocol or Work Instruction	A written instruction to carry out a specific task or activity or job
Record:	Document (electronic or print), product or sample statement, which will confirm that a procedure (or part of the procedure) has been carried out.
Somaclonal variation	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.
Stock Culture	Tissue culture derived from mother plant
Standard Operating Procedures (SOPs)	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 of NCS-TCP, 2013, Department of Biotechnology, New Delihi

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 contract scientist and two laboratory technical assistant 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 ^{0}C & -20 ^{0}C)
- Distilled/RO/Millipore Unit
- Hot Plate/Magnetic Stirrer
- Autoclave
- Microwave Oven
- Vertex Mixer
- pH/EC Meter
- Refrigerator (4 ^{0}C)
- Tissue Grinder/Comodrill/Pestle & Mortar
- Incubator Shaker
- Ice Maker
- Microcentrifuge/Minicentrifuge
- Laminar Air Flow Cabinet
- Fume Hood
- Water Bath with Thermostat (30 to 100 ^{0}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)

- 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

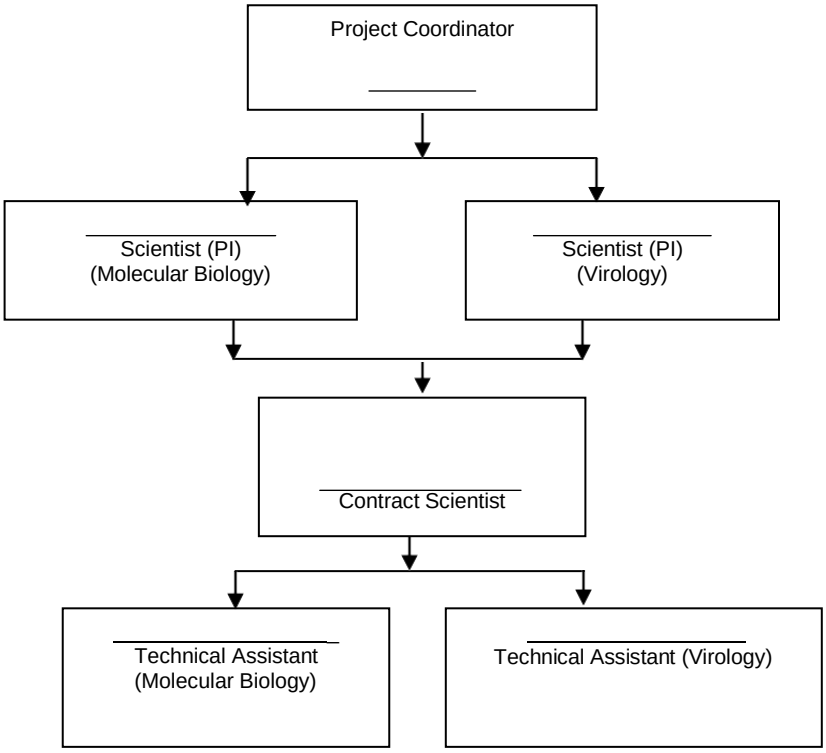
1.5.2.3. Genetic fidelity testing equipments & supplies:

- 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. Project Coordinator:

The Project Coordinator 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 assistants (contractual)/ scientists (contractual)/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

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 technical assistant 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 technical assistant 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. Technical Assistant (Molecular Biology/Virology):

The technical assistant 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.

3.1. Receipt/Registration of Application:

3.1. 1: Plant Tissue/Stock Culture

3.1.1(a) Accredited Test Laboratory will receive intimation for virus testing of mother plant /stock culture in **Annexure- 3A(i)** from a tissue culture production facility at least two weeks prior to receipt of samples . The process flow for testing of plant tissue/stock culture is at **Appendix A**.

3.1.1(b) The intimation {**Annexure- 3A (i)**} received at Accredited Test Laboratory will be verified and an acknowledgement covering the fee details would be sent to the company. This step would ensure the preparedness of ATL and guidance to company in order to send application/sample.

3.1.1(c) Samples would be received along with "Application for virus indexing of plant tissue/stock culture(s)" in the prescribed format {**Annexure 3A (ii**)}.

3.1.1(d) The application received at Accredited Test Laboratory will be verified and ATL would assign **20 digits sample registration number** to each sample as described in the **Annexure 3A(ii).** Unique sample registration number would be maintained thought-out the testing/processing of application

3.1.1(e) The details of the application will be entered in a **Job card (Annexure-3A (iii))** for sample testing of plant tissue/stock culture. The Job card will be forwarded by the Scientist (Virology) to technical person for conducting test.

3.1.2: Tissue Culture Raised Plants

3.1.2(a) Accredited Test Laboratory will receive intimation for virus/ genetic fidelity/genetic uniformity testing for batch certification of tissue culture plants in **Annexure-3B (i)** from the recognized tissue culture production facility at least two weeks prior to receipt of samples. Process flow for batch certification of tissue culture raised plants is at **Appendix 3B**.

3.1.2(b) The **intimation (Annexure-3B(i))** received at Accredited Test Laboratory will be verified and an acknowledgement covering the fee details would be sent to the company. This step would ensure the preparedness of ATL and guidance to company in order to send application/sample.

3.1.2(c) Application for certification of tissue culture plants would be accepted only from the recognized tissue culture facility and only in the case when the batch of tissue culture plants has been produced from indexed stock cultures/mother plants

3.1.2(d) Samples would be received along with "Application for virus/ genetic fidelity testing for batch certification of tissue culture plants" in the prescribed format **(Annexure 3B (ii)).**
Or
Representative of ATL will visit the hardening center of recognized tissue culture production facility for the collection of samples along with prescribed format **(Annexure 3B (ii)).**

3.1.2(e) The application received at Accredited Test Laboratory will be verified and ATL would assign **40 digits batch registration numbe**r to each sample as described in the **Annexure 3B(ii).** Unique batch registration number would be maintained thought-out the testing/processing of application.

3.1.2(f) The details of the application will be entered in a Job card **(Annexure-3B (iii))** for batch certification of tissue culture raised plants. The Job card will be forwarded to the Scientist (Virology/Molecular Biology), as the case may be, for virus/ genetic fidelity/uniformity testing.

3.2. Sampling of tissue culture plants:

3.2.1 Plant Tissue/Stock Culture

3.2.1(a) Each sample should have at least 0.5 gm of tissue per virus per test for all known viruses to be tested.

3.2.1(b) All mother plant tissue/stock culture must be indexed for all known viruses listed in the **Annexure 5B** (Also available in the NCS-TCP website (www.dbtncstcp.nic.in).

3.2.1(c) Ideally all mother plant tissue/stock cultures should be tested. If the number of mother plant tissue/stock culture is large, the samples from batches consisting of a maximum of 10 mother plants/stock cultures may be pooled for testing. In such cases –

(i) The tissue culture unit must maintain proper record of individual mother plants/stock cultures of each batch, so that individual mother plants/stock cultures or smaller batches could be tested, in cases where the pooled samples are found positive for infection, so that only the cultures from infected mother plant/stock culture are discarded.

(ii) If testing is not done as envisaged in 3.2.1 (c) (i), all the cultures generated from the infected mother plants/stock cultures will have to be discarded.

3.2.1(d) The applicant company (seeking recognition/renewal of recognition under NCS-TCP also needs to test the starter material/ stock culture prior to its large scale multiplication in addition to test conducted by its supplier

3.2.1 (d) Virus indexing of mother plants/stock culture can be done by Accredited Test laboratories or any Government Institute or University having the facilities and expertise for virus indexing.

3.2.2 Tissue Culture Raised Plants

3.2.2(a) Each sample should have at least 0.5gm of tissue per virus per test for all known viruses to be tested and an additional 1.0gm for genetic fidelity/uniformity testing.

3.2.2(b) Tissue culture raised plants must be indexed for all known viruses listed in the **Annexure 5B** (Also available in the NCS-TCP website (www.dbtncstcp.nic.in).

3.2.2(c) In case of tissue culture raised plants (ex-agar plants/hardened plants), the following scale of sampling for virus and or/quality (genetic fidelity/ uniformity) 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

3.2.2(d) 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/ fidelity/ uniformity testing without contamination.

3.2.2(e) 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.

3.2.2(f) Every 20th samples of certified batches of tissue culture raised plants will be forwarded to RL by ATLs for random sample testing.

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 Plant Tissue/Stock Culture

3.4.1a. Each sample for plant tissue/stock culture will be allotted 20 digit sample registration number (3 digits for registration number of tissue culture production facility /2 digits for plant species as listed at Appendix 1/2 digits for variety/2 digits for registration number of ATL /6 digits for date/month/year of sample received by ATL/1 digit for nature of sample/1 digit for mother plant/stock culture/last three digits for sample no) to facilitate proper identification of the sample through out the testing process. Sample registration number will be entered by accredited test laboratory and will be maintained throughout testing.

3.4.1 b Each sample should be divided into three sub samples. One of the sub-samples will be maintained as reference sample for minimum one month and other two sub-samples will be utilized for testing, as the case may be, along with a job card (Annexure-3 A(iii). The replicate samples will be either stored in vacuum desicator with anhydrous calcium chloride ($Ca\ Cl_2$) or freeze dry or store at -80 ^{0}C in a deep freezer at the Accredited Test Laboratory.

3.4.2 Tissue Culture Raised Plants

3.4.2a. Each sample for tissue culture raised plant will be allotted **40 digits batch registration number** (the initial 20 digits would be the sample registration number of the plant tissue/stock culture from which the batch has been derived. Additional 20 digits would comprise 4 digits for batch number/4 digits for batch size/3 digits for number of samples/ 6 digits for date/month/year of sample received for batch certification/1 digit for stage

(ex-agar/hardened)/2 digits for ATL to facilitate proper identification of the sample through out the testing process. Batch registration number will be entered by Accredited Test Laboratory and will be maintained throughout testing.

3.4.2 b In case the stock culture/mother plant tissue has been tested by non accredited test laboratory, the ATL who has received samples for certification would assign the initial 20 digits sample registration number as a part of batch registration number. In initial 20 digits (sample registration number), two digits designated for ATL should be allotted [00]

3.4.3 c Each sample should be divided into three sub samples. One of the sub-samples will be maintained as reference sample for minimum one month and other two sub-samples will be utilized for testing, as the case may be, along with a job card (Annexure-3B(iii)). The replicate samples will be either stored in vacuum desicator with anhydrous calcium chloride (Ca Cl2) or freeze dry or store at -80 0C in a deep freezer at the Accredited Test Laboratory.

Annexure-3A (i) Payement details need to be edited

Intimation form for Virus Indexing of Plant Tissue/Stock Culture(s)

(The intimation should reach the Accredited Test Laboratory at least two weeks before the sample(s) is (are) sent)

1. Name/ Address of the tissue culture production facility:	
2. Recognition details under NCS-TCP	Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):	
4. Details of plant sample to be tested: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested*)	
5. Number of sample to be tested	**Plant species**
6. Any additional information:	

Date:

_____

(Signature/Name of Applicant)

*List of viruses to be tested for plant species available at NCS-TCP website (http://www.dbtncstcp.nic.in)

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ACKNOWLEDGEMENT

This is to acknowledge the receipt of "Intimation" for testing of plant tissue/ stock culture(s) from the tissue culture production facility: (Name/Address) …….. Dated..... We request you to kindly send the samples along with the "Application Form for Virus Indexing of tissue stock culture(s) and testing fee of Rs: ……...../- (in word……) through Online transfer (as per account details appended below) for virus indexing for all the known viruses

Date:

Place: ----------

Signature & Name of Director/HOD of Accredited Test Laboratory

Note: Kindly enclose the self-addressed and stamped envelope with intimation form.

Account Details:

Beneficiary Name:	NIPGR- NCS-TCP
Account No.:	40603535988
Type of account:	Saving Account
Bank:	State Bank of India
Branch:	Jawaharlal Nehru University
Address:	JNU Old Campus New Delhi-110067
IFSC code:	SBIN0001624
Email:	ncs-tcp@nipgr.ac.in

Intimation form for Virus Indexing of Plant Tissue/Stock Culture(s)

(The intimation should reach the Accredited Test Laboratory at least two weeks before the sample(s) is (are) sent)

1. Name/ Address of the tissue culture production facility:	
2. Recognition details under NCS-TCP	Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):	
4. Details of plant sample to be tested: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested*)	
5. Number of sample to be tested	**Plant species**
6. Any additional information:	
Date: (Signature/Name of Applicant)	

*List of viruses to be tested for plant species available at NCS-TCP website (http://www.dbtncstcp.nic.in)

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ACKNOWLEDGEMENT

This is to acknowledge the receipt of "*Intimation" for testing of plant tissue/* stock culture(s) from *the tissue culture production facility: (Name/Address)* …….. *Dated* …….. We request you to kindly send the samples along with the "Application Form for Virus Indexing of tissue stock culture(s) and testing fee of Rs: ……...../- (in word……) through Online transfer (as per account details appended below) for virus indexing for all the known viruses

Date:
Place:

Signature & Name of Director/HOD of
Accredited Test Laboratory

Note: Kindly enclose the self addressed and stamped envelope with intimation form.

Annexure-3A (ii)

Application for Virus Indexing of Plant Tissue/Stock Culture (s)

1. Name/ Address of the tissue culture production facility:	
2. Recognition details under NCS-TCP:	Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):	

4. Details of plant sample to be tested: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested*)

Sample No.	**Plant species**	**Variety**	***Nature of sample** **Leaf : 1** **Stem : 2** **Rhizome: 3** **Tuber : 4**	**20 digits Sample registration Number**** (to be allotted by ATLs) **TCPF** (Registration no.)	**Species**	**Variety**	**ATL** (Registration no.)	**Date/Month/Year of sample received** DD MM YY	**Nature of samples** **Leaf : 1** **Stem : 2** **Rhizome : 3** **Tuber : 4**	**MP: 1 SC: 2**	**Sample No.**

5. Sample drawn by:	Accredited Test Lab ☐ Company ☐	
If Samples drawn by ATL: _____ **(Signature/Name/Designation Stamp/Date of Authorized person of/by Accredited Test Laboratory)**	If Samples drawn by Company: _____ **(Signature/Name/Designation of the representative, Tissue Culture Production Facility)**	

In the presence of: _____ **(Signature/Name of authorized person from Tissue Culture Production Facility)**	In the presence of: _____ **(Signature/Name of authorized person from Tissue Culture Production Facility)**	
6. Particulars of payment of testing fees:		
i. Amount in Rs:		
ii. Online transfer acknowledgement no.		
iii. Date of transfer:		
7. Any additional information:		

For Office (Testing Facility) Use

Date of receipt of application:				
Check list	Status		Scrutinized by	
Application complete	Yes	No		
Sample Received with appropriate quantity	Yes	No		
Payment of Fees	Yes	No		
Action Taken: ---------- HOD of laboratory/Date)			---------- (Signature/Name of Director or Accredited Test	

***** Please indicate the type of tissue being sent for testing and its source. For example tissue could be leaf, stem, rhizome, tuber from a field grown plant identified as mother plant or it could be from tissue culture. In case cultures indicate stage of culture cycle.

**** Sample registration number:** Unique 20 digits code number consists of following digits

3 digits for registration number of tissue culture production facility /2 digits for plant species as listed at **Appendix 1**/2 digits for variety/2 digits for registration number of ATL /6 digits for date/month/year of sample received by ATL/1 digit for nature of sample/1 digit for mother plant/stock culture/last three digits for sample no. **.** Sample registration number will be entered by accredited test laboratory and will be maintained throughout testing.

Code to be assigned to different commercially important plant species

Code No.	Plant Species		Code No.	Plant Species
01	Aloe Vera		41	Philodendron
02	Alpine		42	Pineapple
03	Alstroemeria		43	Pointed Gourd
04	Anthurium		44	Pongamia
05	Apple		45	Populus
06	Bamboo		46	Potato
07	Banana		47	Rose
08	Black Pepper		48	Sandalwood
09	Calathea		49	Spathiphyllum
10	Cardamom		50	Stevia
11	Carnation		51	Strawberry
12	Casuarina		52	Sugarcane
13	Cattleya		53	Syngonium
14	Chrysanthemum		54	Teak
15	Citrus		55	Turmeric
16	Coccinea		56	Vanda
17	Cordylines		57	Vanilla
18	Cymbidium		58	Yucca
19	Dahlia		59	Zantedeschia
20	Date Palm		60	Others
21	Dendrobium			
22	Eucalyptus			
23	Ficus			
24	Fig			
25	Gerbera			
26	Ginger			
27	Gladiolus			
28	Grape			
29	Gypsophyllum			
30	Hosta			
31	Jatropha			
32	Lavander			
33	Lemon			
34	Lilium			
35	Limonium			
36	Mangium			
37	Neem			
38	Paphiopedilum			
39	Paulonia			
40	Phalaenopsis			

Annexure-3A (iii)

Job Card For Sample testing of plant tissue/stock culture(s)

Name of the ATL: ....
Address: ....
Accreditation Number:.................................

1. Plant Species to be tested:																													
2. Total Number of samples:																													
3. Particulars of testing																				**Viruses to be tested**									
Sample Registration number (20 digits)																													
																				Testing Protocol : ELISA					**PCR**				
																				Name of virus					**Name of Virus**				

4. Date of testing of samples:	
5. Sample tested by: (Name of technical person)	
6. Remarks (Condition of sample/packing): ________________________ **Signature of Scientist-incharge**	

Annexure-3B (i) Account details need to be edited

Intimation form for (Virus/ genetic fidelity) Testing for Batch Certification of Tissue Culture Raised Plants

(To be submitted only by tissue culture production facility recognized under NCS-TCP. The application should reach the ATL at least two weeks before the sample(s) is (are) sent)

1. Name and Address of the recognized tissue culture production facility:	
2. Recognition details under NCS-TCP	Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):	

4. Details of tissue culture plants required to be sampled: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested* and an additional 1.0 gm for genetic fidelity testing. Sample(s) from mother plant/stock culture is (are) also to be sent for genetic fidelity testing)

Number of sample to be tested.	Plant species	Batch number	Batch size

5. Is the above batch derived from indexed stock cultures/mother plant: Yes/No

If yes!

5.1 Please indicate the sample registration number of stock culture from which the batch has been derived in case the indexing has been done by ATL under NCSTCP:

5.2: If test of stock culture has not been done by ATL then give the details of indexing lab:

6. Any additional information:

Date: _____

(Signature/Name of Applicant)

*List of viruses to be tested for plant species available at NCS-TCP website (http://www.dbtncstcp.nic.in)

.....cut here.....

ACKNOWLEDGEMENT

This is to acknowledge the receipt of Intimation for testing and batch certification of tissue culture raised plants from tissue culture production facility: (Name/Address) ..... We request you to kindly send the samples along with the application for testing certification of tissue culture raised plants and fee Rs.....(in word... .....) through Online transfer (as per account details appended below)

Date:

Place:

Signature/Name of Director, Institute

or

HOD of Accredited Test Laboratory

Note: Self-addressed and stamped envelope to be enclosed with the intimation form.

Account Details:

Beneficiary Name:	NIPGR- NCS-TCP
Account No.:	40603535988
Type of account:	Saving Account
Bank:	State Bank of India
Branch:	Jawaharlal Nehru University
Address:	JNU Old Campus New Delhi-110067
IFSC code:	SBIN0001624
Email:	ncs-tcp@nipgr.ac.in

Intimation form for (Virus/ genetic fidelity) Testing for Batch Certification of Tissue Culture Raised Plants

(To be submitted only by tissue culture production facility recognized under NCS-TCP. The application should reach the ATL at least two weeks before the sample(s) is (are) sent)

1. Name and Address of the recognized tissue culture production facility:			
2. Recognition details under NCS-TCP			Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):			
4. Details of tissue culture plants required to be sampled: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested* and an additional 1.0 gm for genetic fidelity testing. Sample(s) from mother plant/stock culture is (are) also to be sent for genetic fidelity testing)			
Number of sample to be tested.	**Plant species**	**Batch number**	**Batch size**
5. Is the above batch derived from indexed stock cultures/mother plant: Yes/No **If yes!** **5.1 Please indicate the sample registration number of stock culture from which the batch has been derived in case the indexing has been done by ATL under NCSTCP:** **5.2: If test of stock culture has not been done by ATL then give the details of indexing lab:**			
6. Any additional information: **Date:** _____ (Signature/Name of Applicant)			

*List of viruses to be tested for plant species available at NCS-TCP website (http://www.dbtncstcp.nic.in)

……cut here……..

ACKNOWLEDGEMENT

This is to acknowledge the receipt of Intimation for testing and batch certification of tissue culture raised plants from tissue culture production facility: (Name/Address) …….. We request you to kindly send the samples along with the application for testing certification of tissue culture raised plants and fee Rs……(in word……) through Online transfer (as per account details appended below)

Date:
Place:

Signature/Name of Director, Institute or
HOD of Accredited Test Laboratory

Note: Self addressed and stamped envelope to be enclosed with the intimation form.

Annexure-3B (ii)

Application for (Virus/ genetic fidelity) Testing for Batch Certification of Tissue Culture Raised Plants

1. Name and Address of the recognized tissue culture production facility:	
2. Recognition details under NCS-TCP	Registration No. Certificate No. Validity of Certificate:
3. Name of authorized person & contact details (Telephone/ Fax/Mobile/E-Mail):	
4. Date of Sampling:	
5. Number of samples drawn *:	

6.1 Please indicate the 20 digits sample registration number of the plant tissue/stock culture from which the batch has been derived in case the testing has been done under NCS-TCP:

6.2. If testing of plant tissue/stock culture has not been done under NCS-TCP, give the details of indexing lab

(please enclose a copy of test report)

7. Details of tissue culture plants required to be sampled: (Each sample should have at least 0.5gm tissue/virus/test for all the known virus to be tested*)

Batch No.	Batch size	Plant species	Variety	Stage of TC Plants	****40 Batch Registration Number (to be filled by ATLs)**	
					20 digits sample reg. no. from which batch is derived	**4 digits for batch number + 4 digits for batch size + 3 digits for number of samples + 6 digits for date, month and year of sample received for batch certification + 1 digit for stage (ex-agar/hardened)+2 digits for ATL (20 digits)**
					Total 40 digits for batch registration number	
					(20 digits sample reg. no. from which batch is derived)	

8. Purpose of testing/certification:	[ ] Domestic market [ ] Export **Tick out in appropriate box**
9. Testing For:	Virus Indexing [ ] Genetic Fidelity [ ]

If Samples drawn by ATL:	If Samples drawn by company:
_____ **(Signature/Name/Designation Stamp/Date of Authorised person of/by Accredited Test Laboratory)**	_____ **(Signature/Name/Designation of the representative, Tissue Culture Production Facility)**

In the presence of: _____ **(Signature/Name of authorized person from Tissue Culture Production Facility)**	In the presence of: _____ **(Signature/Name of authorized person from Tissue Culture Production Facility)**

11. Particulars of payment of testing fees:			
Amount in Rs:			
Online transfer acknowledgement no.			
Date of transfer:			
12. Any additional information:			
For Office (Testing Facility) Use			
Date of receipt of application:			
Check list	Status		Scrutinized by
Application complete	Yes	No	
Sample received in appropriate quantities	Yes	No	
Payment of Fees	Yes	No	
Action Taken: ---------- (Signature/Name of Director or HOD of Accredited Test laboratory/Date)			
**** Batch Registration Number will be entered by Accredited test laboratory and will be maintained through out certification.**			

*__Sampling method for TC raised plants__

Batch size	**Number of tissue culture plants to be sampled (sample size)**
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

**** Batch Registration Number:**

The initial 20 digits of the batch registration number would be the sample registration number of the plant tissue/stock culture from which the batch has been derived. Additional 20 digits would consist the following information:

4 digits for batch number + 4 digits for batch size + 3 digits for number of samples + 6 digits for date, month and year of sample received for batch certification + 1 digit for stage (ex- agar/hardened) +2 digits for ATL

Annexure-3B (iii)

Job Card for sample testing of TC raised plants

Name of the ATL: .....

Address: .....

Accreditation Number:.....

1. Plant Species to be tested:																		
2. Total Number of samples:																		
3. Batch Registration number (40 digits):																		
	Viruses tested																Genetic Fidelity testing	
Sample no.	Testing Protocol																True to type	Genetically variable
	ELISA								PCR									
	Name of virus								Name of Virus									
4. Date of testing of samples:																		
5. Sample tested by: (Name of technical person)																		
6. Remarks (Condition of sample/packing):																		

_____

Signature of Scientist-incharge

4. Preparation/handling of samples for testing:

4.1. The laboratory technical assistant 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 pippeting 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 pippetings 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.

5.1. The scientist/laboratory technical assistant (plant virology) will use established protocols specified in Annexure-5A for virus testing as approved by the referral Center (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 referral centre (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.

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Annexure-5 A

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 immunobsorbent assay (DAC-ELISA); and (b) double antibody sand-witch 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 micropippette (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
$Na_2HPO_4.2H_2O$	1.44 g or
$Na_2HPO_4.12H_2O$	2.90 g or
Na_2HPO_4	1.50 g
KH_2PO_4	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_2CO_3	1.59 g
$NaHCO_3$	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 to1 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

3.1. Weigh 500 mg test / healthy tissue and grind using autocalved mortar and pestle/tissue grinder in coating buffer containing 2 % (w /v) PVP at a ratio of 1:1 (w / v) at room temperature and low speed centrifuge (ca. 12,000 g) for 2 min.

↓

3.2. Dispense 200µl extract from test as well as healthy samples to each well of the microtitre plate. Cover the plate and incubate at 37°C for 1 h.

↓

3.3. Decant and wash the plate by flooding the wells with PBS-T for about 3 min. Repeat wash and soak operations thrice, and shake out residual liquid draining on a paper towel.

↓

3.4. Dispense 200µl blocking solution (Bovine Serum Albumin, 1 %, w /v) to each well. Incubate at 37°C for 1 h to block polystyrene well reactive surfaces.

↓

3.5. Wash the plate three times as above.

↓

3.6. Dispense 200µl of antiserum (primary antibody) diluted in PBS-TPO to each well. Incubate at 37°C for 2 h.

↓

3.7. Wash the plate three times as above.

↓

3.8. Dispense 200µl anti-rabbit immunoglobulin alkaline phosphatase (universal conjugate, Sigma, USA) diluted in PBS-TPO to each well. Incubate at 37°C for 2 h.

↓

3.9. Wash the plate three times as above.

↓

3.10. Dispense 200µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) (0.5 – 1.0 mg / ml) solution in substrate buffer to each well. Incubate at 37°C for 2 h.

↓

3.11. Measure the intensity of color in each well at 405 nm by using ELISA reader. Compare the absorbance values of the test samples with healthy control. Take a print out for record (Consider samples showing absorbance (OD_{405}) values more than two times of healthy control as positive).

4. Testing Protocol: DAS-ELISA

4.1. Dispense 200 ul specified antibody of desired working dilution in coating buffer to each well and incubate for 1h at 37 °C

↓

4.2. Decant and wash the plate by flooding the wells with PBS-T for about 3 min. Repeat wash and soak operations thrice, and shake out residual liquid draining on a paper towel.

↓

4.3. Weigh 500 mg sample (test / healthy tissue) and grind using autoclaved mortar and pestle in PBS-TPO (1:1 w / v) at room temperature and centrifuge (ca. 12,000 g) for 2 min.

↓

4.4. Dispense 200 µl extract from test as well as healthy samples to each well and incubate at 37 °C for 2h.

↓

4.5. Wash the plate three times as above.

↓

4.6. Dispense 200 µl enzyme labeled (alkaline phosphatase) specific antibody diluted in PBS-TPO to an appropriate dilution and incubate at 37 °C for 2h.

↓

4.7. Wash the plate three times as above.

↓

4.8. Dispense 200 µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) (0.5 – 1.0 mg / ml) solution in substrate buffer to each well and incubate at 37°C for 2h.

↓

4.9. Measure the intensity of color in each well at 405 nm by using ELISA reader. Compare the absorbance values of the test samples with healthy control (Consider samples showing A_{405} nm values more than two times of healthy control as positive).

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:

- Micropippettes (10µl, 50 µl, 100 µl)
- Multichannel Adjustable micropippette (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 $MgCl_2 6H_2O$	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 $CHCl_3$, vortex and centrifuge (ca. 12,000 g) for 2 min.

↓

3.3. Pipette out 200µl clarified sap (upper aqueous layer) into 800µl 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 µl) 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 exits. 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 micropippette (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_2O 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_2O. 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)

Composition of PCR mix		
Reagents	**Volume required (µl) per reaction**	
	Test sample	**Negative control**
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
Total	100	100

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	42ºC	45 min	1
Initial denaturation	94ºC	5 min	1
Denaturation	94ºC	30 s	
Annealing	45-50ºC	1 min	30
Extension	72ºC	1 min	
Final extension	72ºC	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}P DNA probes) as well as non-radioactive probes (chemiluminscent, digoxigenenin-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 H_2O. 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 H_2O. 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_2O. 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
$MgCl_2$	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
$NaH_2PO_4.H_2O$	27.6 g
EDTA	7.4 g

Dissolve in 800 ml H_2O. 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_2O. 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_2O 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_2O in 800 ml H_2O. 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_2O. 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_2O	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:

3.1.1. Put on disposable gloves and cut a piece of membrane (size depends on the number of samples to be tested).

↓

3.1.2. Draw a lattice of 1 cm square with a soft lead pencil and cut one edge to indicate orientation.

↓

3.1.3. Wet the membrane in 20 X SSPE or 20 X SSC for 30 min. and place it on a sheet of whatman-3mm filter paper.

↓

3.1.4. Grind test/healthy samples (~200 mg) in TE buffer (1:1 w/v). Also include a positive and negative control. Press through muslin cloth.

↓

3.1.5. Take 200µl of this extract and denature DNA by adding 50 µl 0.5 N Na OH for 10 min.

↓

3.1.6. Add 25 µl 3 M sodium acetate (pH 5.0) and make dilutions of 1:10 and 1:100 in TE.

↓

3.1.7. Pipette carefully 2.5 µl of each of the samples in each square of the membrane.

↓

3.1.8. Let the spots dry at room temperature and bake the membrane in vacuum oven for 2 h at 80°C.

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_2O	33 µl
Oligolabelling buffer	10 µl
BSA	2 µl
Template	1 µl (20 ng DNA / µl)
32_P-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_2O	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 $1x10^6$ 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:

3.4.1. Remove the membrane from pre hybridization solution and discard the solution into radioactive wastes.

↓

3.4.2. Transfer the membrane to a plastic bag filled with washing solution. Wash twice with 20 X SSC containing 0.1 % SDS at room temperature with constant agitation for 15 – 20 min.

↓

3.4.3. After two washes, place the membrane on a sheet of filter paper and allow it to dry at room temperature.

↓

3.4.4. After drying, place the membrane between 2 layers of thin polythene sheet in a cassette with intensifying screen.

↓

3.4.5. Place X-ray film on the top in dark room, close cassette and place at -70°C overnight. Develop auto radiogram.

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 Viruses and Phytoplasmas reported to naturally infect plants currently covered under National Certification System for Tissue Culture Plants

S. No.	Common Name	Scientific Name	Viruses/ Phytoplasma	No. of viruses to be tested	Tests to Detect listed Virus/ Phytoplasma	
					ELISA	PCR/ RT-PCR
1.	**Alstroemeria**	**Alstromeria spp.**	Alstroemeria mosaic virus (Potyvirus)	3	+	+
			Cucumber mosaic virus (Cucumovirus)		+	+
			Tospovirus			+
2.	**Apple**	**Malus domestica**	Apple chlorotic leaf spot virus (Trichovirus)	5	+	+
			Apple mosaic virus (Ilarvirus)		+	+
			Apple stem grooving virus (Capillovirus)		+	+
			Apple stem pitting virus (Foveavirus)		+	+
			Prunus necrotic ringspot virus (Ilarvirus)		+	+
3.	**Bamboo**	**Bambusa spp.**	Bamboo mosaic virus (Potexvirus)	1	+	+
4.	**Banana**	**Musa spp.**	Banana bract mosaic virus (Potyvirus)	4	+	+
			Banana bunchy top virus (Babuvirus)			+
			Banana streak virus (Badnavirus)		+	+
			Cucumber mosaic virus (Cucumovirus)		+	+
5.	**Black Pepper**	**Piper nigrum**	Cucumber mosaic virus (Cucumovirus)	3	+	+
			Piper yellow mottle virus		+	+

			(Badnavirus)			
			Phytoplasma		+	+
6.	**Calathea**	**Calathea lancifolia**	Nil	Nil	-	-
7.	**Calla lily**	**Zantedeschia aethiopica**	Cucumber mosaic virus (Cucumovirus)	3	+	+
			Dasheen mosaic virus (Potyvirus)		+	+
			Tospovirus			+
8.	**Cardamom Large**	**Amomum subulatum**	Cardamom bushy dwarf virus (Babuvirus)	2	+	+
			Large cardamom chirke virus (Macluravirus)		+	+
9.	**Cardamom Small**	**Elettaria cardamomum**	Banana bract mosaic virus (Potyvirus)	2	+	+
			Cardamom mosaic virus (Macluravirus)		+	+
10.	**Carnation**	**Dianthus caryophyllus**	Carnation etched ring virus (Caulimovirus)	5	+	+
			Carnation latent virus (Carlavirus)		+	+
			Carnation mottle virus (Carmovirus)		+	+
			Carnation necrotic fleck virus (Closterovirus)		+	+
			Carnation vein mottle virus (Potyvirus)		+	+
11.	**Cordyline**	**Cordyline fruticosa**	Nil	Nil	-	-
12.	**Date Palm**	**Phoenix dactylifera**	Phytoplasma	1		+
13.	**Eucalyptus**	**Eucalyptus spp.**	Phytoplasma	1		+
14.	**Fig**	**Ficus spp.**	Cucumber mosaic virus (Cucumovirus)	1	+	+

15.	**Gerbera**	**Gerbera spp.**	Cucumber mosaic virus (Cucumovirus)	3	+	+
			Tobacco rattle virus (Tobravirus)		+	+
			Tospovirus			+
16.	**Ginger**	**Zingiber officinale**	Cucumber mosaic virus (Cucumovirus)	2	+	+
			Ginger chlorotic fleck virus (unassigned Sobemovirus)		+	+
17.	**Gladiolus**	**Gladiolus spp.**	Bean yellow mosaic virus (Potyvirus)	4	+	+
			Cucumber mosaic virus (Cucumovirus)		+	+
			Tobacco rattle virus (Tobravirus)		+	+
			Tobacco ringspot virus (Nepovirus)		+	+
			Tobacco mosaic virus (Tobamovirus)		+	+
18.	**Lily**	**Lilium longifolium**	Cucumber mosaic virus (Cucumovirus)	4	+	+
			Lily mottle virus (Potyvirus)		+	+
			Lily symptomless virus (Carlavirus)		+	+
			Tospovirus			+
19.	**Neem**	**Azadirachta indica**	Nil	Nil	-	-
20.	**Orchids**	**Cymbidium spp., Dendrobium spp., Cattleya spp., Phalaenopsis spp. etc.**	Cymbidium mosaic virus (Potexvirus)	4	+	+
			Odontoglossum ringspot virus (Tobamovirus)		+	+
			Tobacco mosaic virus (Tobamovirus)		+	+
			Tospovirus			+
21.	**Pineapple**	**Ananas comosus**	Pineapple mealybug wilt-associated virus (Ampelovirus)	2		+
			Badnavirus			+
22.	**Pointed gourd**	**Trichosanthes dioica**	Ageratum enation virus (Begomovirus)	1		+

23.	**Potato**	**Solanum tuberosum**	Potato apical leaf curl virus (Tomato leaf curl New Delhi virus)	8		+
			Potato leaf roll virus (Polerovirus)		+	+
			Potato virus A (Potyvirus)		+	+
			Potato virus M (Carlavirus)		+	+
			Potato virus S (Carlavirus)		+	+
			Potato virus X (Potexvirus)		+	+
			Potato virus Y (Potyvirus)		+	+
			Tospovirus			+
24.	**Strawberry**	**Fragaria spp.**	Strawberry latent ringspot virus (Nepovirus)	3	+	+
			Strawberry mild yellow edge-associated virus		+	
			Tobacco streak virus (Ilarvirus)		+	+
25.	**Sugarcane**	**Saccharum officinarum**	Sugarcane bacilliform virus (Badnavirus)	4	+	+
			Sugarcane mosaic virus (Potyvirus)		+	+
			Sugarcane yellow leaf virus (Polerovirus)			+
			Phytoplasma			+
26.	**Syngonium**	**Syngonium spp.**	Tospovirus	1		+
27.	**Teak**	**Tectona grandis**	Cucumber mosaic virus (Cucumovirus)	1	+	+
28.	**Vanilla**	**Vanilla spp.**	Bean common mosaic virus (Potyvirus)	9	+	+
			Bean yellow mosaic virus (Potyvirus)		+	+
			Cowpea aphid borne mosaic virus (Potyvirus)		+	+
			Cucumber mosaic virus (Cucumovirus)		+	+

			Cymbidium mosaic virus (Potexvirus)		+	+
			Dasheen mosaic virus (Potyvirus)		+	+
			Odontoglossum ringspot virus (Tobamovirus)		+	+
			Ornithogalum mosaic virus (Potyvirus)		+	+
			Watermelon mosaic virus (Potyvirus)		+	+
29.	**Yucca**	**Yucca spp.**	Yucca bacilliform virus (Badnavirus)	1		+

ELISA: Enzyme-linked Immunosorbent Assay; **RT-PCR**: Reverse transcription polymerase chain reaction; **PCR:** Polymerase chain reaction

6.1. The scientist/laboratory technical assistant (molecular biology) will use established protocols specified in Anexure-6 for genetic fidelity testing by the National Research Center for Plant Biotechnology, New Delhi.

6.2. If any new protocols used or any deviations from existing protocols will require validation by the national referral laboratory (i.e., National Research Center for Plant Biotechnology, New Delhi) 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 National Research Center for Plant Biotechnology, New Delhi.

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 National Research Center for Plant Biotechnology, New Delhi.

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Annexure-6

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. TATATATATATATATA- represented as $(TA)_8$). An imperfect microsatellite is a repeat motif that is interrupted by base(s) that does not match the motif sequence (e.g. TATATATACTATATA). 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. TATATATATAGTGTGTGTGT).

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 µl of pre-warmed Extraction buffer and 700 µl of 2X CTAB buffer and incubate for 60 min at 60^0C 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 µl of RNase A (10 mg/ml) to final concentration of 50 mg/ml and incubate for 30 min at 37^0 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 ml 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 37ºC 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 4ºC.

(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 4ºC.

(h) Again supernatant is transferred to fresh tube. 2/3rd volume of isopropanol is added and incubated at –20ºC 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^{0} 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 lamda 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 ^{0}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:

$$\text{Amount of DNA (µg / µl)} = \frac{(OD)_{260} * 50 * \text{dilution factor}}{1000}$$

(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 copes 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 in to 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 serves 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 TM that allows an annealing temperature of 55-65^{o}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.

$MgCl_2$ concentration: the optimal $MgCl_2$ concentration is 1.5mM. Mg^{2+} influences enzyme activity and increases the T_M (melting temperature) of double stranded DNA. Excess Mg^{2+} 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 aquacticus that inhabits hot springs.

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

Components	**Final concentration**
10X Buffer	1X
25 mM $MgCl_2$	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):

Components	**Final concentration**
Spermidine	0.4 mM
Triton X-100	0.1%

The PCR amplification conditions for ISSR assay are as follows: an initial denaturation at 94 ^{o}C for 5 minutes, followed by 35-45 cycles of denaturation (at 94 ^{o}C for 45 seconds), annealing* (at 45-55 ^{o}C for 45 seconds) and extension (at 72 ^{o}C for 2 min), and a final extension step at 72 ^{o}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 $MgCl_2$ 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.

Components	**Final concentration**
10X Buffer	1X
25 mM $MgCl_2$	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

Additional components (optional):

Components	**Final concentration**
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 $MgCl_2$ 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:

ISSR Primer	Nucleotide sequence
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′

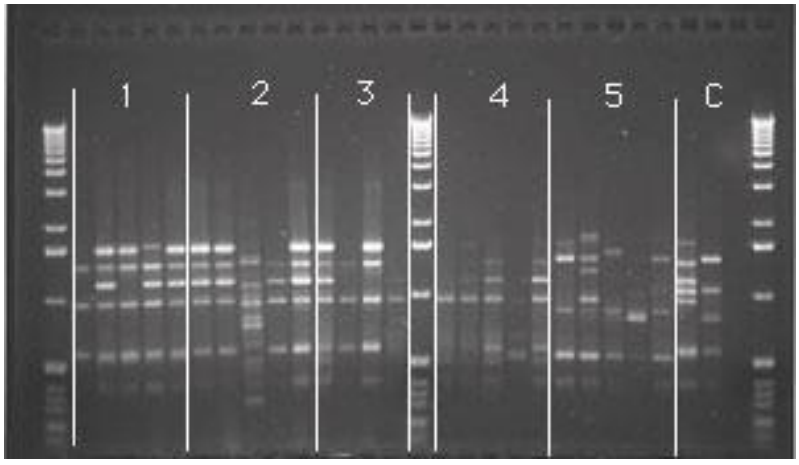
Single letter abbreviations for mixed base positions:

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:

Final Concentration	For 100ml	Stock
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_2O	65.9 ml	--

2X CTAB Solution:

Final Concentration	For 100ml	Stock
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_2O	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 4ºC

[**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 power.]

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_2 EDTA.$_2H_20$)

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 ($Na_2EDTA.2H_2O$)

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 4ºC.

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 ^{o}C for 15 minutes
- Allow it to cool slowly to room temperature (do not snap cool).

7.1. Reporting Results of Testing:

7.1.1 Plant tissue/stock culture

7.1.1(a) The technician assistant (Virology) will complete the testing and enter the results of laboratory testing in the job card within a maximum period of 3-4 working days from the date of receipt of the sample and submit to the scientist (Virology), who will verify the results and sign the job card.

7.1.1(b) Scientist (Virology) will prepare the test report as per the format prescribed at **Annexure-7A (i)** and verify the results and sign the test report for issue within a day.

7.1.1(c) The Scientist (Virology) will enter details of sample and test results in the Master Report of virus indexing of plant tissue/stock culture as per the format prescribed at **Annexure 7A (ii)** prior to dispatch of test report to the tissue culture production facility.

7.1.2 Batch certification of tissue culture plants:

7.1.2(a) The respective technical assistant (Virology/Molecular Biology) will complete the testing and enter the results of laboratory testing in the job card within a maximum period of 3-4 working days from the date of receipt of the samples and submit to the Scientist (Virology/Molecular Biology), who will verify the results and sign the job card.

7.1.2(b) The respective Scientist (Virology/Molecular Biology) will prepare the test report as per the format prescribed at **Annexure- 7 B (i)** and verify the results and sign on the test report for issue within a day.

7.1.2(c) The Scientist (virus/genetic fidelity/uniformity) ATL will enter respective details of sample and test results in the Master Report of virus indexing/genetic fidelity as per the format prescribed at **Annexure 7B (v)**

7.1.2(d) If the test results for the viruses are negative and the results of genetic fidelity/uniformity confirms that plants are true to type, the Project Coordinator of Accredited Test Laboratory will approve the sample and issue a **"Certificate of Quality"** in the format prescribed (MS Excel format) in **Annexure- 7B (ii)** to the concerned tissue culture production facility/hardening unit in the same day.

7.1.2(e) The Accredited Test Laboratory will send a soft copy of "Certificate of Quality" to the NCS-TCP Management Cell (NMC) for printing the required number of certification labels. Data received (in soft copy) from ATLs in the "Certificate of Quality" (MS Excel Format) will be directly imported for printing the content on certificate of Quality with 40 digit 2D barcode. Whenever "certification label" is issued both type of testing (i.e. virus indexing and genetic fidelity/uniformity testing) has to be conducted and samples are found virus free and true to type or genetically uniform

7.1.2(f) The printed certification label with barcode will be dispatched from NCS-TCP Management Cell to ATLs for issuance to respective tissue culture production facilities. Only 10 Certification labels would be issued per "Certificate of Quality" for each batch of tissue culture raised plants

7.1.2(g) ATLs will assess the printed content on certification label and authenticate by signing and placing seal on the printed certification label (with barcode) prior to the issuance to respective tissue culture production facility.

7.1.2(h) The "Certificate of Quality" is issued to 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 Test Laboratory**.

7.1.2(i) "Certificate of Quality" and "Certification Labels" are to be issued if samples of tissue culture raised plants have been tested for both freedom from viruses and true to typeness/ genetic uniformity. However, genetic fidelity testing may not be required in some plant species. In such cases, only "Certificate of Quality" may be issued without "Certification Label" clearly stating that this certificate is only for Quality with respect to freedom for viruses. It may be noted that tissue culture raised plants should be tested for all the known viruses affecting the plant species being tested, as listed in the Standard Operating Procedures (SOPs) for the purpose of issuance of certificate. The "Certificate of Quality" should clearly mention the nature of testing conducted. Whenever "Certification Label" is issued both type of testing (i.e. virus indexing and genetic fidelity testing) has to be conducted and samples are found virus free and true to type

7.1.2(j) Colour of the label shall be diagonally yellow No. 356 (IS 5-1978) and opaline green (IS No. 275) having size of 12 cm x 6 cm. ATL will issue 10 labels with Certificate of Quality and issuance of additional label will be provided only on written request from company without any charges.

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 genetic fidelity testing confirms that plants are not true to type the Director/HOD of Accredited Test Laboratory will issue a letter for "**Not approved for Certification"** in the format prescribed in **Annexure-7B (iv)** to the tissue culture production facility/hardening unit, as the case may be, within the same day of completion of testing, under intimation to National Referral Laboratory and NCS-TCP Management Cell (NIPGR) of Department of Biotechnology. In case of export consignments, a copy of the same will also be forwarded to phytosanitary certificate issuing authority.

7.3.2. On receipt of communication "Not approved for Certification", the concerned tissue culture production facility will take appropriate action as specified and intimate the action taken to concerned accredited testing laboratory.

Annexure-7A (i)

NCS-TCP	_____ _____ (Name & Address and Accreditation Number of Accredited Test Laboratory)	Date:

VIRUS INDEXING REPORT FOR PLANT TISSUE/ STOCK CULTURE(S)

1. Name and Address of Tissue Culture Production Facility:
2. Plant species tested:
3. Total Number of samples:

4. Particulars of testing	Viruses to be tested								
Sample Registration number (20 digits)	Testing Protocol : ELISA					PCR			
	Name of virus					Name of Virus			

Remarks, if any.

(Sign/Name of Scientist (Virology)/date)

(Signature of the Head of ATL/date)

Annexure-7A (ii)

Master Report of virus indexing of plant tissue/stock culture(s)

Name of the ATL: .....
Address: .....
Accreditation Number:.....

S. No.	Sample Registration number	Tissue culture production Facility	Sample details		Number of samples	Date of receipt of samples	Date of reporting	Tested for	Result of testing	Remarks (if any)
			Species	Variety						

Annexure-7B (i)

NCS-TCP	_____ (Name, Address and Accreditation number of Accredited Test Laboratory)	Date:

TEST REPORT FOR TISSUE CULTURE RAISED PLANTS

1. Name & Address of Tissue Culture Production Facility:	
2. Plant species tested:	
3. Total Number of samples:	
4. Batch Registration number:	

5. Testing details

Sample No.	Viruses tested																Genetic Fidelity testing	
	Testing Protocol																	
	ELISA								PCR									
	Name of virus								Name of virus								True to type	Genetically variable

6. Remarks, if any

_____

(Sign/Name of Scientist (Virus/Molecular lab)/date)

(Signature of the Head of ATL/date)

Annexure -7B (ii)

NCS-TCP	**(Name of ATL)**	**Batch Reg. No**	
	(Address)	**Certificate Number.**	
	(Accreditation No.)	**Date of Issue**	

CERTIFICATE OF QUALITY

This is to certify that the tissue culture plants as described hereunder have been tested and found virus-free and genetically uniform, 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 & Technology in exercise of the powers conferred under Section 8 of the Seeds Act, 1966

Place:
Date:

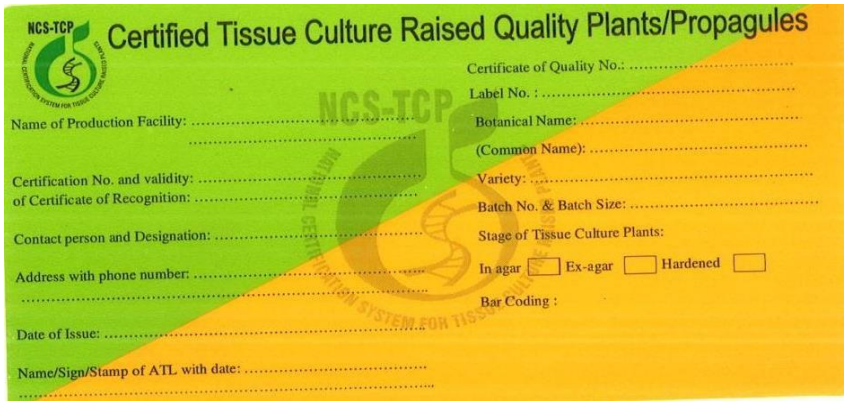
Signature/Name & Designation Stamp
of Certifying Authority

1. Plant Species/Variety/Common			
2. Batch Number			
3. Batch size			
4. Stage of Tissue Culture Plants	☐	In-agar	
	☐	Ex-agar	
	☐	Hardened	
5. Name of Tissue Culture Production Facility			
6. Address of Tissue Culture Production Facility with phone number			
7. Certification No. and validity of Certificate of Recognition			
8. Contact Person and Designation			

Note (i) This certificate is valid till the time of dispatch of plants from the Tissue Culture Production Facility

Signature and seal

Annexure-7B (iii)



Annexure-7B (iv)

Name of the ATL: .....
Address: .....
Accreditation Number:.....

Batch registration Number:
Dated: _____

To:

_____
_____
_____

(Name & Address of Tissue culture production Facility)

Subject: Tissue Culture Raised Plants "Not approved for Certification"

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. Batch No./Batch Size:	
3. Category of tissue culture plants:	
4. Reasons for Disapproval for certification:	
5.: Suggested remedial action	

Place:_____
Date:_____

Signature/Name & Designation Stamp
of Certifying Authority

Copy to:
1. _____
(Name & Address of National Referral Laboratory)
2._____
(Name & Address of NCS-TCP Management Cell (NIPGR)

Signature and seal

Annexure-7B (v)

Master Report of testing/certification of tissue culture plants by Accredited Test Laboratories

Name of the ATL: .....
Address: .....
Accreditation Number:.....

S. No	Batch registration number	Recognized Tissue culture production Facility	Batch Number	Batch size	Tissue culture plants sampled		Number of samples	Date of receipt of samples	Date of reporting	Tested for	Results of testing		Certified / Disapproved	Number of labels issued
					Species	Variety					Virus indexing	Genetic fidelity testing		

8.0 Referral Testing for Plant Tissue/Stock Culture or Batch certification of Tissue culture raised plants

8.1 Every 20th samples of certified batches of tissue culture raised plants received from ATLs will be tested by RL with in prescribed (for random sample testing). In case the result are not in concurrence with the result of ATL the same will be immediately informed to respective ATL for further procedural requirement.

8.2 The tissue culture production facility, if aggrieved by 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 maximum period of 15 days from the receipt of test report. Pending the decision of Appellate Authority the disputed stock culture/plant tissue will be held in the safe custody in the company premise for the maximum period of 30 days and will be not used for production/multiplication. In case of disputed batch, it will also be held in company premise for maximum 30 days and will not be distributed for plantation.

8.3 Appeal for referral testing of disputed sample would be made directly to the NCS-TCP Management Cell (NMC) under intimation to Appellate Authority. NMC will intimate the referral laboratory and direct to the concerned ATL to forward the second sub-sample of disputed sample for referral testing within a period of 7 days.

8.4 On receipt of intimation from NMC, the concerned ATL will forward disputed samples for referral testing by the referral laboratory along with a sample forwardal form for referral testing in **Annexure-8 A** for stock culture/plant tissue and **Annexure 8B** for tissue culture raised plants**.**

8.5 The concerned referral laboratory will undertake a referral testing of disputed sample received from the ATL and intimate the results of referral testing to NMC with a copy to Appellate Authority within a maximum period of one week.

8.6 The Appellate Authority may review the results of referral test report and take a decision in the matter in consultation with Appellate Panel within a maximum period of 7 days from the receipt of test report.

8.7 If the decision of the Appellate Authority is in favour of the company, ATL will be directed to issue a fresh "Certificate of Quality" for tissue culture raised plants and fresh test report in case of stock cultures.

8.8 If the decision of Appellate Authority is against the company, the communication/certificate of ATL i.e. "Tissue culture plants not approved for Certification"/test report earlier issued to the aggrieved company would be effective.

Annexure-8 A

Sample Forwarded to Referral Laboratory

Name of the ATL: .....
Address: .....
Accreditation Number:.....

1. Date:																	
2. Sample forwarded by:																	
3. Sample forwarded to:																	
4. Date on which sample forwarded:																	
5. Particulars of sub-sample forwarded for referral testing:																	
a. Tissue culture Production Unit:																	
c. Date of Sampling:																	
d. Sampled by:																	
6. ATL testing details																	
Sample Registration number (20 digits)	**Testing Protocol**																
	ELISA								**PCR**								
	Name of virus								**Name of virus**								
7. Condition of the sample prior to forwarding (storage, packing etc.):																	
8. To be tested for:																	
9.Reasons for referral testing:	(copy of grievance is to be enclosed)																
10. Remark, if any:																	
11. Sign/Name/Designation of Sender:	In-charge, ATL Signature and seal																

Annexure-8 B

Sample Forwarded to Referral Laboratory

Name of the ATL: .....

Address: .....

Accreditation Number:.....

1 Batch registration Number:																		
2. Sample forwarded by:																		
3. Sample forwarded to:																		
4.Date on which sample forwarded:																		
5. Particulars of sub-sample forwarded for referral testing:																		
a. Tissue culture Production Unit:																		
b. Date of Sampling:																		
c. Sampled by:																		
6. ATL testing details																		
Sample No.	Viruses tested															Genetic fidelity testing		
	Testing Protocol															True to type	Genetically variable	
	ELISA								PCR									
7. Condition of the sample prior to forwarding (storage, packing etc.):																		
8.To be testing for:																		
9. Reasons for referral testing:	(copy of grievance is to be enclosed)																	
10. Remark, if any:																		
11. Sign/Name/Designation of Sender:	In-charge, ATL Signature and seal																	

9.1. The NCS-TCP Management Cell (NMC) 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.

9.3. The NMC 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 at the NCS-TCP 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 NMC of Department of Biotechnology.

10.1. Identification of Equipment:

10.1.1. The laboratory technical assistant (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 technical assistant (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.

Annexure-10 A

Record of Calibrated Equipment

Name of the ATL: ....
Address: ....
Accreditation Number:.................................

S. No.	Equipment	Id. No.	Tolerance	Frequency	Location	Comments & Sign of Scientist

Annexure-10 B

Calibration Report Format

Name of the ATL: .....
Address: .....
Accreditation Number:.....

1. Name of equipment:					
2. Identification number:					
3. Tolerance (if applicable):					
4. Manufacturer:					
5. Frequency of calibration:					
6. Method of Calibration:					
7. Remarks:					
8. Details of Calibration					
S. No.	**Date**	**Time**	**Condition**	**Adjust**	**Initial**
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

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 NCS-TCP Management Cell (NMC), Department of Biotechnology or will establish their own SOPs in line with these established by the NMC. If the SOPs established by accredited testing facility deviate from the one established by the NMC 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 NMC 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 NMC, 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 NMC, Department of Biotechnology or revision of document considered necessary, the required changes will be communicated by the testing facility to the document issuing authority (NIPGR) 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 NMC or introduce new document to the SOPs established by the NMC without any written approval of Document Approving Authority.

11.1.4. As and when any modifications/amendments/revision of documents is brought out, the NMC, 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 technical assistant s/scientists (Virology/Molecular Biology) to facilitate compliance with the Standard Operating Procedures for Accredited Test Laboratory established by the NMC of Department of Biotechnology.

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-11A

Document Change Application

Name of the ATL: .....
Address: .....
Accreditation Number:.....

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

12.1. Training:

12.1.1. NCS-TCP Management Cell (NMC) 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. NMC 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 centres for organizing the training. The national referral centres viz., Advanced Centre for Virus Research, Division of Plant Pathology, IARI, New Delhi or National Research Center for Plant Biotechnology, New Delhi will develop appropriate training modules in consultation with NMC of Department of Biotechnology.

12.1.3. NMC of Department of Biotechnology will identify human resources (trainers/training coordinator) and prepare training schedule (Title of Training Work- Shop, 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 centres viz., Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi or National Research Center for Plant Biotechnology, New Delhi, as the case may be, as per the training schedule finalized by NMC 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 NCS-TCP Management Cell (NMC), Department of Biotechnology.

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 National Research Center for Plant Biotechnology, New Delhi.

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 centres viz., Advanced Centre for Plant Virus Research, Division of Plant Pathology, IARI, New Delhi or National Research Center for Plant Biotechnology, New Delhi, as the case may be and provide the NMC with a list of qualified trainees for record.

12.1.9. The accredited testing facility will maintain a record of technical personnel, who have under gone 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 centres viz., Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi or National Research Center for Plant Biotechnology, New Delhi, respectively, will organize proficiency testing of laboratory technical assistant /scientific experts once in every year to evaluate their competency and consistency in performance of various testing protocols.

12.2.2. The national referral centres viz., Advanced Centre for Plant Virology, Division of Plant Pathology, IARI, New Delhi or National Research Center for Plant Biotechnology, New Delhi, respectively, will submit to the NMC, 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.

13.1. Communication:

13.1.1. The NMC 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 NMC for updating the website.

13.2. Auditing:

13.2.1. The NMC of Department of Biotechnolgy 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 NMC are followed for virus/quality (genetic fidelity) testing and certification of tissue culture plants.

13.2.2. The NMC of Department of Biotechnolgy, 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 NMC of Department of Biotechnology.

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 audting.

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 NMC 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 NMC. 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 NMC, 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 NMC, Department of Biotechnology.

13.2. Review:

13.2.1. The NMC, 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 NMC, Department of Biotechnology will establish a procedure for investigating into non-performing accredited tissue culture testing facilities and recommend to the Department of Biotechnology for appropriate action.