Banana virus indexing ring test 2017

Aim: Ring Test between 10 Institutes to determine the virus indexing capabilities within the *Musa* GR community.

Contact scientist: Dr Kathy Crew, DAF, Brisbane, Australia (kathy.crew@daf.qld.gov.au)

Introduction

Freedom from viruses is a critical component in the supply of clean banana planting material and in the exchange of banana germplasm. Material entering Bioversity's International *Musa* Germplasm Transit Centre (ITC), in Leuven, Belgium, is routinely indexed for viruses by collaborating laboratories, using a standard protocol including symptom observation, IC-PCR and electron microscopy. These and other assays are described in the 3rd edition of the "Technical guidelines for the safe movement of *Musa* germplasm" recently published by Bioversity International (http://www.musalit.org/seeMore.php?id=15942). A ring test is a valuable check on the efficiency of testing methods, and also aspects that need attention.

The workplan for the banana virus indexing ring test, coordinated by the MusaNet Conservation Thematic Group, is attached (Appendix 1).

Invited participants

On 15 October, 2017 letters of invitation to participate in the ring test for banana virus indexing (Appendix 2) and a copy of the standard protocols (Appendix 3) were sent out to the 15 banana virology laboratories listed in Table 1. Interest in participating in the ring test was expressed by 12 of these laboratories, with intention to use the standard protocols, their own protocols, or a mixture or comparison of these protocols. Participation by five laboratories was precluded as biosecurity protocols could not be met.

Table 1. Laboratories invited to participate in the ring test.

Country	Institute	Contact person	Participation	Comments
Australia	DAF	Kathy Crew	yes	Standard assays
Belgium	ULg	Sebastien Massart	yes	Standard assays
Brazil	EMBRAPA	Claudia Fortes Ferreira	Unable to satisfy phytosanitary certificate	Withdrawn
Burundi/Kenya	ISABU	Célestin Niyongere	no reply	
China (Kunming)		Kuanyu Zheng	Unable to satisfy phytosanitary certificate	Withdrawn
China (Taiwan)		Hong-Ji Su / T. H Hung	no reply	John Thomas also emailed
France (Guadeloupe)	CIRAD	Pierre-Yves Teycheney	yes	Compared standard and own lab assays
France (la Reunion)	ANSES	Delphine Masse	yes	Own assays
France (Montpellier)	CIRAD	Marie-line Caruana	yes	Own assays
Fiji	SPC	Amit Sukal	Unable to get import permit in time	Withdrawn
India	ICAR	R. Selvarajan	Unable to get import permit	Own assays
Nigeria	IITA	Lava Kumar	yes	Own assays, no BBrMV
The Philippines	UPLB	Fe Dela Cueva	yes	Mix of own and standard assays
USA	UM	Ben Lockhart	Unable to get import permit in time	Withdrawn
Uganda	NARO	Jerome Kurbiriba	no reply	email bounced, resubmitted through web form

Sample details

The ring test entailed blind tests on up to 15 lyophilised leaf samples infected with a defined range of banana viruses, specifically Banana bunchy top virus (BBTV), Cucumber mosaic virus (CMV), Banana bract mosaic virus (BBrMV), Banana mild mosaic virus (BanMMV), and Banana streak viruses (BSV): Banana streak Cavendish virus (BSCAV), Banana streak Goldfinger virus (BSGFV), Banana streak Imové virus (BSIMV), Banana streak Mysore virus (BSMYV), Banana streak Obino L'Ewai virus (BSOLV).

Six samples for BSV indexing (labelled 1 to 6; see Table 2 for details), eight samples for indexing for other banana viruses (BBTV, CMV, BanMMV, BBrMV; labelled A to G and J), and six samples as positive and negative controls for PCR assays were provided to participating laboratories. Four samples for ISEM analysis were also provided to the laboratories participating in this component of the ring test (Table 3).

Along with lyophilised leaf tissue, aliquots of the immunocapture antibodies and (RT)-PCR primers required for conduct of the standard indexing assays were sent to laboratories using the standard protocols.

Table 2. Lyophilised leaf tissue samples and controls provided to participating laboratories.

Assay	Tube label	Virus	Host cultivar (Group)
BSV	control: IM+GF	BSIMV & BSGFV	IRFA 910 (AAB)
BSV	control: OL+MY+CA	BSOLV & BSMYV & BSCAV	Mixed
BSV, other viruses	control: H (AAA)	Negative	Williams (AAA)
Other viruses	control: BBTV	BBTV	Williams (AAA)
Other viruses	control: CMV	CMV	Williams (AAA)
Other viruses	control: BanMMV	BanMMV	Ducasse (ABB)
Other viruses	control: BBrMV (& BanMMV)	BBrMV & BanMMV	Cardarba (ABB)
BSV	sample 1	BSOLV	TMBx hybrid
BSV	sample 2	BSCAV	Williams (AAA)
BSV	sample 3	BSMYV	Mysore (AAB)
BSV	sample 4	Negative	Williams (AAA)
BSV	sample 5	Negative	Pisang ceylan (AAB)
BSV	sample 6	BSIMV & BSGFV	IRFA 910 (AAB)
Other viruses	sample A	BBTV	Williams (AAA)
Other viruses	sample B	Negative / BSOLV	TMBx hybrid
Other viruses	sample C	CMV	Williams (AAA)
Other viruses	sample D	BBTV weak	Williams (AAA)
Other viruses	sample E	Negative	Ladyfinger (AAB)
Other viruses	sample F	BBTV 1 in 5 dilution	Williams (AAA)
Other viruses	sample G	BanMMV	Ducasse (ABB)
Other viruses	sample J	BBrMV & BanMMV	Cardarba (ABB)
ISEM	sample EM1	BSOLV	TMBx hybrid
ISEM	sample EM2	Negative	Ladyfinger (AAB)
ISEM	sample EM3	BanMMV	Ducasse (ABB)
ISEM	sample EM4	BSOLV & CMV & BanMMV	Mixed, see above

Table 3 details the dispatch and delivery dates for the samples, controls and reagents sent to each participating laboratory. Prior to dispatch to participants, samples and controls were tested by Dr Crew using standard assays to confirm virus status (Appendix 4).

Leaf tissue and reagents were sent by FedEx courier and laboratories advised of the waybill number so transit of their package could be tracked. Sample instruction sheets were included with the packages (Appendix 5), and laboratory contacts emailed with a form on which to return their laboratory's results (Appendix 6).

Details of protocols from laboratories using their own assays are presented in Table 4.

Table 3. Details of dispatch of ring test materials to participating laboratories. *All laboratories received samples for laboratory (molecular/serological) testing.*

Lab	EM	Dispatch date	Delivery date	Comments
A	Yes	27/11/2017	5/12/2017	
B	Yes	5/12/2017	8/12/2017	
C	No	27/11/2017	4/12/2017	Parcel arrived very warm.
D	No	27/11/2017	4/12/2017	Own assay protocols provided after sample dispatch; normally use much larger sample size for assays.
E	Yes	2/01/2018	5/01/2018	
F	No	27/11/2017	6/12/2017	No BBrMV sent.
G	No	28/11/2017	4/12/2017	BSMYV primers sent separately (delivery date 7/12/17).

Table 4. Details of protocols of laboratories using own assays.

Lab	Protocol summary	Comments
C	CIRAD protocol compared with standard international protocol.	Do not routinely test for BSCAV but included in ring test using standard assay.
D	**BBTV:** ELISA using Agdia reagents **CMV:** ELISA using Agdia & PRI reagents **BBrMV:** IC-one step RT-PCR using Agdia antibodies and Bract N2+NR primers **BanMMV:** one-step RT-PCR on RNA extract using ML Caruana's primers **BSV:** IC-PCR using Sediag antibody and standard primers for OL, GF, MY and own primers for IM.	Do not routinely test for BSCAV.
F	**BBTV:** partial R gene and full N gene (own primers) **CMV:** own primers **CI (degenerate potyvirus):** own primers **BanMMV:** BanMMCP2 and Poty1 primers **BSV:** standard primers for BSGFV, BSCAV, BSIMV, BSMYV; also included BadnaFP+RP pair **Also:** Banana microsatellite AGMI 025/026 primer pair.	TNAE template for (RT)-PCRs, including BSV. Did not test for BSOLV. AGMI 025/026 microsatellite assay labelled as BSV assay.
G	Not received (as at 5/6/18).	Normally test for BBTV, CMV, BBrMV. Used to test for BSV but no longer have antibodies.

Results and Discussion

Analyses of the ring test results are summarised below (**Error! Not a valid bookmark self-reference.**), with details presented in Table 6 and Table 7. A de-identified version of this report has been sent to each participating laboratory, with relevant feedback on their specific test results.

Most participant laboratories returned results that were close to those expected. Highest conformity to the expected results were achieved by laboratories using either the standard international indexing methods used for the ITC (and Australian post-entry quarantine) or other validated indexing methods, although the sample quantity required for these latter tests was much greater.

Issues with the testing were mostly minor, and concerned the arrival condition of the sample (eg samples arriving in a warm/hot) or quantity (requiring significant modification to regular sample preparation methods because of insufficient tissue which lead to minor cross-contamination). The participants were aware of these issues and communicated well with the test organiser.

Testing deficits were identified in two laboratories. Identification of these issues will hopefully assist these laboratories to improve their testing procedures (and may explain previous irregularities with testing).

One laboratory experienced a high rate of false positives across a number of tests. Of concern is that this laboratory tested for BSV species using a nucleic acid extract as PCR template, which generated false positives because of the amplification of endogenous sequences. This laboratory also apparently missed testing specifically for BSOLV, and reported the microsatellite marker AGMI 025/026 test as a test for BSV. Additionally, only one of this laboratory's two tests for BBTV appeared specific; a number of false positives for this virus were reported for the non-specific test.

A second laboratory appeared unaware that their BSOLV test was not working. Size separation between the BSV amplicons in the multiplex IC-PCR assay may not be sufficient for all laboratories' visualisation procedures to discriminate between them. It is suggested that an additional non-multiplexed BSOLV positive control be run to monitor for amplification of this virus in the assay, as this species is commonly detected in germplasm indexing.

Several laboratories reported that they do not routinely index for BSCAV, however this is unlikely to be a cause for concern because this BSV species is very rarely detected by laboratories which index germplasm for it. However, the lack of detection of BSCAV by one laboratory highlights that infection by any BSV species outside the common four (BSOLV, BSIMV, BSGFV and BSMYV) can easily be missed if a non-specific assay for BSV is not included in the suite of banana virus indexing assays.

Electron microscopic detection of flexuous rod-shaped particles was achieved by all three laboratories participating in this component of the test. CMV titre appears to have been insufficient for virion detection by microscopy as no laboratories detected this virus. Only one laboratory successfully detected and identified bacilliform particles, which is a concern for detection of BSV species not covered in the standard molecular assay protocols.

Recommendations

1. Five laboratories interested in participating were not able to do so because of issues obtaining the required import documentation from their country's quarantine/biosecurity regulator. Future ring tests should ideally allow 3-6 months for this paperwork to be obtained by the participants before test samples and controls are sent out, and be aware that it imposes both cost to the participant laboratories (financial and salary costs to organise the permit) and test organiser (salary to communicate sample/test details to participants).

2. The technical guidelines list multiple published testing methods and reagent details for each virus. However, as demonstrated for BBTV, while specific and sensitive serological and molecular testing methods are in use by a number of laboratories, some alternative assays are not specific and generate false positives.

Additionally, as demonstrated for BSV indexing, the use of an incorrect template preparation method generated false positives using specific primers because of the amplification of endogenous BSV sequences. Hence, it may be worth updating the technical guidelines to recommended specific protocols (and their details) that have been validated against a wide variety of virus and host cultivar genomic sequence variation. For some viruses, several test formats (eg ELISA and PCR) could be recommended as alternatives.

3. To facilitate improvement of the conduct of banana virus indexing assays, a discussion forum could be established on the MusaNet website. This would allow diagnosticians to raise queries about assay protocols, and share new developments relevant to diagnostic assays.

4. A concerning point identified by the ring test was the difficulty for some laboratories to identify bacilliform (BSV) particles by electron microscopy. This lack of BSV detection may because of difficulty with the sample preparation, or operation or alignment/resolution of the microscope, or because of the experience/familiarity of the operator with the range of plant virus shapes and sizes. Because of the serological and molecular variability between BSV species, non-specific methods of detection form a crucial part of virus indexing of banana germplasm. Investigation of alternative indexing methods which do not rely on prior knowledge of the virus, such as high throughput sequencing with non-specific (but possibly virion-enriching) template preparation methods, is recommended to improve the ability of laboratories without electron microscope assess or expertise to index for BSV species not detected by the routine specific assays. Two cautionary considerations are that, like electron microscopy, high-level expertise and equipment (in this case high-end computing resources) are required, and that the relevant background biological knowledge must be integrated with the interpretation of the bioinformatics.

Table 5. Summary of results of Laboratory (molecular/serological) testing.

Lab	BSV assay	Testing for other viruses	Electron microscopy	Comments
A	Results as expected.	Results as expected.	Results almost as expected. Isometric particles not detected.	
B	Did not detect BSOLV in Sample 1 or control, and confused BSMYV detection with possible BSOLV positive.	Results as expected.	Flexuous rod-shaped particles detected. Bacilliform particles either not detected or confused for flexuous rods. Isometric particles not detected.	BSOLV PCR appears to have failed; problems with size of bands (gel stain issues?)
C	Results almost as expected. One BSV control did not amplify.	Results almost as expected. CMV in sample C and control, and weak BBTV (sample D) were not detected.	Not done.	The parcel arrived quite warm, so virion degradation may explain these results. BBTV and BanMMV detection was more sensitive with the standard international protocol.

Lab	BSV assay	Testing for other viruses	Electron microscopy	Comments
D	Results almost as expected. Did not detect BSCAV in Sample 2 and control.	Results almost as expected. Did not detect BanMMV in all samples with this virus. Unexpected CMV detection in Sample F.	Not done.	Used own testing methods, which do not detect BSCAV. Modification of sample preparation methods likely contributed to false positive. BanMMV primers should be checked against known diversity.
E	Results as expected.	Results almost as expected. Unexpected BanMMV detection in Sample C.	Flexuous rod-shaped particles detected. Bacilliform particles not detected. Isometric particles not detected.	Details of EM testing not provided. Uncertain whether these results are from a leaf/sap dip rather than ISEM.
F	Unexpected BSV detection in healthy/uninfected samples. Additional (or incorrect) BSV species detected in infected samples.	Unexpected BBTV detection in Samples B, C, E and Healthy, CMV and BanMMV controls. Unexpected CMV detection in BanMMV control.	Not done.	Used own testing methods, which do not detect BSOLV. Many false positives for BSV assays; correct primers but used on nucleic acid extract, which may also detect endogenous virus sequences. BBTV detection using R component primers generated a number of false positives. BBTV detection using N component primers was specific.
G	Not received.	Not received.	Not done.	Tests completed, results not returned to organiser (as at 5/6/18).

Table 6. Laboratory (molecular/serological) indexing results, as reported from participating laboratories to which samples have been sent.
Unexpected results are presented in red.

Laboratory	Virus status	A	B	C	D	E	F	G
Sample receipt		5/12/17	8/12/2017	4/12/17	4/12/17	5/01/2018	6/12/2017	4 & 7/12/2017
Results returned		15/12/17	2/3/18	15/12/17	19/12/17	28/01/2018 & 22/3/18	14/05/2018	Not received (as at 5/06/18)
BSV assay								
control: H (AAA)	uninfected	negative	negative	negative	negative	negative	BSGFV & BSIMV	
control: IM+GF	positive	positive	positive	positive	positive	positive	BSGFV, BSIMV & BSMYV	
control: OL+MY+CA	positive	positive	BSMYV & BSCAV	negative	BSOLV & BSMYV	positive	BSMYV, BSCAV & BSGFV	
sample 1	BSOLV	BSOLV	negative	BSOLV	BSOLV	BSOLV	BSMYV & BSCAV	
sample 2 (AAA)	BSCAV	BSCAV	BSCAV	BSCAV	negative	BSCAV	BSCAV, BSGFV, BSIMV & BSMYV	
sample 3 (AAB)	BSMYV	BSMYV	BSMYV or BSOLV	BSMYV	BSMYV	BSMYV	BSMYV, BSIMV & BSCAV	
sample 4 (AAA)	negative	negative	negative	negative	negative	negative	BSIMV & BSCAV	
sample 5 (AAB)	negative	negative	negative	negative	negative	negative	BSMYV & BSCAV	
sample 6(AAB)	BSIMV & BSGFV	BSGFV & BSIMV	BSGFV & BSIMV	BSGFV & BSIMV	BSGFV & BSIMV	BSGFV & BSIMV	BSGFV, BSIMV, BSMYV & BSCAV	
Other viruses assay								
control: H (AAA)	uninfected	negative	negative	negative	negative	negative	BBTV (R only)	
control: BBTV	BBTV	BBTV	BBTV	BBTV	BBTV	BBTV	BBTV	
control: CMV	CMV	CMV	CMV	negative	CMV	CMV	CMV & BBTV(R only)	
control: BanMMV	BanMMV	BanMMV	BanMMV	BanMMV	BanMMV	nd	BanMMV, CMV & BBTV (R only)	
control: BBrMV	BBrMV	BBrMV	BBrMV	BBrMV	BBrMV	BBrMV	Not sent	
sample A	BBTV	BBTV	BBTV	BBTV	BBTV	BBTV	BBTV	
sample B	negative	negative	negative	negative	negative	negative	BBTV (R only)	
sample C	CMV	CMV	CMV	negative	CMV	CMV & BanMMV	CMV & BBTV (R only)	
sample D	BBTV weak	BBTV	BBTV	negative	BBTV	BBTV	BBTV	
sample E	negative	negative	negative	negative	negative	negative	BBTV (R only)	
sample F	BBTV 1/5 dilution	BBTV	BBTV	BBTV	BBTV & CMV	BBTV	BBTV	
sample G	BanMMV	BanMMV	BanMMV	BanMMV	negative	BanMMV	BanMMV	
sample J	BBrMV & BanMMV	BBrMV & BanMMV	BBrMV & BanMMV	BBrMV & BanMMV	BBrMV & BanMMV	BBrMV	Not sent	

Table 7. Electron microscopy indexing results, as reported from participating laboratories to which samples have been sent.
Unexpected results are presented in red.

Laboratory	Virus status	A	B	E
Sample receipt		5/12/2017	8/12/2017	5/01/2018
Results returned		15/12/2017	2/3/2018	28/01/2018
sample EM1	BSOLV	Bacilliform particles	? flexuous rod-shaped particles	Negative
sample EM2	Negative	Negative	Negative	Negative
sample EM3	BanMMV	Flexuous rod-shaped particles	Flexuous rod-shaped particles	Flexuous rod-shaped particles
sample EM4	BSOLV & BanMMV & CMV	Bacilliform particles & flexuous rod-shaped particles. No CMV	Flexuous rod-shaped particles No bacilliform particles or CMV	Flexuous rod-shaped particles No bacilliform particles or CMV

Appendices

Appendix 1. Workplan for the banana virus indexing ring test 2017.
Appendix 2. Letter of invitation to participate in the banana virus indexing ring test 2017.
Appendix 3. Protocol outlining the standard banana virus indexing assays.
Appendix 4. Verification of the material and reagents provided to laboratories.
Appendix 5. Sample instructions, included with parcel of samples and reagents.
Appendix 6. Form for submission of indexing results.

Appendix 1. Workplan for the banana virus indexing ring test 2017.

Aim: Ring Test between 10 Institutes to determine the virus indexing capabilities within the *Musa* GR community.
Contact scientist: Dr Kathy Crew

Methodology

A ring test will be organised to assess detection of a number of banana viruses, specifically Banana bunchy top virus (BBTV), Cucumber mosaic virus (CMV), Banana bract mosaic virus (BBrMV), Banana mild mosaic virus (BanMMV), and Banana streak viruses (BSV).

The ring test will consist of two components: molecular/serological assays (approximately 15 samples) and immunosorbent electron microscopy (ISEM; 6-8 samples). Up to 10 laboratories will participate in the molecular/serological assays component. Four or five laboratories (ITC virus indexing centres or prospective centres) are anticipated to be included in the ISEM component.

Test samples for the molecular/serological assays are planned to consist of single and mixed virus combinations, covering all viruses, and a variety of healthy samples covering AAA, AAB and ABB genotypes. ISEM samples will cover BBrMV, BanMMV, BSV, CMV, healthy banana and mixed virus samples.

Methods for the molecular/serological assays and ISEM will be provided to each participating laboratory. For laboratories wishing to use these methods, indexing reagents (antibodies and primers) and positive controls will be provided along with the test samples. Laboratories not using the standard methods will need to provide a summary of their assay protocol ahead of test sample provision. Handling of the samples in accordance with biosecurity requirements will be emphasised in the correspondence with the laboratories.

Blind samples as freeze-dried leaf tissue will be sent out to participating laboratories in October, 2017. Laboratories will have four weeks from sample receipt to conduct the indexing assays, and six weeks for ISEM samples. Individual laboratories will be provided with feedback on their reported results. Results from all laboratories will be collated, and collated data distributed to participating laboratories will be presented in an anonymous fashion. Strictly confidential actual results will be reported to Bioversity International.

Major expenses for conduct of this research are staff salaries, for experimental design, correspondence with participating laboratories, preparation and checking of samples, collation of results, and analysis and reporting, and courier costs.

Laboratories suggested to participate:

Australia – Brisbane*† (previous VIC)
Belgium – Gembloux*† (current VIC)
China – Kunming*†
China – Taiwan*†
France – CIRAD-Montpellier‡
France – CIRAD-Guadeloupe
France – ANSES-La Reunion
India
Philippines
Fiji – SPC
Brazil – EMBRAPA
Nigeria – IITA
Uganda
USA

* molecular/serological assays must be conducted according to the current VIC protocols for full indexing
† ISEM component must be conducted according to the current VIC protocols for full indexing
‡ invite participation in the ISEM component

Appendix 2. Letter of invitation to participate in the banana virus indexing ring test 2017.

Dear (Facility Manager),

Through the MusaNet Conservation Thematic Group, we are holding a ring test on banana virus detection. Freedom from viruses is a critical component in the supply of clean planting material and in the exchange of germplasm. Material entering the Bioversity International Transit Centre, KUL, Belgium, is routinely indexed for viruses by collaborating laboratories, using a standard protocol including IC-PCR, electron microscopy and symptom observation. These and other assays are described in, the 3rd edition of the "Technical guidelines for the safe movement of Musa germplasm" recently published by Bioversity International (http://www.musalit.org/seeMore.php?id=15942). A ring test is a valuable check on the efficiency of testing methods, and also aspects that need attention.

We invite you to participate in this exercise, using the routine assays used by Bioversity International, or alternative assays of your choice.

The ring test is scheduled to be completed by early-December 2017, and will entail blind tests on up to 15 lyophilised leaf samples infected with a defined range of banana viruses, specifically Banana bunchy top virus (BBTV), Cucumber mosaic virus (CMV), Banana bract mosaic virus (BBrMV), Banana mild mosaic virus (BanMMV), and Banana streak viruses (BSV).

For laboratories using the standard protocols, methods and specialised indexing reagents (antibodies and primers) can be provided along with the test samples. Positive controls will also be provided. Laboratories using alternative assays are requested to provide a summary of their protocol prior to sample dispatch. A payment from Bioversity International to cover indexing costs (up to US $500) will be made available to laboratories that submit test results within four/six weeks of their receipt. Individual labs will be informed of their own results, but overall test results will be presented anonymously.

Please let me know as soon as you are able if you would like to participate in this ring test. We can then discuss the assays you propose to use and any assistance you may need in preparation.

Best regards,
Kathy Crew, contact scientist
John Thomas, co-chair of the Musanet Conservation Thematic Group

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Mail: Ecosciences Precinct, GPO Box 267, Brisbane QLD 4001

Appendix 3. Protocol outlining the standard banana virus indexing assays.

Participant laboratory's details

Please complete the details below and return to Kathy Crew (kathy.crew@daf.qld.gov.au).

Laboratory details	
Organisation/Laboratory name	
Contact name	
Contact email	
Contact phone number	
Full laboratory address (for courier)	
What are the receiving country's import documentation/requirements?	
Indexing methods	
Standard protocols	(yes or no)
If yes, brand of PCR reagents*	
Are primers/antibodies required?	
If not using standard protocols, please summarise your methods	BSVs: BBTV: CMV: BBrMV: BanMMV:

* Please note that Invitrogen PCR reagents are not a requirement of the standard assay, however adjustment for $MgCl_2$ concentration and the amount of the DNA polymerase may be needed. Please check the details for your laboratory's specific system.

Please ensure that your laboratory handles these samples in accordance with local biosecurity/quarantine requirements.

Buffer details

Carbonate Coating Buffer, pH 9.6
For 1 litre of solution:

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g

Dissolve chemicals in about 900 mL of dH_2O.
Check pH is 9.6. If pH needs to be adjusted slightly, use HCl or NaOH.
Then make up to 1 Litre with dH_2O.
Store at 5°C.

5× PBS (Phosphate buffered saline)
For 1 litre of solution:

NaCl	40.0	g
KH_2PO_4	1.0	g
Na_2HPO_4	5.75	g
KCl	1.0	g

Dissolve chemicals in about 900 mL of dH_2O.
Adjust to pH 7.4 using 3 M NaOH.
Then make up to 1 Litre with dH_2O.
Store at 5°C.

1× PBS-Tween
Dilute 5× PBS stock solution to 1× PBS in dH_2O.
Add 0.05% (0.5 mL/Litre) of Tween20 and mix using a stirring bar.
Store 1× PBS-Tween at 5°C.

CMV ELISA Extraction Buffer
For 1 litre of solution:

0.05 M tri sodium citrate	14.7	g
0.5 mM EDTA	0.186	g
0.05% Tween20	0.5	mL
1% Skim milk powder*	10	g
0.5% monothiolglycerol	5	mL

Dissolve chemicals in about 900 mL of dH_2O.
Adjust to pH 8 using citric acid.
Then make up to 1 Litre with dH_2O.

* Options for preparing buffer depending on subsequent storage.
Note 1: Add skim milk AFTER AUTOCLAVING – otherwise it will burn.

Note 2: Can be prepared without autoclaving, in which case, freeze 10-50 mL aliquots and store at -20°C. Do not thaw more than once. Thawed buffer can be stored at 5°C.

Standard protocols for banana virus indexing laboratory tests

BSV IC-PCR with DNaseI treatment

Immunocapture

1. Coat tubes with 25 µL of a 1/200 dilution of Agdia SCBV antibodies and BSMYV IgG (1 mg/mL) at 5 ug/mL (1/200 dilution) in carbonate coating buffer for
   1 h at 37°C OR 3 h at room temperature OR overnight at 5°C.
2. Wash tubes for 3 x 3 minutes with PBS-T.
3. Add 25 µL of clarified leaf tissue ground 1/10 (w/v) in CMV extraction buffer to tubes and incubate for 2 h at 37°C OR 4 h at room temperature OR overnight at 5°C.
4. Wash tubes for 3 x 3 minutes with PBS-T and rinse with sterile distilled water.
   Ensure tubes are empty of liquid.

DNaseI treatment

5. Add 3 Units of DNaseI (Invitrogen stock is 300 U/µL) in 300 µL 1× buffer per well.
   1× buffer is made up fresh as below:

99.64	µL	1 M Tris pH 7.5
25.00	µL	1 M $MgCl_2$
5.00	µL	1 M $CaCl_2$
9,870.36	µL	water

6. Incubate at room temperature for 30 minutes.
7. Wash tubes for 3 x 3 minutes with PBS-T and rinse with sterile distilled water.

Ensure tubes are empty of liquid.

PCR

8. Add 25 µL PCR master mix to each well and run PCR program on thermocycler.

PCR Reaction Mix	**1×**	**×____reactions**
Sterile distilled H_2O	18.55 µL	µL
10 × PCR buffer (Invitrogen)	2.5 µL	µL
50 mM $MgCl_2$ (Invitrogen)	0.75 µL	µL
10 mM dNTP mix	0.5 µL	µL
12 Primer mix	2.5 µL	µL
Taq (5U/ul) (Invitrogen)	0.2 µL	µL
Total:	25.0 µL	µL

Primer mix:

Water	88	µL
100 µM Red Dacca F	1	µL
100 µM Red Dacca R	1	µL
100 µM Cavendish F	1	µL
100 µM Cavendish R	1	µL
100 µM IRFA F	1	µL
100 µM IRFA R	1	µL
100 µM Goldfinger F	1	µL
100 µM Goldfinger R	1	µL
100 µM Mysore F	2	µL
100 µM Mysore R	2	µL

PCR program:
94°C for 30 seconds
35 cycles of:
  94°C for 10 seconds
  60°C for 30 seconds
  72°C for 1 minute
72°C for 10 minutes

9. Electrophorese 8 µL of PCR product through 1.5% agarose-TBE.
10. Stain, visualise and photograph gel.

PCR product sizes

BSV species	Primer pair	Product size
BSCAV	Cavendish F / Cavendish R	782 bp
BSMYV	Mysore F / Mysore R	589 bp
BSOLV	Red Dacca F / Red Dacca R	522 bp
BSGFV	Goldfinger F / Goldfinger R	476 bp
BSIMV	IRFA F / IRFA R	400 bp

Multiplex IC-(RT)-PCR

Immunocapture

1. Coat tubes with 25 µL of a mixture of antibodies combined in carbonate coating buffer for 1 h at 37°C OR 3 h at room temperature OR overnight at 5°C.
   - BBTV PC Ig (1 mg/mL) at 1 µg/mL 1/1000 dilution
   - CMV (Sediag) 1/1000 dilution
   - BBrMV IgG (1 mg/mL) at 5 µg/mL 1/200 dilution
   - BanMMV (Ducasse) (1 mg/mL) at 5 µg/mL 1/200 dilution
2. Wash tubes for 3 x 3 minutes with PBS-T.
3. Add 25 µL of clarified leaf tissue ground 1/10 (w/v) in CMV extraction buffer to tubes and incubate for 2 h at 37°C OR 4 h at room temperature OR overnight at 5°C.
4. Wash tubes for 3 x 3 minutes with PBS-T and rinse with sterile distilled water. Ensure tubes are empty of liquid.

cDNA synthesis and PCR

5. Add cDNA mastermix subtotals to each tube and incubate following the protocol in the table below.
6. Prepare the PCR mastermix and aliquot into clean PCR tubes.
7. Add product of cDNA synthesis reaction (includes BBTV DNA) to PCR mastermix and run PCR program on thermocycler.

cDNA mastermixes for _____ reactions			**PCR Mastermix for ____ reactions**		
Reagent	**Calculation**	**Volume x __ reactions**	**Reagent**	**Calculation**	**Volume x__ reactions**
Sterile distilled H_2O	11.0 µl	µl	Sterile distilled H_2O	13.96 µl	µl
20 µM Poty1	0.75 µl	µl	10 × PCR buffer (Invitrogen)	2.0 µl	µl
20 µM CMV3'	0.75 µl	µl	50 mM $MgCl_2$ (Invitrogen)	0.7 µl	µl
SUBTOTAL	**12.5 µl**	**µl**	10 mM dNTP mix	0.4 µl	µl
→ 80°C for 10 min, ice			10 µM BBT1	0.4 µl	µl
			10 µM BBT2	0.4 µl	µl
			20 µM CMV3'	0.34 µl	µl
5 x first strand buffer	4.0 µl	µl	20 µM CMV5'	0.4 µl	µl
0.1 M DTT	2.0 µl	µl	20 µM Poty1	0.4 µl	µl
10 mM dNTP mix	1.0 µl	µl	20 µM Bract 1	0.4 µl	µl
RNaseOUT (40U/µl)	0.25 µl	µl	20 µM Bract 2	0.4 µl	µl
Superscript III (200U/µl)	0.25 µl	µl	*Taq* (Invitrogen)	0.2 µl	µl
SUBTOTAL	**7.5 µl**	**µl**	**SUBTOTAL**	**18.0 µl**	**µl**
→ 50°C for 45 min,			Template cDNA	2.0 µl	
→ 70°C for 15 min, ice			**TOTAL**	20.0 µl	

PCR product sizes:

BBT1 / BBT2:	349 bp
CMV3' / CMV5':	500 bp
Bract1 / Bract2:	604 bp
Poty1 / Bract1:	700 bp

PCR program:

94°C for 1 minute
35 cycles of:
94°C for 20 seconds
60°C for 20 seconds
72°C for 40 seconds
72°C for 3 minutes

9. Electrophorese 8 µL of PCR product through 1.5% agarose-TBE.
10. Stain, visualise and photograph gel.
11. Prepare and run the BanMMV PCR using the product of this cDNA reaction as template.

BanMMV PCR

PCR

PCR Mastermix for _____ reactions		
Reagent	**Calculation**	**Volume x__ reactions**
Sterile distilled H_2O	16.8 µl	µl
10× PCR buffer (Invitrogen)	2.5 µl	µl
50 mM $MgCl_2$ (Invitrogen)	1.0 µl	µl
10 mM dNTP mix	0.5 µl	µl
10 mM Poty 1	1.0 µl	µl
20 mM BanMMCP2	1.0 µl	µl
Taq (Invitrogen)	0.2 µl	µl
SUBTOTAL	23.0 µl	µl
Template cDNA	2.0 µl	
TOTAL	25.0 µl	

PCR program:

94°C for 1 minute

35 cycles of:

94°C for 20 seconds

60°C for 20 seconds

72°C for 20 seconds (OK to use 40 seconds if running alongside multiplex PCR)

72°C for 3 minutes

PCR product sizes:

BanMMCP2 / Poty1 280 bp

12. Electrophorese 8 µL of PCR product through 1.5% agarose-TBE.
13. Stain, visualise and photograph gel.

BANANA VIRUS MINIPREPS

(Based on the method of Ben Lockhart)

Materials

0.2 M potassium phosphate buffer pH 7.0
- Mix 0.2 M K_2HPO_4 and 0.2 M KH_2PO_4 while stirring and checking pH, until required pH is reached

Extraction buffer
- 0.2 M potassium phosphate buffer pH 7.0
- 15 mM EDTA
- 2% PVP
- 2% PEG (mw 6,000 to 8,000)
- 0.4% Na_2SO_3 (add on day of use)

33% Triton X-100

30% sucrose in 0.2 M potassium phosphate buffer pH 7.0

10 mM potassium phosphate buffer pH 7.0

Chloroform

Mortars and pestles

Acid-washed sand

Measuring cylinder (10 mL) or equivalent

Funnels

Cheesecloth / muslin

Ice in deep esky

Highspeed centrifuge tubes – capless polypropylene

Ultracentrifuge tubes - capless polycarbonate

Parafilm

Vortex

1mL and 100µL pipette and tips

Microfuge tubes (1.5 mL)

Microfuge

Method

Grind tissue and clarify extract:

1. Grind 2 g of leaf lamina and midrib tissue with a mortar and pestle and acid-washed sand in 6 mL extraction buffer.
2. Filter through cheesecloth or muslin.
3. Centrifuge at 12,000 **g** (10,000 rpm) for 15 min at 5ºC.
4. Decant supernatant into a 10 mL ultracentrifuge tube.

Lyse cell membranes:

5. Add 120 µl of 33% Triton X-100, cover tube with parafilm and vortex for 1 min to dissolve.

Sucrose cushion ultracentrifugation:

6. Underlay a 1 mL cushion of 30% sucrose in 0.2 M potassium phosphate buffer pH 7.0.
7. Centrifuge at 163,000 **g** (50,000 rpm) for 30 min at 5ºC.
8. Remove the supernatant and wipe inside of tube carefully.

Chloroform clarification

9. Resuspend the pellet in 100 µl of 10 mM potassium phosphate pH 7.0, transfer to a microcentrifuge tube.
10. Add 30 µl of chloroform, vortex for 30 sec.
11. Centrifuge for 5 min at ca. 10,000 – 15,000 rpm.
12. Collect the supernatant and freeze at –70ºC.
13. View under an electron microscope, using phosphotungstic acid or ammonium molybdate stain.

Can be scaled down or up, proportionally.

Banana virus immunosorbent electron microscopy (ISEM)

Materials

3 mm copper EM grids, coated with nicollodon and carbon-stabilised
1% ammonium molybdate (AM) pH 6.8 – 7.0
10 mM Na phosphate buffer, pH 7.0 (PB)
2% glutaraldehyde (25% stock stored at -20°C)
Jeweller's forceps
Humid chamber (eg large petri dish with parafilm and wet tissue)
ELISA plate or similar
Filter paper

Coating antibody mixture (in PB):

SCBV coating antibody (purified IgG), Agdia	1/200	
BSMYV Ig, J.Thomas' lab, 1 mg/mL stock	1/200	(5 µg/mL)
BBrMV Ig, J.Thomas' lab, 1 mg/mL stock	1/200	(5 µg/mL)
BanMMV Ig, J.Thomas' lab, 1 mg/mL stock	1/200	(5 µg/mL)
CMV coating antibody (purified IgG), Sediag	1/500	

Method

1. Place grid, film side down, on a 10 µL drop of antibody mixture and incubate in a humid chamber for 30-60 min at room temperature.
2. Wash grid with about 20 drops PB or on ELISA plate well full of PB for 5 min x 2, then touch dry with filter paper.
3. Place grid film side down on a 10 µL drop of viral miniprep, prepared previously.
4. Incubate at room temperature for 1-4 hr or overnight at 4°C.
5. Wash grid with about 20 drops PB or on ELISA plate well full of PB for 5 min x 2, then touch dry with filter paper.
6. Working in the fume cupboard, place grid film side down on a 10 µL drop of 2% glutaraldehyde.
7. Incubate at room temperature for 5-10 min.
8. Wash grid with about 20 drops PB or on ELISA plate well full of PB for 5 min x 2, then touch dry with filter paper.
9. Stain with ~5 drops of 1% ammonium molybdate.
10. Touch dry with filter paper, ensuring all free liquid is gone.
11. View in EM, photograph particles found.

Banana viruses

BSV	bacilliform	120-150 x 30 nm
BBrMV	flexuous rod	ca. 725 × 12 nm
BanMMV	flexuous rod	ca. 600 x 12-13 nm
CMV	isometric	28-30 nm
BBTV	isometric	18-20 nm(almost impossible to see in miniprep)
ABTV	isometric	18-20 nm(almost impossible to see in miniprep)
SCMV	flexuous rod	ca. 750 x 12 nm
BVX	flexuous rod	600-1000 x 10-15 nm
Unidentified isometric particles		28-30 nm, 35-40 nm
Unidentified closterovirus particles		650-2000 x 12nm

Appendix 5. Sample instructions, included with parcel of samples and reagents.

27 November 2017

Dear participating laboratory,

Please find enclosed the following reagents and samples for testing for banana viruses, Banana bunchy top virus (BBTV), Banana bract mosaic virus (BBrMV), Banana mild mosaic virus (BanMMV), Cucumber mosaic virus (CMV), Banana streak OL virus (BSOLV), Banana streak IM virus (BSIMV), Banana streak GF virus (BSGFV), Banana streak MY virus (BSMYV) and Banana streak OL virus (BSCAV).

It is recommended to include your laboratory's positive and negative controls with the tests, if available.

A results sheet with be sent out by email. I would appreciate the return of your PCR indexing results for these samples to kathy.crew@daf.qld.gov.au by **Friday, 22 December, 2017** so the report to Bioversity International can be written before the end of the year. ISEM results should be submitted by Friday, 19 January 2018.

Please also contact Nicolas Roux at Bioversity International (n.roux@cgiar.org) regarding payment for this indexing. Note that Nicolas must receive your organisation's invoice requesting payment by **Friday, 15 December 2017**.

Please briefly centrifuge the antibodies and primers, then check their volume ahead of use.
If necessary, add sterile distilled water to bring the volume up to that listed below.
Store antibodies at 4°C and primers at -20°C.

Purified antibodies from rabbits

15 µL SCBV	Agdia coating antibody to Sugarcane bacilliform virus, produced in USA
15 µL BSMYV	1 mg/mL, produced by A. Geering *et al.*, Brisbane, Australia
15 µL BanMMV	1 mg/mL, produced by J. Thomas *et al.*, Brisbane, Australia
15 µL BBrMV	1 mg/mL, produced by J. Thomas *et al.*, Brisbane, Australia
10 µL BBTV	1 mg/mL, produced by J. Thomas *et al.*, Brisbane, Australia
10 µL CMV	Sediag coating antibody, produced in France

Oligonucleotide primers for PCR assays

BBTV	10 µL 100 µM BBT1	CTCGTCATGTGCAAGGTTATGTCG	Thompson and Dietzgen, 1995
BBTV	10 µL 100 µM BBT2	GAAGTTCTCCAGCTATTCATCGCC	Thompson and Dietzgen, 1995
CMV	20 µL 100 µM CMV3'	TTTTAGCCGTAAGCTGGATGGACAACCC	Bariana *et al.*, 1994
CMV	20 µL 100 µM CMV5'	TATGATAAGAAGCTTGTTTCGCGCA	Bariana *et al.*, 1994
BBrMV	20 µL 100 µM Bract1	GACATCACCAAATTTGAATGGCACATGG	Rodoni *et al.*, 1997
BBrMV	20 µL 100 µM Bract2	CCATTATCACTCGATCAATACCTCACAG	Rodoni *et al.*, 1997
polyA tail	20 µL 100 µM Poty1	GGATCCCGGGTTTTTTTTTTTTTTTTV	Based on Gibbs and Mackenzie, 1997
BanMMV	20 µL 100 µM BanMMCP2	TGCCAACTGAYGARGAGCTRAATGC	Thomas, 2015
BSOLV	10 µL 100 µM Red Dacca F	ATCTGAAGGTGTGTTGATCAATGC	Geering *et al.*, 2000
BSOLV	10 µL 100 µM Red Dacca R	GCTCACTCCGCATCTTATCAGTC	Geering *et al.*, 2000
BSIMV	10 µL 100 µM IRFA F	TGCCAACGAATACTACATCAAC	Thomas, 2015
BSIMV	10 µL 100 µM IRFA R	CACCCAGACTTTTCTTTCTAGC	Thomas, 2015
BSGFV	10 µL 100 µM Goldfinger F	ACGAACTATCACGACTTGTTCAAGC	Geering *et al.*, 2000
BSGFV	10 µL 100 µM Goldfinger R	TCGGTGGAATAGTCCTGAGTCTTC	Geering *et al.*, 2000
BSMYV	20 µL 100 µM Mysore F	TAAAAGCACAGCTCAGAACAAACC	Geering *et al.*, 2000
BSMYV	20 µL 100 µM Mysore R	CTCCGTGATTCTTCGTGGTC	Geering *et al.*, 2000
BSCAV	10 µL 100 µM Cavendish F	AGGATTGGATGTGAAGTTTGAGC	Thomas, 2015
BSCAV	10 µL 100 µM Cavendish R	ACCAATAATGCAAGGGACGC	Thomas, 2015

Store lyophilized leaf tissues samples at -20°C, in accordance with your local quarantine requirements.

Six samples as positive and negative controls for PCR assays
Labelled BBTV, CMV, BBrMV+BanMMV, IM+GF (BSIMV+BSGFV), OL+MY+CA (BSOLV+BSMYV+BSCAV) and Healthy (AAA).
Each sample consists of 0.05 g lyophilized leaf tissue.

Six samples for BSV indexing
Labelled 1 to 6.
Each sample consists of 0.05 g lyophilized leaf tissue.
Please return your results for these samples to kathy.crew@daf.qld.gov.au by Friday 22/12/2017

Eight samples for indexing for other banana viruses (BBTV, CMV, BanMMV, BBrMV)
Labelled A to G, and J.
Each sample consists of 0.05 g lyophilized leaf tissue.
Please return your results for these samples to kathy.crew@daf.qld.gov.au by Friday 22/12/2017

Four samples for VMP+ISEM indexing
Labelled EM1 to EM4.
Each sample consists of 0.3 g lyophilized leaf tissue.
Please return your results for these samples to kathy.crew@daf.qld.gov.au by Friday 19/01/2018

Sincerely,
Dr Kathy Crew
Plant Pathologist (Virology)
Queensland Department of Agriculture and Fisheries
Ecosciences Precinct
Courier: Joe Baker St B3 loading dock, Dutton Park QLD 4102 Australia
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