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## **Proposed testing requirements for the verification of ToBRFV and ToMMV status in tomato and capsicum seed for export to Australia**

### **Proposed updates to approved protocols**

- Laboratories are required to use two reverse-transcription qPCR protocols to verify the status of ToBRFV and ToMMV in each seed lot.
- For ToBRFV, the CaTa28 protocol must be used as one of the two qPCR protocols, and the CSP1325 protocol or the qs1/p1/qas2 protocol must be used as the second protocol (Table 1).
- For ToMMV, the CaTa9 must be used as one of the two qPCR protocols, and the CSP1572 protocol or the ToMMV2 protocol must be used as the second protocol (Table 1). If any approved qPCR yields a Ct score equal to or below the Ct cut-off for that qPCR, as indicated in Table 1, ToBRFV or ToMMV is considered to have been detected in the seed lot (a positive detection).
- References to these approved protocols are listed in Table 2. Please note that the department's requirements for these protocols and for reporting results vary from some of the parameters indicated in the references.
- No other tests are approved for verifying the status of a seed lot regarding these two viruses.
- Protocols that are no longer approved for testing for the two viruses are listed in Table 3.
- Laboratories may re-test RNA extracts from subsamples to resolve anomalous qPCR results but may only do so following the rules stipulated in the 'Re-testing and replicates' section provided (below).
- Laboratories shall not discount a positive result obtained from an approved qPCR test on the grounds of any other testing or any other consideration, except re-testing following the stipulated rules.
- Extraction and amplification controls must be run for every sample and should include an RNA target. In order to provide assurance of appropriate sensitivity, at least one weak positive control should be used that produces a Ct score of 30 or higher.

### Re-testing guideline

- A laboratory may consider an amplification result to be potentially anomalous, for the purpose of re-testing, if:
  - the amplification curve deviates substantially from the expected sigmoidal shape, or
  - the amplification produces a Ct score greater than 32 and the laboratory considers this result may be a false positive.
- If an amplification result is considered potentially anomalous (as defined above) the RNA extract from the subsample may be re-tested.
- Re-testing and investigation of a potentially anomalous amplification must be conducted on the same RNA extract.
- If an RNA extract is re-tested, at least two more replicate qPCRs must be done using the same qPCR protocol.
- Upon re-testing, if a replicate produces a Ct score below the cut-off, then the subsample will be considered to be positive.
- An anomalous/ambiguous result from a qPCR cannot be superseded by testing another seed sample or subsample.
- Any anomalous finding that cannot be satisfactorily resolved should be considered to be a positive.

### Record keeping

- The department may conduct re-testing of seed lots or trace back investigations of seed lots, and will expect that when requested, laboratories will be able provide records of previous testing, or other information to assist with these investigations.
- Laboratories must keep copies of the protocols they use and any validation and verification reports relevant to the tests, and quality control records, and be prepared to provide these documents to the department for review if asked.
- Laboratories should keep records of all testing done to meet the Australian requirements for at least 3 years, including Ct scores, records of decisions, and results from controls, and provide these records and all test results for the seed lots to the department if asked. If possible, images of amplification curves should also be kept.
- If a laboratory considers an anomalous result to be resolved, and subsequently that a seed lot is negative for the virus, the laboratory must record the anomalous result(s) and record the specific reason(s) why the anomalous result was considered not to be a detection of the virus. Laboratories should be prepared to provide the records of decisions, including decisions to re-test, the reasoning, and all test results for the seed lot in question to the department if asked.

**Table 1. Approved Protocols and result interpretation for ToBRFV and ToMMV**

Virus	Protocol	Primers and probe	Ct (Cq) cut-off
ToBRFV	CaTa28 reverse-transcription qPCR  cycles <ul style="list-style-type: none"> <li>• 50 °C for 10 min</li> <li>• 95 °C for 3 min</li> <li>• 40 cycles of 95 °C for 10 sec and 60 °C for 60 sec.</li> </ul>	CaTa28 forward GGTGGTGTCAGTGTCTGTTT CaTa28 reverse GCGTCCTTGGTAGTGATGTT CaTa28 6FAM-AGAGAATGGAGAGAGCGGACGAGG- BHQ1	≤38
	CSP1325 reverse-transcription qPCR  cycles: <ul style="list-style-type: none"> <li>• 48 °C for 30 min</li> <li>• 94 °C for 5 min</li> <li>• 40 cycles of 94 °C for 10 sec and 60 °C for 30 sec.</li> </ul>	CSP1325 forward CATTGAAAGTGCATCCGGTTT CSP1325 reverse GTACCACGTGTGTTTGCAGACA CSP1325 VIC*-ATGGTCCTCTGCACCTGCATCTTGAGA – BHQ1 *can be FAM	≤36
	qs1/p1/qas2 Menzel and Winter reverse-transcription qPCR  cycles <ul style="list-style-type: none"> <li>• 48°C for 30 min</li> <li>• 94°C for 5 min</li> <li>• 40 cycles of 94°C for 10 sec and 60°C for 30 sec.</li> </ul>	ToBRFV qs1 forward CAATCAGAGCACATTTGAAAGTGCA ToBRFV qas2 reverse CAGACACAATCTGTTATTTAAGCATC ToBRFV p16 6-FAM-ACAATGGTCCTCTGCACCTG- BHQ1	≤36

Virus	Protocol	Primers and probe	Ct (Cq) cut-off
ToMMV	CaTa9 reverse-transcription qPCR  cycles <ul style="list-style-type: none"> <li>• 48 °C for 30 min</li> <li>• 94 °C for 5 min</li> <li>• 40 cycles of 94 °C for 10 sec and 60 °C for 30 sec.</li> </ul>	CaTa9 forward ATGTGGAGGAACCCTCTATGA CaTa9 reverse AATCTCCTCGCTCCTTGTAAC CaTa9P ROX-TCAATGGCCCGTGGTGAGTTACAA-BHQ1	≤37
	CSP1572 reverse-transcription qPCR  cycles: <ul style="list-style-type: none"> <li>• 48 °C for 30 min</li> <li>• 94 °C for 5 min</li> <li>• 40 cycles of 94 °C for 10 sec and 60 °C for 30 sec.</li> </ul>	CSP1572 forward CATTTGAAAGTGCATCCGGTTT CSP1572 reverse GTACCACGTGTGTTTGACAGACA CSP1572P 6FAM-TGCCACTCGCAGAGTGGACGATGCTACG-BHQ1	≤37
	ToMMV2 reverse-transcription qPCR  cycles <ul style="list-style-type: none"> <li>• 50°C for 10 min</li> <li>• 95°C for 3 min</li> <li>• 40 cycles of 95°C for 10 sec and 60°C for 1 min.</li> </ul>	ToMMV2 forward GAAACATTGGATGCCACTCG ToMMV 2 reverse CTCTGGTTGTAGAAACCTGTTCC ToMMV2p FAM-CGATGCTACGGTTGCGATCAGGTC BHQ1	≤37

Table 2. References to protocols

Protocol	Reference
CaTa28	<p>Berendsen SMH, Tavares C, Hiddink G, and Woudenberg JHC (2020). Detection of Tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed by SE-PCR. ISHI-Veg, Validation Report. <a href="https://worldseed.org/wp-content/uploads/2020/09/2020_Validation-Report-_ToBRFV_Tomato-Pepper_W.pdf">https://worldseed.org/wp-content/uploads/2020/09/2020_Validation-Report-_ToBRFV_Tomato-Pepper_W.pdf</a></p> <p>ISHI-Veg 2019. Detection of Infectious <i>Tomato brown rugose fruit virus</i> (ToBRFV) in Tomato and Pepper Seed. <a href="https://www.worldseed.org/wp-content/uploads/2019/09/Tomato-ToBRFV_2019.09.pdf">https://www.worldseed.org/wp-content/uploads/2019/09/Tomato-ToBRFV_2019.09.pdf</a></p>
CSP1325	<p>Berendsen SMH, Tavares C, Hiddink G, and Woudenberg JHC (2020). Detection of Tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed by SE-PCR. ISHI-Veg, Validation Report. <a href="https://worldseed.org/wp-content/uploads/2020/09/2020_Validation-Report-_ToBRFV_Tomato-Pepper_W.pdf">https://worldseed.org/wp-content/uploads/2020/09/2020_Validation-Report-_ToBRFV_Tomato-Pepper_W.pdf</a></p>
Menzel and Winter	<p>Menzel W, and Winter S. (2021). Identification of novel and known tobamoviruses in tomato and other solanaceous crops using a new pair of generic primers and development of a specific RT-qPCR for ToBRFV. <i>Acta Horticulturae</i>. 1316, 143-148 DOI: 10.17660/ActaHortic.2021.1316.20</p> <p>EPPO (2021), PM 7/146 (1) Tomato brown rugose fruit virus. EPPO Bulletin 51: 178-197. <a href="https://doi.org/10.1111/epp.12723">https://doi.org/10.1111/epp.12723</a></p>
CaTa9	<p>Mehle, N et al. (2024). ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study. <a href="#">Euphresco DROP - ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study</a></p>
CSP1572	<p>Mehle, N et al. (2024). ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study. <a href="#">Euphresco DROP - ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study</a></p>
ToMMV2	<p>Mehle, N et al. (2024). ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study. <a href="#">Euphresco DROP - ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study</a></p>

**Table 3. PCR protocols removed from the approved list**

Protocol and Primers	Reason
<p>Levitzky et al. 2019</p> <p>F GAAGAAGTTGTTGATGAGTTCAT R GATTTAAGTGGAGGGAAAAACAC</p> <p>Reference Levitzky, N, Smith, E, Lachman, O, Luria, N, Mizrahi, Y, Bakelman, H, Sela, N, Laskar, O, Milrot, E &amp; Dombrovsky, A, 2019, 'The bumblebee <i>Bombus terrestris</i> carries a primary inoculum of <i>Tomato brown rugose fruit virus</i> contributing to disease spread in tomatoes' <i>PloS one</i>, volume14, issue 1, p.e0210871.</p>	<p>The protocol of Levitzky et al 2019 is not suitable for the detection of ToBRFV and ToMMV as it is not sufficiently sensitive and uses a group specific primer set.</p>
<p>Alkowni et al. 2019</p> <p>F AATGTCCATGTTTGTACGCC R CGAATGTGATTTAAACTGTGAAT</p> <p>Reference Alkowni, A, Alabdallah, O &amp; Fadda, Z 2019, 'Molecular identification of <i>tomato brown rugose fruit virus</i> in tomato in Palestine' <i>Journal of Plant Pathology</i>, available at DOI 10.1007/s42161-019-00240-7.</p>	<p>The protocol of Alkowni et al 2019 is not suitable for the detection of ToBRFV as it is not sufficiently sensitive.</p>