Tobamovirus Group PCR Primer Mix

Catalog number: PCR 95100/0025

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Lot	Item	Quantity / Volume
_____________	Lyophilized forward and reverse primers	1 tube / enough for 25 reactions
_____________	Sterile PCR quality water	1 tube / 0.8 ml
	Instructions	1

This product is for research purposes only.

STORAGE

Store lyophilized primer mix at -20°C, stable for 1 year. When the primer mix is reconstituted it is recommended to use in one experiment. The effect of freezing and thawing on the reconstituted primers is not documented.

YOU WILL NEED

The RT-PCR process using virus group primers was optimized with the following equipment and reagents. It is recommended to use the same reagents to obtain successful results.

EQUIPMENT AND CONSUMABLES

- Pipettes, aerosol barrier/filter pipette tips, gloves, tube openers, sterile microcentrifuge tubes, sterile PCR tubes, PCR tube rack, microcentrifuge tube rack, marking pen, thermal cycler, and ice block

RNA EXTRACTION

- Qiagen RNeasy® Plant Mini Kit……………………………………….Qiagen®, catalog number 74904
- Qiagen RNase-Free DNase Set…………………………………….Qiagen®, catalog number 79254

RT & PCR REAGENTS

- RNase inhibitor 40 U/µl …………………………………………….. Fisher Scientific®, catalog number BP32225
- M-MLV Reverse Transcriptase 200 U/µl ……………………..….. Fisher Scientific®, catalog number PR M1705
  Promega®, catalog number M1705
- Random hexamers [pd(N)$_6$]……………………………………... Applied Biosystems®, catalog number N808-0127
- Hot start DNA Polymerase
  - AmpliTaq Gold® ……………………………………………… Applied Biosystems®, catalog number N808-0241
  - Thermo-Start ®………………………………………………... ABgene®, catalog number AB-0908
- PCR Buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl) 10X ………. Applied Biosystems®, catalog number N808-0010
  ABgene®, catalog number AB-0194
- $MgCl_2$ 25 mM ……………………………………………………... Applied Biosystems® catalog number N808-0010
  ABgene®, catalog number AB-0194
- dNTPs 100 mM ……………………………………………………..…. Promega®, catalog number U1330 - dilute before using
  To make 10 mM dNTP mixture: 200 µl of 100 mM dGTP, 200 µl of 100 mM dCTP, 200 µl of 100 mM dATP, 200 µl of 100 mM dTTP, 1.2 ml HPLC grade H_2O FINAL VOLUME = 2 ml
  To make 2.5 mM dNTP mixture: Dilute the 10 mM dNTP.

ELECTROPHORESIS

- Power supply, electrophoresis unit, agarose, TAE or TBE buffer, molecular weight marker, loading dye, and ethidium bromide for staining the gel

INTENDED USE

The Tobamovirus group test PCR* primers offer a sensitive diagnostic method to detect members of the *Tobamovirus* genus. The PCR primers are based on conserved genome regions and can detect unidentified viruses and isolate members of the *Tobamovirus* genus. This test can be used as an aid for identification of viral etiology of unknown plant diseases, test for known viruses when other tests are not available and can be used to confirm results from other test methods.

Disclaimer of License:
* This product is optimized for use in the Polymerase Chain Reaction (PCR) process covered by patents owned by Hoffmann-La Roche, Inc. and F. Hoffmann-La Roche Ltd. Purchase of this product does not include the license under these patents to use the PCR process. This product is recommended for use in conjunction with an authorized thermal cycler.

REFERENCES

Maroon, C.J.M. and Zavriev, S. 2002. PCR-based tests for the detection of tobamoviruses and carlaviruses. Acta Hort. (ISHS) 568:117-122. http://www.actahort.org/books/568/568_16.htm

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

TEST PROCEDURE

PRECAUTIONS

- Wear gloves at all times
- It is very important to use RNase-free materials and solutions throughout the procedure.
- Work in a dust-free area designated for PCR use only.
- Wipe down lab bench with 70% ethanol or 10% bleach before use.
- Use aerosol barrier/filter pipette tips.
- For preparation of RT and PCR master mixes, use PCR designated pipettes that have not been exposed to positive samples or other nucleic acids.
- Perform DNase treatment during RNA extraction to remove DNA.
- Prepare master mixes in a separate area from sample preparation and PCR product analysis.
- Use of a hot start DNA polymerase is strongly recommended.
- If using a thermal cycler without a heated lid, use a mineral oil overlay to prevent evaporation.

SAMPLE EXTRACTION

Extract total RNA from your samples. Include positive and negative controls. RNA used for RT-PCR must be of high quality and free of genomic DNA. The use of Qiagen RNeasy® Plant Mini Kit and Qiagen® RNase-Free DNase Set is strongly recommended.

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REVERSE TRANSCRIPTION (RT)

1. Label PCR tubes for each sample, positive control, negative control, and water control.
2. Do calculations for RT master mix. Prepare enough master mix for each sample, controls, and 1 extra reaction.
3. Thaw PCR Buffer, $MgCl_2$, dNTP mix, and random hexamers in PCR prep area. Mix and briefly centrifuge each reagent before using.
4. Remove RNase inhibitor and M-MLV Reverse Transcriptase from freezer just before needed. Keep on ice. Mix and briefly centrifuge each reagent before using.
5. Prepare master mix of reagents (all except RNA) and aliquot 15 µl into labeled PCR tubes while in the PCR prep area.
6. In an area away from the PCR prep area, add 5 µl of RNA (0.5 to 1µg) to aliquotted RT master mix. Mix contents, spin briefly, and let tubes stand at room temperature for 10 minutes.
7. Place tubes in a PCR machine and run the following:

   42°C, 30 minutes
   99°C, 5 minutes
   5°C, 5 minutes
   5°C, hold until next step or store at -20°C

RT MASTER MIX

RT MIX Reagents (stock concentrations)	Volume per reaction	Final concentration
PCR quality water	5.25 µl	
10X PCR Buffer II	2.00 µl	1X
25 mM $MgCl_2$	4.00 µl	5 mM $MgCl_2$
10 mM dNTP mix	2.00 µl	1 mM each dNTP
50 µM random hexamers [pd(N)$_6$]	1.00 µl	2.5 µM random hexamers [pd(N)$_6$]
40 U/µl RNase inhibitor**	0.50 µl	20 U RNase inhibitor/ reaction
200 U/µl M-MLV Reverse Transcriptase**	0.25 µl	50 U M-MLV Reverse Transcriptase/ reaction
Amount of master mix to use per tube	**15.00 µl**	
Total RNA	**5.00 µl**	~0.5 to 1 µg/ reaction
Final volume	**20.00 µl**	

** Concentration may vary depending on manufacturer. Adjust amounts of RNase inhibitor, M-MLV Reverse Transcriptase and water accordingly.

POLYMERASE CHAIN REACTION (PCR)

1. Label PCR tubes for each sample, positive control, negative control, RT water control, and PCR water control.
2. Do calculations for PCR master mix. Prepare enough master mix for each sample, controls, and 1 extra reaction.
3. Thaw PCR Buffer, $MgCl_2$, and dNTP mix in PCR prep area. Mix and briefly centrifuge each reagent before using.
4. Briefly centrifuge tube of lyophilized primer mix to remove any primer from the lid before opening. Reconstitute lyophilized primer mix with 135 µl of PCR quality water (included).
5. Remove hot start DNA polymerase from freezer just before needed. Keep on ice. Mix and briefly centrifuge Taq polymerase before using.
6. Prepare master mix of reagents (all except cDNA) and aliquot 22.5 µl into labeled PCR tubes while in the PCR prep area.

8. In an area away from the PCR prep area, add 2.5 µl of cDNA to each tube of PCR master mix. Mix contents and spin briefly.
9. Place tubes in a PCR machine. Run the following program:

   94°C, 10 minutes
   94°C, 1 minute }
   50°C, 3 minutes } 35 cycles
   72°C, 3 minutes }
   72°C, 10 minutes
   4°C, hold

PCR MASTER MIX

PCR MIX Reagents (stock concentrations)	Volume per reaction	Final concentration
PCR quality water	11.625 µl	
10X PCR Buffer II	2.50 µl	1X
25 mM $MgCl_2$	1.25 µl	1.25 mM $MgCl_2$
2.5 mM dNTP mix	2.00 µl	0.2 mM each dNTP
Primer mix	5.00 µl	0.4 µM primers
5 U/µl hot start DNA Polymerase**	0.125 µl	0.625 U Taq/ reaction
Amount of master mix to use per tube	**22.50 µl**	
cDNA (from RT reaction)	**2.50 µl**	
Final volume	**25.00 µl**	

** Concentration may vary depending on manufacturer. Adjust amounts of Taq and water accordingly.

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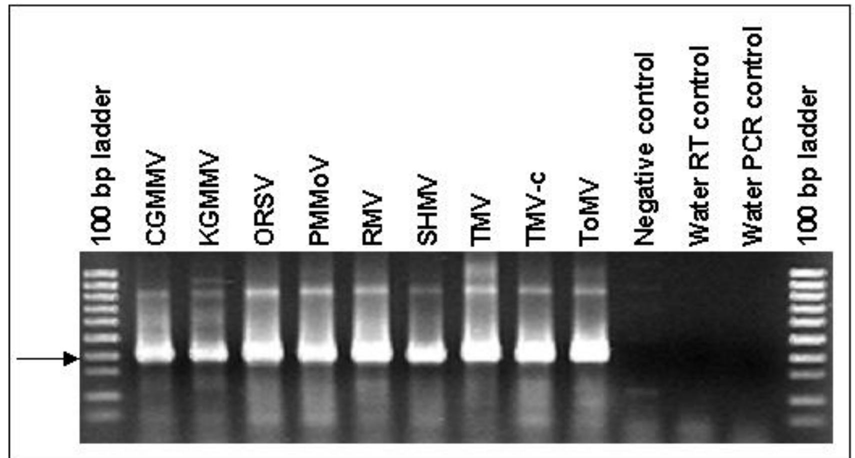
ELECTROPHORESIS

1. Mix PCR product with gel loading buffer.
2. Run 10 µl of PCR product on a 1.5% agarose gel made up in TAE or TBE buffer. Load appropriate molecular weight marker.
3. Run gel at 85 volts for 1½ hours.
4. Wearing gloves, stain gel with ethidium bromide at a concentration of 0.5 µg/ml in water or electrophoresis buffer (Sambrook et al., 1989). (Caution: ethidium bromide is a strong mutagen.)
5. View on UV transilluminator.



Lane 1:	100 bp ladder	
Lane 2:	CGMMV -	Cucumber green mottle mosaic virus
Lane 3:	KGMMV -	Kyuri green mild mottle virus
Lane 4:	ORSV -	Odontoglossum ringspot virus
Lane 5:	PMMoV -	Pepper mild mottle virus
Lane 6:	RMV -	Ribgrass mosaic virus
Lane 7:	SHMV -	Sunn-hemp mosaic virus
Lane 8:	TMV -	Tobacco mosaic virus
Lane 9:	TMV-c -	Tobacco mosaic virus – common strain
Lane 10:	ToMV -	Tomato mosaic virus
Lane 11:	Negative control	
Lane 12:	Water RT control	
Lane 13:	Water PCR control	
Lane 14:	100 bp ladder	

This list includes known tobamoviruses that were available for testing. The list does not represent all members of the *Tobamovirus* group. As other virus members are acquired and tested they will be added to the list.

RESULTS

- The expected PCR product size is about **370 to 400bp.**
- The test can detect members of the group that have not been identified or have not yet been sequenced and may produce a slightly different PCR product size in these cases.
- Positive controls should be the correct size and easily detectable.
- Negative control should not be detectable.
- Water controls should not be detectable.

LIMITATIONS

The following is a description of factors that could limit test performance or interfere with proper test results.

- Lyophilized primers are stable for 1 year from date received when stored at -20°C.
- It is recommended to use the reconstituted primer mix in one experiment. If subsequent experiments will be performed, the primer mix should be aliquotted and stored at -20°C. The effect of freezing and thawing on the reconstituted primers is not documented.
- Success of RT-PCR is dependent upon the quality of the RNA.
- DNA contamination of RNA extracts may cause false positives or multiple-banded reactions.
- The test can detect members of the group, but it cannot specifically identify individual pathogens.