

Carmovirus-Pelarspovirus Group PCR Primer Mix

Catalog number: PCR 94000/0025

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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
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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

- RNasin® Plus Ribonuclease Inhibitor 40 U/μL Promega® catalog number N261A
- M-MLV Reverse Transcriptase 200 U/μL Promega® catalog number M1705
- Random hexamers [d(N)₆] 50 μM..... Invitrogen™ catalog number N808-0127
- AccuPrime™ Taq DNA Polymerase..... Invitrogen™ catalog number 12339
- GeneAmp® PCR Buffer II 10X..... Applied Biosystems® catalog number N8080010
- GeneAmp® MgCl₂ 25 mM..... Applied Biosystems® catalog number N8080010
- 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 H₂O 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, ethidium bromide for staining the gel, and a UV transilluminator.

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INTENDED USE

The Carmovirus-Pelarspovirus Group PCR primers offer a sensitive diagnostic method to detect members of the *Alphacarmovirus*, *Betacarmovirus*, *Gammacarmovirus*, and the *Pelarspovirus* genera of the *Procedovirinae* subfamily. The primer sequences are based on conserved genome regions and can detect characterized and unassigned members of the *Alphacarmovirus*, *Betacarmovirus*, *Gammacarmovirus*, and the *Pelarspovirus* genera.

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.

This test also detects Maize chlorotic mottle virus (a member of the *Machlomovirus* genus) and Tobacco necrosis virus A (a member of the *Alphanecrovirus* genus). For a full list of confirmed and predicted reactions, see the product webpage.

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 cyclers.

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.
- 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.

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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, MgCl₂, dNTP mixture, and random hexamers [d(N)₆] in PCR prep area. Mix and briefly centrifuge each reagent before using.
4. Remove Ribonuclease inhibitor and M-MLV Reverse Transcriptase from freezer just before needed. Keep on ice. Gently mix (do not vortex) 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 1 µg) to aliquotted RT master mix. Gently mix contents (do not vortex), 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

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, MgCl₂, and dNTP mixture 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 AccuPrime™ Taq DNA polymerase from freezer just before needed. Keep on ice. Gently mix (do not vortex) and briefly centrifuge Taq DNA 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.
7. In an area away from the PCR prep area, add 2.5 µL of cDNA to each tube of PCR master mix. Gently mix contents (do not vortex) and spin briefly.
8. Place tubes in a PCR machine. Run the following program:
 - 94 °C, 10 minutes
 - 94 °C, 1 minute
 - 56 °C, 1 minute
 - 72 °C, 3 minutes
 - 72 °C, 10 minutes
 - 4 °C, hold

} 35 cycles

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 mixture	2.00 µL	1 mM each dNTP
50 µM random hexamers [d(N) ₆]	1.00 µL	2.5 µM random hexamers [pd(N) ₆]
40 U/µL Ribonuclease inhibitor	0.50 µL	20 U Ribonuclease 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	

PCR MASTER MIX

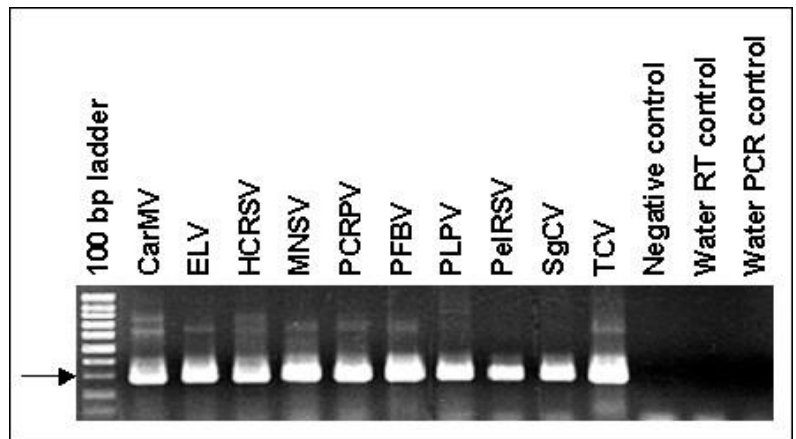
PCR MIX Reagents (stock concentrations)	Volume per reaction	Final concentration
PCR quality water	11.437 µL	
10X PCR Buffer II	2.50 µL	1X
25 mM MgCl ₂	1.25 µL	1.25 mM MgCl ₂
2.5 mM dNTP mixture	2.00 µL	0.2 mM each dNTP
Primer mix	5.00 µL	0.4 µM primers
5 U/µL Taq DNA Polymerase	0.313 µL	1.565 U Taq/ reaction
Amount of master mix to use per tube	22.50 µL	
cDNA (from RT reaction)	2.50 µL	
Final volume	25.00 µL	

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ELECTROPHORESIS

1. Mix PCR product with gel loading dye.
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 1.0 $\mu\text{g}/\text{mL}$ 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: CarMV - Carnation mottle virus
Lane 3: ELV - Elderberry latent virus
Lane 4: HCRSV - Hibiscus chlorotic ringspot virus
Lane 5: MNSV - Melon necrotic spot virus
Lane 6: PCRPV - Pelargonium chlorotic ring pattern virus
Lane 7: PFBV - Pelargonium flower break virus
Lane 8: PLPV - Pelargonium line pattern virus
Lane 9: PeIRSV - Pelargonium ringspot virus
Lane 10: SgCV - Saguaro cactus virus
Lane 11: TCV - Turnip crinkle virus
Lane 12: Negative control
Lane 13: Water RT control
Lane 14: Water PCR control

RESULTS

- The expected PCR product size is about **300 bp**.
- 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.

The list above includes several virus species that have been experimentally detected with our Carmovirus-Pelarspovirus PCR and confirmed by sequencing. For a full list of confirmed and predicted virus detections, see the product webpage.

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 $-20\text{ }^{\circ}\text{C}$.
- 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 $-20\text{ }^{\circ}\text{C}$. 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.
- Using reagents other than those described in this user guide can significantly affect sensitivity and specificity of the product.
- The test can detect members of the group, but it cannot specifically identify individual pathogens.