List of contents

Lot number	Item	Quantity
_____	Nylon membrane	1
_____	CChMVd Extraction Buffer	140 mL
_____	RNAse-free Water	6 mL
_____	Sodium Acetate (NaAc)	7 mL
_____	Binding Buffer	2 X 97.5 mL
_____	Nonionic Cellulose	7 g
_____	Elution Buffer	25 mL
_____	Instructions	1
_____	Membrane Submission Form	1
	The above items should be stored at room temperature (18 - 30 °C).	

Introduction

This kit includes materials and protocols necessary to extract plant samples and to spot a membrane for *Chrysanthemum chlorotic mottle viroid* (CChMVd) nucleic acid hybridization.

Not included but required

- NAK Permit Packet (catalog NAK 07500) for Foreign Shipments only
- microcentrifuge
- pipettor
- vortex
- microcentrifuge tubes (1.5 mL)
- water-saturated phenol (pH 4.2 to 4.5)
- 2-mercaptoethanol
- 95 - 100 % ethanol
- sterile distilled water
- gloves

Preparing for the test

Familiarize yourself with the kit components. Check that all components are present in the kit.

Store membrane in the protective folder in a cool, dry place until ready to use.

A line divides the reserved column for internal use from the other columns. Do not spot anything on the reserved column.

When the kit is first used it is necessary to add 52.5 mL of 95 - 100 % ethanol to each bottle of Binding Buffer. Mark the label on the Binding Buffer bottles once the ethanol has been added.

2-mercaptoethanol must be added to the CChMVd Extraction Buffer before each use. You will need 1.25 mL of CChMVd Extraction Buffer for each sample. To prepare, measure out only the amount of Extraction Buffer needed for the day and add 2-mercaptoethanol to a final concentration of 0.2 % (v/v).

A 70 % ethanol solution will also be necessary in step 22. You will need 1.0 mL of 70 % ethanol for each sample. To prepare, dilute 95 - 100 % ethanol with the appropriate volume of sterile distilled water.

Precautions

Gloves should be worn at all times to protect against RNAse contamination and for safety.

Handle the membrane only with clean forceps and rubber or plastic gloves that have been thoroughly cleaned and dried. Touching the membrane can cause non-specific blotches or spots on the final film.

It is crucial that the extraction protocol be followed exactly. Altering or straying from any of the steps will jeopardize the success of the test. If you have any questions about using this kit, please contact Agdia, Inc. by phone (574-264-2014 or 800-622-4342) or by email (info@agdia.com).

Nucleic Acid Extraction

Step 1. Weigh out approximately 0.25 g of leaf tissue.

Step 2. Process samples by expressing sap using a juice press or by grinding tissue with a mortar and pestle. To the expressed sap or ground tissue, add 1.25 mL Extraction Buffer (note: be sure 2-mercaptoethanol was added to the Extraction Buffer. See preparing for the test on page 1). Further grind tissue samples until completely macerated.

Step 3. Transfer 1 mL of the homogenate to labeled microcentrifuge tubes.

Step 4. Centrifuge tubes for 20 minutes at 8,000 rpm.

Step 5. Transfer 500 µL of supernatant to new labeled tubes.

Step 6. Add 1 volume (500 µL) of water-saturated phenol to each tube. Vortex or mix vigorously.

Step 7. Centrifuge tubes for 5 minutes at 12,000 rpm. Transfer aqueous phase (should be about 500 µL) to new labeled microcentrifuge tubes.

Nucleic Acid Precipitation

Step 8. Add 1/10 volume sodium acetate (NaAc) plus 2 volumes 95-100% ethanol to each tube. For example, 500 µL of aqueous sample would require 50 µL of NaAc and 1100 µL of 95-100% ethanol. Mix by inverting the capped tubes a few times.

Step 9. Store overnight at -10 to -30 °C or at least 30 minutes at -70 to -90 °C.

Step 10. Centrifuge tubes for 15 - 20 minutes at 12,000 rpm. Remove and discard supernatant. (To save time, you can prepare tubes for the next section while centrifuging. Follow step 12 to prepare the next set of tubes.)

Step 11. Resuspend each remaining pellet in 50 µL of RNAse-free Water. Mix by vortexing or pipetting up and down.

Cleaning up Nucleic Acids

Step 12. Label new microcentrifuge tubes. Add 50 mg Nonionic Cellulose and 700 µL of Binding Buffer (be sure that ethanol has been added to the Binding Buffer) to the new tubes. Vortex 1 - 2 minutes. Let tubes stand for about 5 minutes.

Step 13. Centrifuge tubes for 5 minutes at 12,000 rpm. Remove and discard supernatant.

Step 14. Add 50 µL of resuspended pellet from Step 11 and 700 µL of Binding Buffer to tube from step 13. Vortex 1 - 2 minutes. Let stand for about 5 minutes.

Step 15. Centrifuge tubes for 5 minutes at 12,000 rpm. Remove and discard supernatant.

Step 16. Add 700 µL of Binding Buffer to each tube. Vortex 1 - 2 minutes. Let stand for 5 minutes.

Step 17. Centrifuge tubes for 5 minutes at 12,000 rpm. Remove and discard supernatant.

Step 18. Add 200 µL of Elution Buffer to each tube. Vortex 1 - 2 minutes. Let stand for 5 minutes.

Step 19. Centrifuge tubes for 5 minutes at 12,000 rpm. Transfer 100 µL of supernatant to a new labeled tube. Be sure not to pipette any of the cellulose with the supernatant. Cellulose can interfere with the test and cause poor results.

Concentrating Nucleic Acids

Step 20. Add 1/10 volume NaAc and 2 volumes 95-100 % ethanol to each tube. For example, 100 µL of supernatant would require 10 µL of NaAc and 220 µL of ethanol. Mix by inverting capped tubes a few times.

Step 21. Incubate overnight at -10 to -30 °C or at least 30 minutes at -70 to -90 °C.

Step 22. Centrifuge for 10 to 15 minutes at 12,000 rpm. Remove and discard supernatant.

Step 23. Add 1 mL of 70 % ethanol to each tube (see preparing for the test on page 1). Note: Do not mix or vortex.

Step 24. Centrifuge for 10 minutes at 12,000 rpm. Remove and discard supernatant. Air dry pellets. (approximately 10 to 15 minutes)

Step 25. Resuspend each pellet in 10 µL RNAse-free Water by pipetting up and down.

Spot extracts

For your reference, complete a loading diagram to show the locations of your samples.

Wearing gloves, open the clear plastic sleeve protecting the membrane and fold it under for spotting. Do not remove the membrane from the sleeve. Avoid touching the membrane as it can cause non-specific marks which can obstruct sample spots on the final film.

Following the loading diagram, pipette 2 µL of the resuspended pellet onto selected spots of the membrane. Allow the membrane to air dry, then close the jacket and replace the membrane in its protective folder.

Store your nucleic acid extracts at -10 to -30 °C or -70 to -90 °C until you receive results. If you need to retest any samples, you can use the stored nucleic acid samples or extract from fresh material.

Submitting the Membrane Back to Agdia

U.S. Customers

Complete **Membrane Submission Form A** (included with your kit). Return it to Agdia with your spotted membrane by the expiration date on the plastic sleeve. Not completing and including this form could delay your results.

International Customers

IMPORTANT: Customers from outside the United States must submit their membranes to Agdia using a special NAK permit packet. This packet facilitates the transfer of your membranes from your country of origin, through a USDA APHIS inspection station, and finally to Agdia. This process is required under U.S. law. Permit packets can be ordered from Agdia for a fee. If Red/White labels have expired, please

contact Agdia for replacement. Any membranes submitted to Agdia without using the permit packet cannot be processed and will be destroyed upon receipt.

Complete **Membrane Submission Form B** (included with your kit). Return it to Agdia with your spotted membrane by the expiration date on the plastic sleeve. Not completing and including this form could delay your results.

Please Note:

- Do not cut and submit part of a membrane. Only entire membranes will be accepted for processing.
- A partially spotted membrane may be held for completion of spotting later.
- Agdia does not guarantee results for membranes submitted after the expiration date.

Loading diagram for nucleic acid hybridization assay

Membrane number _____ Date _____ Samples spotted by _____

	Agdia controls	1	2	3	4	5	6	7	8	9	10	11	12
A													
B													
C													
D													
E													
F													
G													
H													

agdia®

Note: The shaded column is reserved for our controls. Do not apply samples to these spots.