I. Introduction

The purpose of this work instruction is to describe the subsampling and grinding procedure of cucurbit seeds samples for ELISA and RNA extraction for CGMMV detection. This method has been tested on cucumber, watermelon, cantaloupe/melon, squash and pumpkin seeds.

Due to the uneven distribution of the virus in a seed lot and samples, the following sample size and number of subsamples are crucial to achieve reliable detection when testing seed lots. The sample size and number of subsamples are derived from recommendations of the International Seed Testing Association 7-026 protocol: to ensure a 95 % probability that infestations of 0.15 % or higher are detected it is necessary to test 20 subsamples of 100 seeds each for a total of 2000 seeds per sample.

Each seed sample received is tested as a set of twenty (20) subsamples of 100 seeds each.

II. Related Work Instructions

WI-B-T-4-4 ELISA for Screening Detection of Cucumber Green Mottle Mosaic Virus in cucurbit seeds

WI-B-T-2-20 Total RNA Extraction from cucurbit seeds for Cucumber Green Mottle Mosaic Virus (CGMMV) detection using TRIzol LS reagent.

III. Equipment and Materials

A. Equipment

a. Blender-Oster Brand: e.g. https://www.target.com/p/oster-174-simple-blend-153-100-blender/-/A-51199418?preselect=14714388#lnk=sametab
b. Blender Bottom: e.g. http://www.goodmans.net/i/2597/blender-jar-bottom-cap-for-for-oster-osterizer-ble.htm
c. Blender Seals: e.g. http://www.goodmans.net/i/2596/rubber-o-ring-gasket-seal-for-oster-osterizer-3-pa.htm
d. Blender blades: e.g.http://www.goodmans.net/i/2589/ice-crusher-blender-blade-cutter-for-oster-osteriz.htm
e. Or Blender Bottom, Blender seals and blades may be bought as a set: Blender Accessory Kit (blade; blender seals; base) GSA advantage Mfr Part No.: 006010015NP0

B. Materials

a. Mason-style jar (4 oz) (Ball quilted crystal jelly jar with lid and band-regular mouth): e.g. https://www.amazon.com/dp/B00B80TK2K/ref=twister_B01MSZCPVF?_encoding=UTF8&psc=1
b. Gloves, non-powder (any vendor)
c. Weigh boats (any vendor)
d. Paper mat or towels, absorbent (any vendor)
e. Disposable, absorbent bench underpads (any vendor)

IV. Sample and subsample preparation

Wearing gloves when handling samples and changing them as often as possible is strongly recommended, especially if gloves had direct contact with seed material. Changing gloves between individual samples in high throughput situations may not be practical so in these situations, changing gloves can be done between each set of twenty subsamples derived from a single sample/lot. Gloves must be worn when handling suspect positive samples.

1. Seed samples should be stored in a cool, dry place before processing.
2. Each sample of 2000 seeds (or more) should be thoroughly mixed before subsampling. This can be achieved by placing the seeds into a Ziploc bag and inverting it several times to achieve good mixing.
3. Count twenty (20) subsamples of 100 seeds each or use the table below to make the subsamples by weight or volume.
4. Store the remaining seeds in a cool, dry place in case confirmatory testing is needed for this sample.

Table 1. Approximate weight and volume of selected cucurbit seeds.

Seed	Variety	100 seeds	
		Weight (g)	Volume (mL)*
Cucumber	Northern Pickling	2.9	5.5
Cantaloupe	Sweet Granite	2.3	5-5.6
Watermelon	Sugar Baby	4.4	9-9.5
Pumpkin	Champion	18.8	50
Squash	Yellow Crookneck	7.1	15-17.5

- *Volume as measured in a 15ml Falcon tube*

V. Assembly of Oster accessories and jar

Note: *Use of cut-resistant gloves and goggles is recommended for handling the blades and performing grinding*

1. Prepare the parts by cleaning jars, blade and Oster base (Figure 1) by rinsing in water then running through the dishwasher. Clean the rubber seal by hand washing only. Air dry all parts.

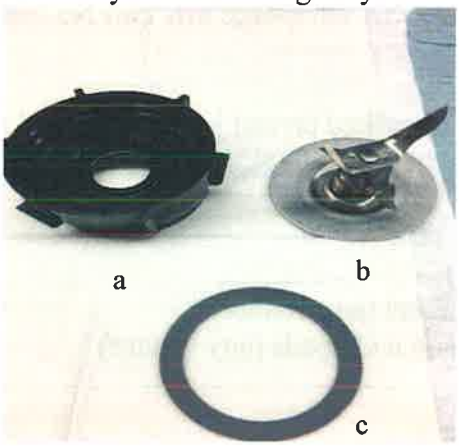

Figure 1. Blender accessories, base (a), blade (b) and rubber seal (c).

2. Examine jars for any cracks or chip. Discard jars that are damaged.

3. Make sure the blades will not touch the sides of glass jar. Adjustment can be made by bending blade upwards using pliers (Figure 2). IMPORTANT: Use cut-resistant gloves and goggles when doing this.

Figure 2. Bending of the blades

4. Place 100 seeds in jar. Table 1 shows the average weight and volume of selected cucurbit seeds.

5. Assemble the base, blade, rubber seal and jar; the seal sits on top of the blade so that the blade and the mouth of the jar do not touch. The mouth of the jar screws on to the base. (Figure 3)

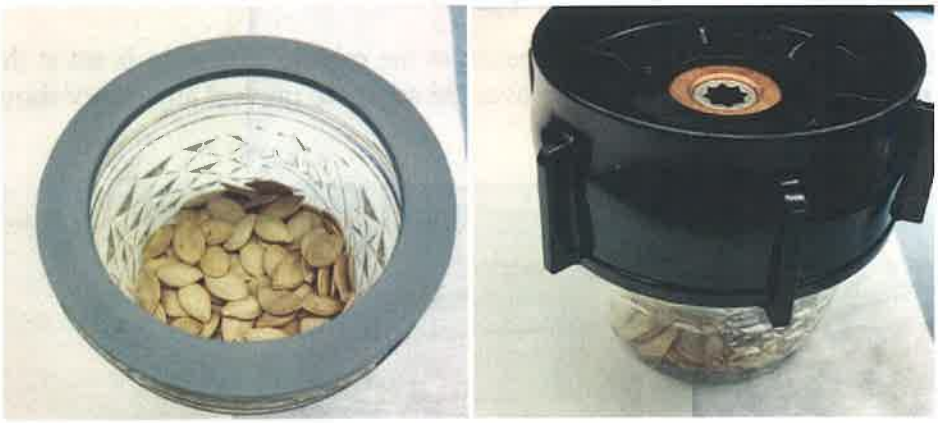

Figure 3. Assembly of base, blade, rubber seal and jar.

6. Attach jar to blender bottom and turn it clockwise to ensure fit. (Figure 4)

Figure 4. Attaching the jar to the blender bottom.

7. Examine if blades touch the sides of the jar. Repeat step 3 if blades touch the sides of the jar.

VI. Grinding

1. Grinding time is 1 minute with a 10 second pause in the middle. Grinding is set at the highest setting (Ice Crush). Place plastic container, large enough over the set-up to prevent any injury should glass break during the grinding. (Figure 5)

Figure 5. The jar-blender bottom assembly is covered by a 3L plastic beaker as a precautionary measure if glass jar breaks during grinding. Alternatively, a large plastic yogurt container may also be used for this purpose.

2. To remove jar after grinding, lift the jar assembly from the blender bottom. Once detached, unscrew the glass jar from the base and blade, with the glass jar on the bottom to collect the ground sample. Figure 6 shows the consistency of ground seed (fine ground). The grinding setting provided was found to work for all seeds tested (Table 1). The grinding time may be increased if a fine powder consistency is not achieved with this setting, however, the effect of prolonged grinding has not been evaluated for its effect on sample degradation.

Figure 6. Ground cucumber seeds with a fine powder consistency.

3. Once the jar is opened, use a plastic spatula to scrape off the ground material off the jar's walls. Mix grindage well and proceed with ELISA or RNA extraction.
4. Any remaining grindage should be stored at -20 °C (short term) or long term -80°C (long-term). Transferring to a 17ml Falcon tube is recommended prior to storage.
5. Once done with grinding, clean jars and blender parts as previously described.

VII. References

1. **International Rules for Seed Testing 2017: 7-026** Detection of *Squash mosaic virus*, *Cucumber green mottle mosaic virus* and *Melon necrotic spot virus* in cucurbit seed. International Seed Testing Association publication (https://www.seedtest.org/en/home.html).

Document Revision History

Status	Document Revision Number	Effective Date	Description
Original	Original	See Date of Electronic Signature	For Training ONLY: to baseline the work instruction for screening laboratories.

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Trizol LS protocol for CGMMV extraction from seeds (Unofficial Protocol)

Prepare: 75% ethanol (for 20 mL: 15 ml 100% ethanol+ 5mL MG water)

Centrifuge: set at 4˚C

Heat block: set at 55˚C

Note: Add reagents and open sample tubes in the fume hood up to step 10 to ensure that no hazardous fumes are released.

1) Add 1 mL Trizol LS reagent to 200 microliters of homogenized sample in a 2 ml tube.
2) Homogenize the sample by pipetting up and down several times
3) Incubate for 5 minutes at room temperature
4) Add 0.3 mL chloroform
5) Incubate for 2-3 minutes
6) Centrifuge the sample for 15 minutes at 12,000Xg at 4˚C. The mixture separates into a lower red phenol chloroform, and interphase, and a colorless upper aqueous phase. (If no separation occurs, mix the sample by gently inverting tube several times then centrifuge again)
7) Transfer the aqueous phase containing the RNA to a new 1.5 mL tube by angling the tube at 45˚ and pipetting the solution out
8) Add 0.7 mL of isopropanol to the aqueous phase
9) Incubate for 10 minutes at room temperature
10) Centrifuge for 10 minutes at 12,000Xg at 4˚C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube
11) Discard the supernatant with a pipettor
12) Resuspend the pellet in 1.3 mL of 75% ethanol
13) Vortex the sample briefly (5-10 seconds) then centrifuge for 5 minutes at 7500Xg at 4˚C
14) Discard the supernatant with a pipettor; make sure that all the ethanol are gone from the sides of the tube as well
15) Air dry the RNA pellet for 10 minutes
16) Resuspend pellet in 30 microliters of MG water by pipetting up and down
17) Incubate in a heat block at 55˚C for 10-15 minutes.
18) Measure concentration using a NanoDrop (RNA setting) then store labeled RNA tubes at -80˚ C