

## ISTA proficiency test for the detection of *Cucumber green mild mottle virus* and *Squash mosaic virus* in *Cucurbitaceae* seeds

### Objective

The aim of this proficiency test was to determine the ability of participating laboratories to detect CGMMV and/or SQMV in naturally contaminated *Cucurbitaceae* seeds and to evaluate their performance.

### Approach

Several naturally contaminated seed lots containing different levels of CGMMV and/or SqMV contamination were characterized to allow a reliable set up for the proficiency test.

Different categories, healthy, medium contamination (MC), and heavily contaminated (HC) samples, were compiled from these seed lots. Prerequisite for the selection of the healthy seed lot is that 6000 seeds (three times the sample size) tested negative for both viruses. Prerequisite for the highly contaminated seed lots is that a 10 fold dilution of the seed lot in healthy seeds is still positive for all sub samples. Both criteria were met for the selected samples and for the medium and highly contaminated samples average high optical density (OD 405) values were obtained for CGMMV and SqMV respectively ( CGMMV: MC 2.20 and HC 3.50, SqMV; MC 0.80 and HC 1.20).

For each participant in total 50 seed samples of each 100 seeds were prepared and labeled with a sample number (Table 1).

**Table 1.** Composition of proficiency test sample set

seed health status	number of subs	ratio (# seeds)	expectation
Healthy (H)	19	zzb658 (100)	negative (100%)
Medium contam. CGMMV	11	zzb658/zzb545 (90/10)	100% CGMMV positive
Medium contam. SqMV	10	zzb658/zzb451 (80/20)	100% SqMV positive
Heavily contam. CGMMV	5	zzb658/zzb636 (90/10)	100% CGMMV positive
Heavily contam. SqMV	5	zzb148 (100)	100% SqMV positive

One of the prepared sets was pretested to determine the homogeneity. A second set was stored at room temperature during the proficiency test and then tested to determine the stability of the viral pathogens in time.

Participants were asked to test the samples according to the standard ISTA protocol applied in their laboratory and to include “in house” positive controls. Two replicate tubes of reference material (RM) with CGMMV and SqMV were provided with an instruction to have an additional positive control. The RM (fine flour of ground CGMMV and SqMV contaminated seed samples) was tested in several replicates to determine whether the distribution of both viruses was homogenously and whether the viruses

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were stable in time (data not shown). Objective of the inclusion of RM was to allow comparison of the sensitivity of the assays between laboratories.

### **Submission of data**

Participants were asked to supply raw results (OD values) and their final interpretation of the results (positive or negative) for each sub-sample in provided excel sheets.

### **Results**

#### **Analysis CGMMV**

##### **Homogeneity test for CGMMV**

Each set consisted of 34 healthy (including 15 samples with SqMV), 11 medium contaminated and five highly contaminated samples (table 1).

In the homogeneity test in the organising laboratory of the 34 presumably healthy samples no CGMMV was detected, all medium (11 out of 11) and all highly (five out of five) were positive for CGMMV (appendix 1). For the homogeneity test all the results were according to the expectations. Therefore the homogeneity of the sets was fit for purpose.

##### **Stability test for CGMMV**

One set was stored at room temperature for three months and then the samples were tested in the organising laboratory. For 47 out of 50 samples the results were in line with the expectations. However in the 34 samples from the presumably CGMMV-free seed lot (ZZB658) there were three lightly positive samples (appendix 1). One explanation is that CGMMV was not homogeneously present in ZZB658 and escaped detection by the very low incidence. This is rather unlikely since CGMMV was not detected in previous tests using more than 8500 seeds of ZZB658 including the homogeneity test. An alternative probably more likely explanation is that somewhere cross contamination occurred between the processing of the samples in the test. The 11 medium contaminated and five highly CGMMV contaminated samples were easily detected with relative high OD405 values. Despite the unexpected results for the healthy samples the stability of the CGMMV contamination in the contaminated samples was high which is in line with the high persistence of tobamoviruses such as CGMMV.

##### **Proficiency test results for CGMMV**

Sixteen laboratories provided CGMMV data (appendix 3). The binominal data and statistical analysis are summarized in table 2.

Three laboratories (6, 8 and 10) did have a perfect score and have an A-rating.

##### **High level:**

A-rating: 5 positive samples

BMP-rating: <5 positive samples

**Medium level:**

A-rating: 11 positive samples  
 BMP-rating: <11 positive samples

**Healthy:**

A-rating: 0 positive samples  
 BMP-rating: >0 positive samples

**Table 2.** Summarised results for the detection of CGMMV positive samples in the proficiency test with three levels of CGMMV contamination

Lab	1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21
Healthy (34)	8	0	3	0	0	0	1	0	0	3	0	2	0	0	0	0
Medium (11)	0	3	6	11	4	11	9	11	5	9	3	4	5	6	3	4
High (5)	1	5	5	5	4	5	5	5	5	5	5	2	5	5	5	5
ISTA Rating*	BMP	BMP	BMP	A	BMP	A	BMP	A	BMP	BMP	BMP	BMP	BMP	BMP	BMP	BMP

\* ISTA proposed rating is categorised as A, B, C and BMP (Below Minimum Performance)

**Conclusion for CGMMV PT**

Three laboratories (6, 8 and 10) performed the CGMMV ELISA very well and 13 laboratories performed below minimum performance (BMP).

**Analysis SqMV**

**Homogeneity test for SqMV**

Each set consisted of 35 “healthy” (including 16 samples with CGMMV), 10 medium contaminated and five highly SqMV contaminated samples (table 1).

In the homogeneity test in the organising laboratory no SqMV was detected in the presumably 35 “healthy” samples. SqMV was easily detected in the five SqMV contaminated samples. For the medium contaminated samples nine out of 10 were detected (appendix 2). For the SqMV homogeneity test, the result was unexpected for the medium contaminated (one negative in the homogeneity test) samples but all other samples did fit the expectations. The data were used in the ISTA seedcalc8.0 tool to estimate the probability and determine ISTA ratings (Table 3-4, Fig.1).

**Table 3.** Estimated incidence of SqMV in the medium contaminated seed lot using seedcalc8.0 using a confidence level of 95%

# of Seed Pools	10	<i>Measured property on seed pools</i>	Computed % in sample	2,28 %
# of Seeds per Pool	100		Desired Confidence Level	95 %
Total Seeds Tested	1000			
# Deviants Pools	9		Upper Bound of True % Impurity	5,14
(95% confident that the lot impurity is below 5,14%.)				

**Table 4.** Estimated probability of finding 0-10 SqMV positives in the medium contaminated samples with SqMV using seedcalc8.0

		<i>k</i>	Prob (%) of <i>k</i> positive out of <i>n</i>
# of seeds per sample ( <i>m</i> )	100	0	0,0000%
True contamination rate ( $\pi$ )	5,1400%	1	0,0000%
		2	0,0000%
Total # of samples ( <i>n</i> )	10	3	0,0000%
		4	0,0000%
		5	0,0000%
		6	0,0000%
		7	0,0015%
		8	0,1127%
		9	4,8786%
		10	95,0071%

Based on the negative result of one sample from the medium category in the homogeneity assay a wider range applies for the ISTA rating.

**High level:**

A-rating: 5 positive samples  
 BMP-rating: <5 positive samples

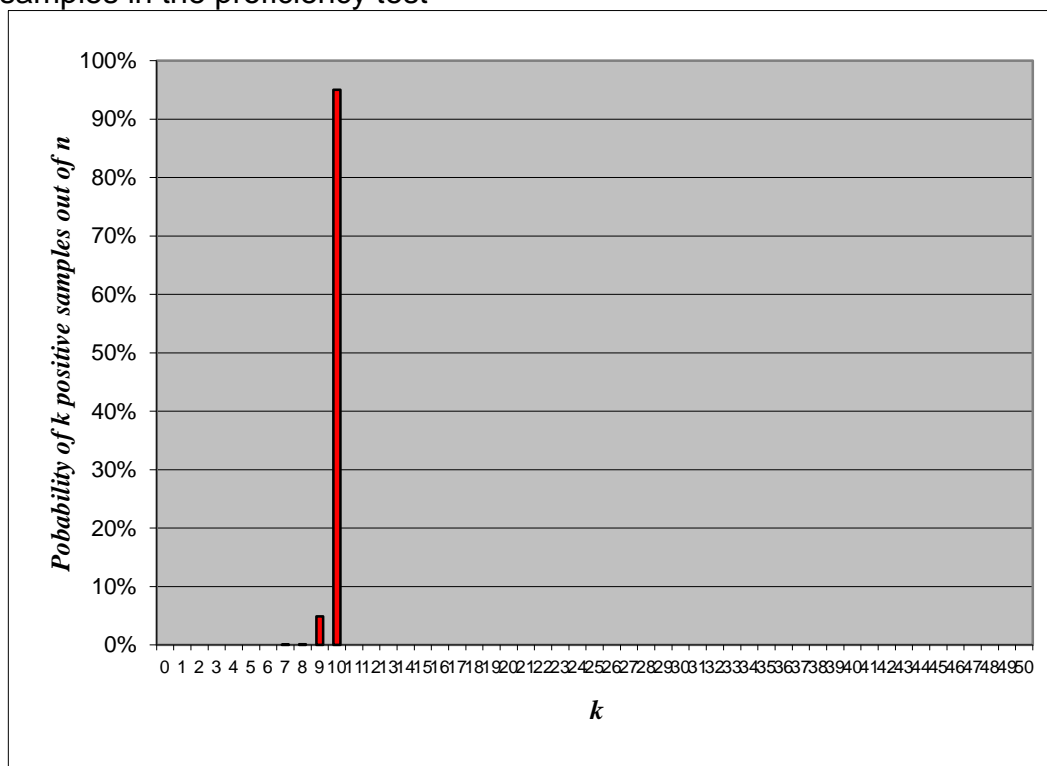
**Medium level:**

A-rating: 10 positive samples  
 B-rating: 9 positive samples  
 BMP-rating: <9 positive samples

**Healthy:**

A-rating: 0 positive samples  
 BMP-rating: >0 positive samples

**Fig. 1.** Probability of finding 0-10 positives of the medium SqMV contaminated samples in the proficiency test



### Stability test for SqMV

After storage at room temperature for two months all presumably SqMV contaminated samples tested positive for SqMV showing that the set was stable and fit for purpose. Even for the medium contaminated samples all reacted well above the ELISA decision threshold and 10 out of 10 samples were positive for SqMV (Appendix 2).

### Proficiency test results for SqMV

Due to the homogeneity of the medium contaminated seed lot some more variation was expected in the outcome of the SqMV proficiency test. Still 10 out of 16 laboratories were able to detect at least 9 positives in the medium category (Table 5, Appendix 4A). Eight laboratories did have a BMP rating since less than 9 samples were found in the medium category and/or highly contaminated samples were missed. Also some false positive results in laboratories 5 and 7 did lead to the BMP rating.

**Table 5.** Summarised results for the detection of SqMV positive samples in the proficiency test with three levels of SqMV contamination

Lab	1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21
Healthy (35)	2	0	3	0	1	0	0	0	0	0	0	0	0	0	0	1
Medium (10)	6	10	10	9	10	10	9	10	7	8	10	7	10	10	8	4
High (5)	0	5	5	5	5	5	5	5	5	5	5	4	5	5	5	4
ISTA Rating*	BMP	A	BMP	B	BMP	A	B	A	BMP	BMP	A	BMP	A	A	BMP	BMP

\* ISTA proposed rating is categorised as A, B, C and BMP (Below Minimum Performance)

## **Conclusion for SqMV PT**

For SqMV most laboratories (10/16) were able to detect this virus consistently. Four laboratories did find false positive samples amongst the healthy samples. Maybe cross contaminations did occur in these laboratories.

## **Discussion**

The average ELISA values per contamination were highly variable between laboratories for both CGMMV and SqMV (appendix 3B and 4B). Part of the variation could be explained by variation in virus load that is normal for natural contaminated seed lots. However clearly some laboratories did have consistently lower (laboratory 13 and 15) or higher (laboratory 10 and 16) ELISA values indicating that the sensitivity of the assays is highly variable,

Especially for CGMMV several laboratories were not able to detect this virus consistently (13/16). One possible explanation for the large number of false negatives is that some laboratories were using antisera that are less suitable for the matrix seeds. More than 10 different antiserum combinations were used in this comparative test.

For CGMMV five laboratories did have an issue with false positives. Cross contamination between samples could be an explanation for this result. Some laboratories (6, 8 and 10) performed well for both viruses.

## **Overall conclusions**

The performance of the participating laboratories was highly variable and especially for CGMMV many laboratories did score a BMP.

## **Appendices**

**Appendix 1.** Average OD405 ELISA data (2 replicates) of 50 samples in CGMMV homogeneity and stability test

**Appendix 2.** Average OD405 ELISA data (2 replicates) of 50 samples in SqMV homogeneity and stability test

**Appendix 3A.** Detection of CGMMV in PT by different laboratories

**Appendix 3B.** Average OD for three CGMMV contamination levels in PT

**Appendix 4A.** Detection of SqMV in PT by different laboratories

**Appendix 4B.** Average OD for three SqMV contamination levels in PT

**Appendix 5A.** Detection of CGMMV in undiluted and 10 fold dilutions of GMMV/SqMV contaminated reference material (RM) in PT

**Appendix 5B.** Detection of SqMV in undiluted and 10 fold dilutions of CGMMV/SqMV contaminated reference material (RM) in PT

## ISTA Seed Health Proficiency test for method SH7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

**Appendix 1.** Average OD405 ELISA data (2 replicates) of 50 samples in CGMMV homogeneity and stability test (red colour samples were regarded as positives). Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with CGMMV, respectively.

CGMMV		dec-15 july2015	
Contam. level	sample	Stability	homogeneity
		Xb	Y
1	1	0,051	0,034
1	2	0,064	0,037
1	3	0,054	0,035
1	15	0,051	0,038
1	16	0,052	0,036
1	17	0,049	0,030
1	18	0,051	0,038
1	19	0,057	0,036
1	20	0,060	0,037
1	21	0,062	0,035
1	22	0,063	0,040
1	23	0,064	0,037
1	24	0,061	0,036
1	25	0,063	0,031
1	26	0,058	0,031
1	27	0,061	0,032
1	28	0,061	0,037
1	29	0,052	0,038
1	30	0,051	0,034
1	31	0,047	0,035
1	32	0,047	0,037
1	38	0,152*	0,027
1	39	0,139*	0,028
1	40	0,054	0,034
1	41	0,056	0,037
1	42	0,044	0,036
1	43	0,038	0,034
1	44	0,048	0,035
1	45	0,037	0,035
1	46	0,044	0,035
1	47	0,2685*	0,039
1	48	0,050	0,039
1	49	0,044	0,035
1	50	0,041	0,037
2	4	0,729	0,210
2	5	2,909	0,445
2	6	3,049	0,466
2	7	0,458	0,435
2	8	2,559	0,401
2	9	3,114	0,288
2	10	0,642	0,255
2	11	1,147	0,306
2	12	0,593	0,251
2	13	0,554	0,267
2	14	2,275	0,220
3	33	3,308	0,669
3	34	3,150	0,663
3	35	2,902	0,586
3	36	2,708	0,613
3	37	3,080	0,629

\* 3 false positives in stability check

## ISTA Seed Health Proficiency test for method SH7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

**Appendix 2.** Average OD405 ELISA data (2 replicates) of 50 samples in SqMV homogeneity and stability test (red colour samples were regarded as positives). Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with CGMMV, resp.

SqMV			
dec-15 july 2015			
Contam. level	sample	Stability	homogeneity
		Xb	Y
1	1	0,059	0,036
1	2	0,045	0,038
1	3	0,057	0,038
1	4	0,065	0,040
1	5	0,055	0,038
1	6	0,053	0,036
1	7	0,065	0,044
1	8	0,068	0,037
1	9	0,077	0,036
1	10	0,069	0,038
1	11	0,070	0,037
1	12	0,070	0,038
1	13	0,064	0,037
1	14	0,061	0,037
1	15	0,058	0,033
1	16	0,059	0,040
1	17	0,060	0,043
1	18	0,058	0,038
1	29	0,061	0,038
1	30	0,057	0,037
1	31	0,049	0,037
1	32	0,047	0,039
1	33	0,053	0,030
1	34	0,072	0,034
1	35	0,048	0,034
1	36	0,046	0,037
1	37	0,049	0,037
1	38	0,047	0,044
1	39	0,052	0,037
1	40	0,044	0,034
1	41	0,047	0,036
1	47	0,050	0,039
1	48	0,048	0,038
1	49	0,039	0,035
1	50	0,047	0,040
2	19	1,603	0,074*
2	20	1,507	0,671
2	21	3,516	1,032
2	22	3,571	0,951
2	23	3,549	1,140
2	24	2,726	0,912
2	25	3,583	1,521
2	26	3,429	1,079
2	27	3,618	0,757
2	28	3,491	0,647
3	42	3,550	1,614
3	43	3,551	1,517
3	44	3,595	1,291
3	45	3,546	1,422
3	46	3,520	1,455

\* Regarded as SqMV negative



## ISTA Seed Health Proficiency test for method SH7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

**Appendix 3A.** Detection of CGGMV in PT by different laboratories (red colored samples were regarded as positives). Average OD405 ELISA data (2 replicates) of 50 samples. Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with CGMMV, resp.

CGMMV Contam. level	sample	Laboratory															
		1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21
1	1	0,335	0,139	0,050	0,084	0,008	0,074	0,000	0,029	0,108	0,110	0,116	0,086	0,141	0,105	0,130	0,116
1	2	1,050	0,183	0,053	0,081	-0,025	0,078	-0,007	0,027	0,112	0,129	0,140	0,083	0,137	0,108	0,122	0,114
1	3	0,590	0,150	0,052	0,092	-0,034	0,072	-0,005	0,028	0,109	0,107	0,105	0,088	0,135	0,103	0,093	0,114
1	15	0,155	0,143	0,072	0,082	0,033	0,063	0,032	0,036	0,114	0,247	0,170	0,093	0,140	0,105	0,098	0,115
1	16	0,204	0,146	0,047	0,083	-0,001	0,072	0,034	0,032	0,105	0,380	0,150	0,080	0,167	0,104	0,075	0,128
1	17	0,575	0,121	0,055	0,073	0,005	0,080	0,012	0,032	0,115	0,130	0,138	0,080	0,149	0,104	0,076	0,112
1	18	0,300	0,142	0,055	0,077	-0,014	0,073	0,038	0,031	0,106	0,113	0,146	0,083	0,228	0,099	0,097	0,110
1	19	1,265	0,127	0,047	0,082	-0,020	0,071	0,004	0,040	0,102	0,133	0,120	0,097	0,127	0,114	0,078	0,126
1	20	1,590	0,149	0,043	0,083	-0,144	0,071	0,005	0,036	0,101	0,267	0,152	0,093	0,127	0,106	0,035	0,108
1	21	1,100	0,145	0,048	0,088	-0,017	0,070	0,006	0,035	0,102	0,138	0,143	0,082	0,130	0,105	0,065	0,108
1	22	0,290	0,151	0,049	0,086	-0,001	0,073	0,003	0,039	0,098	0,112	0,149	0,110	0,127	0,101	0,030	0,162
1	23	1,205	0,110	0,052	0,084	-0,013	0,070	0,003	0,032	0,103	0,146	0,135	0,105	0,135	0,127	0,060	0,108
1	24	0,043	0,110	0,047	0,084	-0,005	0,079	-0,004	0,025	0,103	0,153	0,125	0,154	0,135	0,101	0,032	0,105
1	25	0,390	0,111	0,050	0,091	0,002	0,071	-0,001	0,034	0,106	0,144	0,121	0,102	0,149	0,097	0,070	0,104
1	26	0,435	0,129	0,055	0,089	-0,013	0,068	0,004	0,034	0,104	0,129	0,160	0,204	0,240	0,106	0,030	0,103
1	27	1,405	0,167	0,056	0,085	-0,021	0,065	-0,002	0,033	0,105	0,112	0,133	0,073	0,257	0,095	0,070	0,105
1	28	1,415	0,127	0,071	0,090	-0,023	0,069	0,000	0,034	0,110	0,149	0,136	0,093	0,131	0,145	0,043	0,107
1	29	0,036	0,124	0,067	0,079	-0,008	0,071	0,001	0,032	0,110	0,134	0,175	0,125	0,128	0,113	0,093	0,105
1	30	0,060	0,128	0,099	0,079	-0,006	0,074	0,002	0,032	0,113	0,135	0,157	0,082	0,145	0,096	0,059	0,104
1	31	0,230	0,143	0,114	0,081	-0,003	0,078	0,002	0,035	0,114	0,150	0,189	0,072	0,147	0,101	0,114	0,124
1	32	0,315	0,164	0,246	0,080	-0,001	0,073	-0,001	0,029	0,119	0,150	0,177	0,112	0,189	0,102	0,083	0,146
1	38	0,290	0,151	0,050	0,088	-0,037	0,068	0,101	0,032	0,123	0,136	0,201	0,102	0,182	0,119	0,138	0,102
1	39	1,305	0,175	0,051	0,084	-0,029	0,065	0,067	0,030	0,101	0,133	0,186	0,092	0,130	0,107	0,116	0,104
1	40	0,980	0,162	0,045	0,088	-0,024	0,067	0,059	0,027	0,095	0,146	0,214	0,182	0,133	0,091	0,090	0,106
1	41	0,089	0,119	0,049	0,075	-0,031	0,066	0,047	0,029	0,112	0,129	0,207	0,106	0,140	0,099	0,117	0,103
1	42	0,375	0,099	0,037	0,070	-0,042	0,063	0,027	0,007	0,105	0,124	0,154	0,097	0,145	0,103	0,021	0,096
1	43	0,120	0,112	0,037	0,070	-0,035	0,062	0,021	0,007	0,096	0,133	0,134	0,115	0,225	0,096	0,028	0,096
1	44	0,330	0,106	0,033	0,071	-0,025	0,064	0,016	0,008	0,098	0,136	0,147	0,089	0,263	0,091	0,024	0,096
1	45	0,550	0,093	0,032	0,062	-0,020	0,063	0,016	0,007	0,106	0,163	0,160	0,140	0,126	0,093	0,022	0,102
1	46	0,770	0,114	0,044	0,064	-0,012	0,062	0,017	0,007	0,097	0,147	0,155	0,088	0,120	0,095	0,027	0,097
1	47	0,079	0,135	0,035	0,067	-0,006	0,072	0,020	0,029	0,114	0,15	0,350	0,104	0,125	0,099	0,096	0,108
1	48	0,147	0,122	0,052	0,071	0,008	0,074	0,019	0,028	0,120	0,152	0,186	0,123	0,132	0,133	0,088	0,114
1	49	0,051	0,147	0,045	0,075	-0,001	0,088	0,016	0,033	0,125	0,154	0,220	0,151	0,164	0,108	0,089	0,108
1	50	0,006	0,143	0,044	0,074	-0,003	0,079	0,013	0,031	0,122	0,144	0,200	0,105	0,183	0,113	0,102	0,106
2	4	0,590	0,183	0,302	0,536	1,164	0,389	0,085	0,836	0,164	0,175	0,118	0,124	0,219	0,107	0,498	0,217
2	5	0,145	0,197	0,062	3,091	1,697	0,364	0,043	0,149	1,544	0,632	0,255	0,110	0,166	0,639	0,622	0,222
2	6	0,050	0,188	0,066	0,590	0,013	0,399	0,510	0,327	1,851	0,361	0,615	0,338	2,919	0,787	0,141	0,240
2	7	0,115	0,223	0,572	0,514	-0,007	1,686	0,586	0,164	0,862	0,313	0,124	0,186	1,973	0,776	0,221	0,232
2	8	0,016	0,266	0,131	0,589	0,061	0,251	0,131	0,143	0,177	0,431	0,129	0,103	0,897	0,121	0,094	1,592
2	9	0,195	0,146	0,367	0,632	0,029	1,550	0,164	0,200	0,872	0,170	0,619	0,181	0,213	0,291	0,201	0,214
2	10	0,210	0,250	0,222	0,559	1,283	0,410	0,119	0,312	2,177	0,262	0,639	0,167	0,359	0,119	0,125	0,218
2	11	0,052	0,265	0,069	3,281	1,455	0,361	0,296	0,212	0,195	0,284	0,124	0,210	0,191	0,134	0,147	0,224
2	12	0,015	0,195	0,068	3,320	0,047	0,668	0,135	0,198	0,206	0,224	0,129	0,121	0,854	0,859	0,187	0,414
2	13	0,530	0,172	0,888	0,609	0,043	0,557	1,430	1,021	0,152	0,218	0,177	0,088	2,182	1,112	0,147	1,247
2	14	0,200	2,048	0,073	1,184	0,055	0,398	0,687	0,159	0,185	0,227	0,184	0,107	0,174	0,743	0,648	0,274
3	33	0,018	2,643	1,741	3,504	1,029	1,062	0,999	3,166	2,366	0,620	1,255	0,243	4,000	1,210	2,660	1,900
3	34	0,175	2,884	1,922	3,561	0,988	1,115	0,891	3,209	3,008	0,795	1,565	0,127	3,977	1,457	2,295	2,099
3	35	1,100	2,805	1,964	3,586	0,028	1,123	0,958	3,173	2,258	0,681	1,410	0,206	3,927	1,455	2,678	2,302
3	36	0,430	1,986	1,904	3,557	1,031	1,268	1,132	3,274	2,681	0,679	1,670	0,135	4,000	1,519	2,175	2,288
3	37	0,110	2,528	2,409	3,233	1,097	1,192	0,857	3,169	2,361	0,480	1,625	0,156	3,881	1,343	2,635	1,978

# ISTA Seed Health Proficiency test for method SH7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

**Appendix 3B.** Average OD for three CGMMV contamination levels in PT. Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with CGMMV, resp.

CGMMV contam. level	n	Average OD value per Laboratory																	Mean
		1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21		
1	34	0,53	0,13	0,06	0,08	-0,02	0,07	0,02	0,03	0,11	0,15	0,16	0,11	0,16	0,11	0,07	0,11	0,12	
2	11	0,19	0,38	0,26	1,35	0,53	0,64	0,38	0,34	0,76	0,30	0,28	0,16	0,92	0,52	0,28	0,46	0,48	
3	5	0,37	2,57	1,99	3,49	0,83	1,15	0,97	3,20	2,53	0,65	1,51	0,17	3,96	1,40	2,49	2,11	1,84	

**Appendix 4A.** Detection of SqMV in PT by different laboratories (red colored samples were regarded as positives). Average OD405 ELISA data (2 replicates) of 50 samples. Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with SqMV, resp.

SqMV Contam. level	sample	Laboratory																
		1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21	
1	1	1,200	0,097	0,170	0,064	-0,019	0,086	0,007	0,022	0,129	0,090	0,072	0,117	0,122	0,113	0,120	0,169	
1	2	0,063	0,101	0,064	0,063	0,001	0,078	0,004	0,019	0,107	0,104	0,068	0,110	0,117	0,094	0,113	0,202	
1	3	0,240	0,106	0,069	0,066	-0,011	0,082	0,006	0,014	0,111	0,140	0,064	0,127	0,113	0,103	0,120	0,212	
1	4	0,300	0,099	0,054	0,063	0,001	0,088	0,003	0,022	0,108	0,106	0,079	0,126	0,212	0,112	0,169	0,197	
1	5	0,423	0,101	0,046	0,060	0,017	0,079	0,004	0,016	0,107	0,143	0,082	0,152	0,212	0,114	0,182	0,192	
1	6	0,615	0,102	0,041	0,059	0,047	0,095	0,008	0,014	0,112	0,119	0,085	0,145	0,210	0,122	0,177	0,220	
1	7	0,687	0,104	0,042	0,060	0,024	0,080	0,003	0,022	0,104	0,141	0,087	0,136	0,215	0,118	0,176	0,180	
1	8	0,054	0,095	0,039	0,057	0,013	0,081	0,002	0,025	0,112	0,127	0,097	0,137	0,217	0,107	0,177	0,149	
1	9	0,365	0,098	0,041	0,063	0,029	0,079	0,003	0,013	0,109	0,126	0,089	0,156	0,206	0,114	0,181	0,156	
1	10	0,170	0,109	0,040	0,064	0,039	0,076	0,005	0,020	0,106	0,112	0,089	0,115	0,220	0,111	0,168	0,190	
1	11	0,038	0,111	0,038	0,067	0,000	0,079	0,005	0,014	0,111	0,106	0,082	0,134	0,237	0,111	0,185	0,168	
1	12	0,135	0,117	0,037	0,062	0,013	0,080	0,004	0,013	0,115	0,127	0,080	0,175	0,237	0,114	0,206	0,198	
1	13	0,145	0,105	0,039	0,062	0,030	0,080	0,003	0,023	0,115	0,114	0,091	0,120	0,214	0,105	0,167	0,182	
1	14	0,411	0,103	0,038	0,063	0,060	0,071	0,003	0,014	0,112	0,137	0,085	0,123	0,226	0,109	0,174	0,167	
1	15	0,315	0,103	0,037	0,064	0,039	0,054	0,004	0,015	0,108	0,096	0,068	0,124	0,146	0,089	0,135	0,189	
1	16	0,690	0,097	0,035	0,061	0,016	0,060	0,007	0,023	0,114	0,107	0,071	0,123	0,130	0,115	0,126	0,173	
1	17	0,083	0,107	0,039	0,059	0,016	0,070	0,006	0,016	0,110	0,106	0,070	0,169	0,120	0,091	0,144	0,198	
1	18	0,255	0,123	0,038	0,060	0,019	0,067	0,005	0,015	0,111	0,105	0,065	0,135	0,142	0,082	0,139	0,212	
1	29	0,030	0,097	0,036	0,065	-0,027	0,073	0,000	0,017	0,111	0,110	0,054	0,133	0,145	0,128	0,139	0,172	
1	30	0,190	0,108	0,032	0,065	-0,035	0,071	0,002	0,014	0,107	0,113	0,079	0,142	0,152	0,100	0,147	0,161	
1	31	0,205	0,095	0,038	0,063	-0,034	0,072	0,005	0,012	0,115	0,107	0,063	0,129	0,140	0,094	0,134	0,174	
1	32	0,275	0,102	0,039	0,063	-0,026	0,091	0,003	0,019	0,112	0,104	0,061	0,126	0,113	0,094	0,120	0,185	
1	33	0,170	0,093	0,041	0,071	0,024	0,081	-0,001	0,015	0,128	0,110	0,079	0,170	0,265	0,120	0,218	0,164	
1	34	0,041	0,101	0,039	0,065	-0,010	0,070	-0,001	0,014	0,114	0,104	0,072	0,143	0,275	0,116	0,209	0,154	
1	35	0,315	0,103	0,035	0,064	1,084	0,097	0,002	0,021	0,113	0,114	0,077	0,182	0,292	0,118	0,237	0,178	
1	36	0,675	0,101	0,066	0,062	-0,015	0,077	0,004	0,015	0,111	0,112	0,078	0,135	0,230	0,118	0,183	0,185	
1	37	0,110	0,098	0,042	0,061	0,017	0,077	0,001	0,013	0,112	0,113	0,101	0,157	0,257	0,122	0,207	0,186	
1	38	0,425	0,098	0,041	0,060	-0,018	0,070	-0,001	0,015	0,111	0,105	0,054	0,118	0,136	0,121	0,127	0,171	
1	39	1,545	0,100	0,049	0,061	-0,018	0,072	-0,001	0,013	0,109	0,107	0,050	0,131	0,179	0,098	0,155	0,172	
1	40	0,885	0,099	0,063	0,060	-0,014	0,071	0,001	0,013	0,109	0,105	0,058	0,171	0,146	0,093	0,158	0,162	
1	41	0,155	0,087	0,116	0,066	-0,021	0,071	0,003	0,019	0,108	0,095	0,058	0,116	0,275	0,094	0,195	0,198	
1	47	0,061	0,106	2,563	0,061	0,003	0,069	0,005	0,014	0,111	0,102	0,070	0,155	0,180	0,091	0,167	0,196	
1	48	0,184	0,092	0,043	0,060	-0,014	0,076	0,005	0,013	0,108	0,100	0,060	0,166	0,147	0,091	0,156	1,782	
1	49	0,060	0,094	0,034	0,063	0,005	0,080	0,004	0,020	0,102	0,102	0,064	0,154	0,160	0,092	0,157	0,184	
1	50	0,007	0,095	0,030	0,063	0,002	0,081	0,005	0,016	0,111	0,103	0,075	0,183	0,130	0,089	0,157	0,190	
2	19	1,330	1,862	2,775	3,238	1,646	1,337	0,325	3,116	0,550	0,109	2,140	1,454	3,985	0,696	2,719	0,265	
2	20	1,240	0,505	0,977	3,326	1,457	1,508	0,386	3,136	0,304	0,353	3,945	0,286	1,960	1,264	1,123	0,145	
2	21	1,085	0,649	1,066	3,158	0,932	1,705	0,037	3,336	0,844	0,161	2,760	2,616	3,950	0,965	3,283	0,296	
2	22	0,540	0,385	1,383	0,100	0,402	1,996	0,063	3,337	0,588	0,284	2,855	0,197	2,417	0,681	1,307	0,202	
2	23	1,050	1,724	3,000	3,409	1,433	1,029	0,994	3,265	0,322	0,737	3,895	0,525	1,571	1,503	1,048	0,420	
2	24	0,069	1,915	0,556	1,281	1,451	1,703	2,160	3,455	0,416	0,656	3,940	0,257	4,000	1,412	2,129	0,300	
2	25	0,135	1,511	2,867	2,818	1,580	0,757	0,067	2,972	0,171	0,220	3,810	1,943	1,949	0,919	1,946	0,228	
2	26	0,800	2,101	1,296	3,484	1,109	0,391	0,636	3,119	0,195	0,361	3,475	0,533	2,391	1,525	1,462	0,188	
2	27	1,495	0,611	3,000	3,441	1,154	1,841	1,306	3,172	0,170	0,722	3,415	0,662	3,960	1,553	2,311	0,158	
2	28	1,295	0,772	3,000	2,661	0,977	2,027	0,443	3,034	0,289	0,552	1,195	0,690	2,631	1,692	1,660	0,147	
3	42	0,590	2,146	2,477	3,215	0,424	1,727	2,095	3,180	0,288	0,582	3,485	2,093	3,716	1,627	2,905	0,363	
3	43	0,275	1,703	1,198	3,634	0,989	2,350	2,402	3,042	0,388	0,599	3,695	1,563	3,988	1,720	2,775	0,211	
3	44	0,200	2,047	2,746	3,480	0,811	1,347	2,578	3,123	0,501	0,769	3,970	1,440	4,000	1,718	2,720	0,534	
3	45	0,280	1,842	2,635	2,651	0,958	0,998	1,591	3,212	0,437	0,672	3,670	0,197	3,945	1,556	2,071	0,582	
3	46	0,745	1,829	0,121	3,125	1,104	2,244	1,532	3,174	0,437	0,707	3,500	1,449	3,986	1,372	2,717	0,348	

## ISTA Seed Health Proficiency test for method SH7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

**Appendix 4B.** Average OD for three SqMV contamination levels in PT. Average OD405 ELISA data (2 replicates) of 50 samples. Contamination level 1, 2 and 3 were healthy, medium and heavily contaminated with SqMV, resp.

SqMV Contam. level	n	Average OD value per Laboratory															Mean	
		1	2	5	6	7	8	9	10	12	13	14	15	16	17	18		21
1	35	0,329	0,101	0,120	0,062	0,035	0,077	0,003	0,016	0,111	0,112	0,074	0,141	0,186	0,106	0,163	0,228	0,117
2	10	0,904	1,204	1,992	2,691	1,214	1,429	0,641	3,194	0,385	0,416	3,143	0,916	2,881	1,221	1,899	0,235	1,523
3	5	0,418	1,913	1,835	3,221	0,857	1,733	2,040	3,146	0,410	0,666	3,664	1,348	3,927	1,598	2,638	0,408	1,864

**Appendix 5A.** Detection of CGMMV in undiluted and 10 fold dilutions of GMMV/SqMV contaminated reference material (RM) in PT

CGMMV	1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21	Mean
Provided RM (A) undiluted	no data	2,94	no data	3,23		0,97	0,96	3,19	2,28	0,72	1,97	0,21	3,95	1,08	3,02	2,67	2,09
RM (A) 10x diluted		2,14			1,51	0,93	0,45	2,18	1,57	0,43	0,61	0,18	3,12	0,93	2,40	2,38	1,45
RM (A) 100x diluted		0,61			0,32	0,66	0,13	0,71	0,37	0,30	0,15	0,17	1,17	0,28	0,69	0,90	0,50
RM (A) 1000x diluted		0,16			0,04	0,33	0,05	0,17	0,17	0,19	0,10	0,10	0,28	0,10	0,23	0,30	0,17
Provided RM (B) undiluted		2,92		3,28	0,83	1,13	0,97	3,12	2,30	no data	1,96	0,19	3,95	1,18	2,83	2,75	2,11
RM (B) 10x diluted		1,97			0,45	0,96	0,46	2,05	0,90		0,60	0,14	3,10	0,86	1,97	2,26	1,31
RM (B) 100x diluted		0,56			0,06	0,51	0,13	0,67	0,43		0,13	0,14	1,22	0,32	0,53	0,93	0,47
RM (B) 1000x diluted		0,15			-0,03	0,34	0,06	0,16	0,18		0,14	0,10	0,29	0,10	0,18	0,35	0,17
NC extraction buffer		0,09		0,07	0,00	0,06	0,00	0,02	0,10	0,10	0,13	0,10	0,10	0,09	0,09	0,10	0,08
NC extraction buffer		0,09		0,07	0,00	0,06		0,02	0,10		0,15	0,10	0,11	0,10			0,08

**Appendix 5B.** Detection of SqMV in undiluted and 10 fold dilutions of CGMMV/SqMV contaminated reference material (RM) in PT

SqMV	1	2	5	6	7	8	9	10	12	13	14	15	16	17	18	21	Mean
Provided RM (A) undiluted		0,86		2,13		0,43	0,15	3,02	0,41	0,09	2,92	0,58	4,00	1,09	2,43	0,46	1,43
RM (A) 10x diluted		0,14			0,16	0,14	0,02	0,80	0,23	0,47	0,27	0,15	4,00	0,53	0,32	0,13	0,57
RM (A) 100x diluted		0,11			0,04	0,07	0,00	0,11	0,14	0,21	0,10	0,14	3,49	0,16	0,08	0,11	0,37
RM (A) 1000x diluted		0,10			0,00	0,06	0,00	0,02	0,13	0,12	0,07	0,14	0,76	0,09	0,04	0,18	0,13
Provided RM (B) undiluted		1,08		1,94	0,42	0,33	0,20	3,07	0,38		3,31	0,76	4,00	1,09	3,02	0,42	1,54
RM (B) 10x diluted		0,15			0,06	0,10	0,02	0,72	0,23		0,26	0,17	4,00	0,46	0,50	0,13	0,57
RM (B) 100x diluted		0,10			-0,03	0,06	0,00	0,09	0,15		0,09	0,14	2,42	0,16	0,09	0,11	0,28
RM (B) 1000x diluted		0,09			-0,05	0,06	0,00	0,02	0,14		0,07	0,17	0,56	0,09	0,04	0,22	0,12
NC extraction buffer		0,09		0,06	0,00	0,06	0,00	0,02	0,11	0,10	0,06	0,12	0,10	0,08	0,10	0,21	0,08
NC extraction buffer		0,09		0,06	0,00	0,06		0,01	0,10		0,06	0,11	0,11	0,09			0,07