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Improvements in challenging diagnostic of *Pepino mosaic virus* and Potato spindle tuber viroid in tomato seeds for better sensitivity

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Pepino mosaic virus (PepMV)

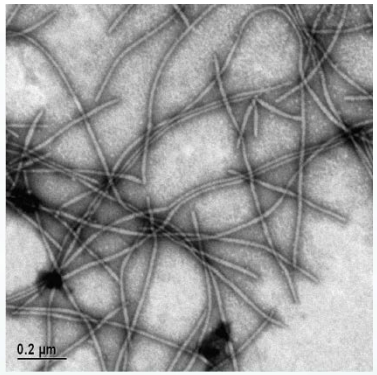
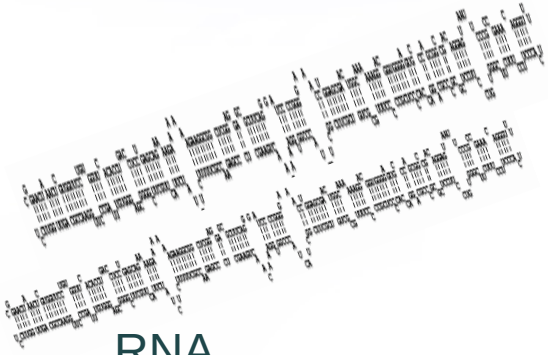


Photo: Dr. Magda Tušek Žnidarič, NIB

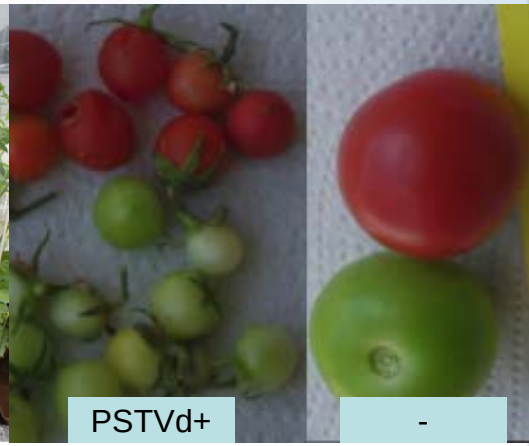
Photo: Dr. Inge Hanssen, Scientia Terrae, Belgium

Potexvirus; main strains: Peruvian, EU-tomato, US1/Ch1, Chile-2, PES

Potato spindle tuber viroid (PSTVd)

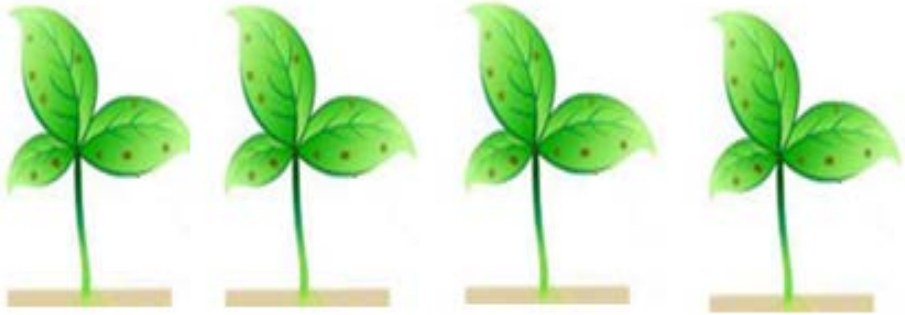


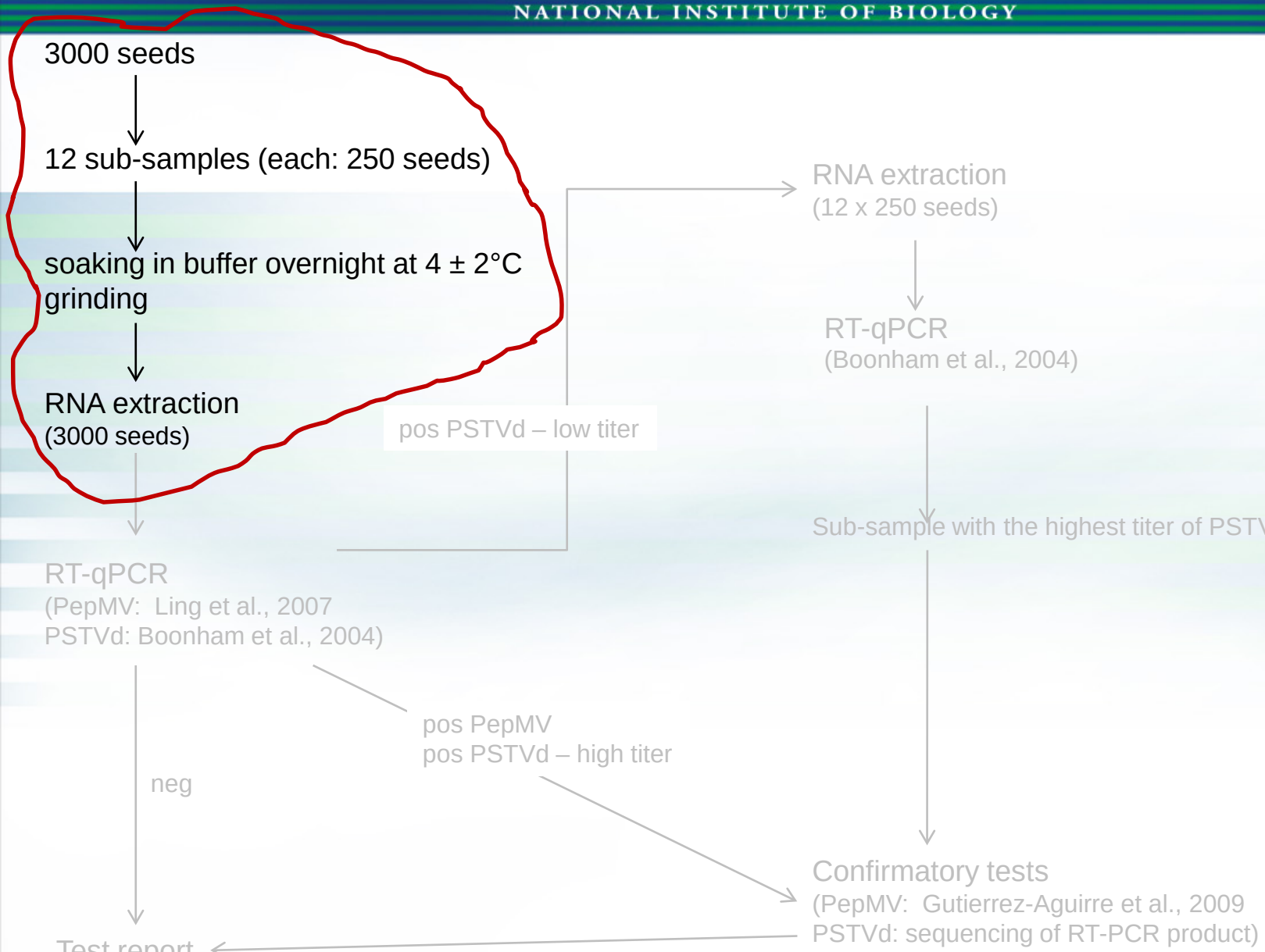
RNA
(356-361 nukleotidov)



Sensitive methods for their detection!!!

-stable
-transmitted readiliy between plants by crop handling, e.g., via contaminated tools and hands
- survival and transmission in water (Mehle et al., Applied and Envirnmental Microbiology, 2014: 1455-1462)




3000 seeds
12 sub-samples (each: 250 seeds)
soaking in buffer overnight at 4 ± 2°C
grinding
RNA extraction
(3000 seeds)
RT-qPCR
(PepMV: Ling et al., 2007
PSTVd: Boonham et al., 2004)
neg
Test report
pos PSTVd – low titer
RNA extraction
(12 x 250 seeds)
RT-qPCR
(Boonham et al., 2004)
Sub-sample with the highest titer of PSTVd
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Confirmatory tests
(PepMV: Gutierrez-Aguirre et al., 2009
PSTVd: sequencing of RT-PCR product)

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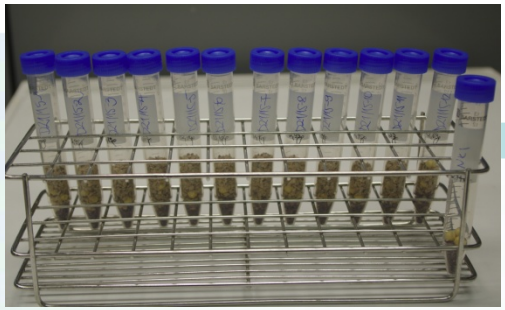
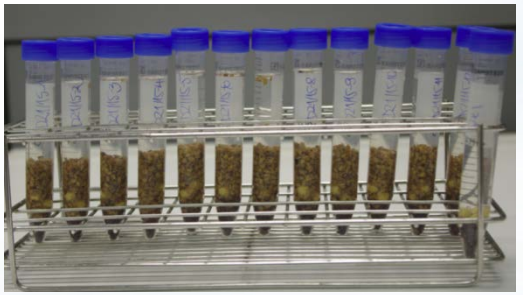
Sample preparation

3000 seeds



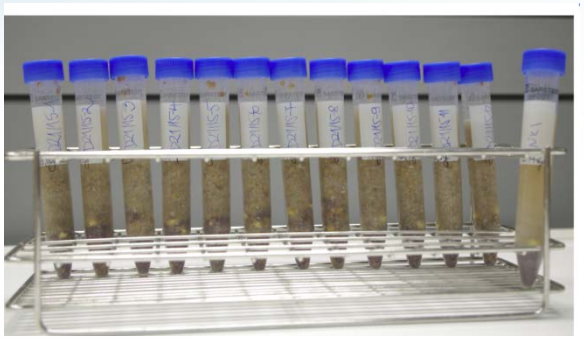
12 sub-samples of 250 seeds

+ 10 mL of 0.1M phosphate buffer
(Na_2HPO_4/KH_2PO_4 pH 7.2)



4 ± 2°C overnight

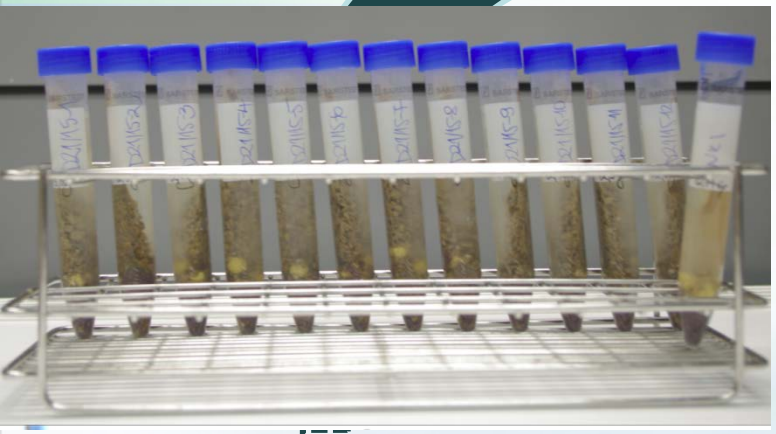
5 m/s, 40 sec



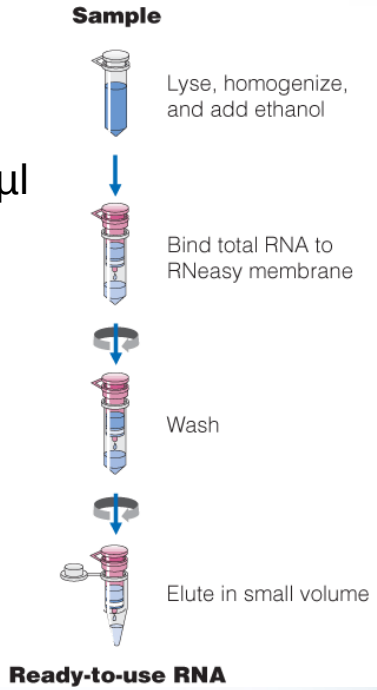
Centrifuge:
(10 min,
10.000 g, 4°C)

RNA extraction

(12 x 50µl)



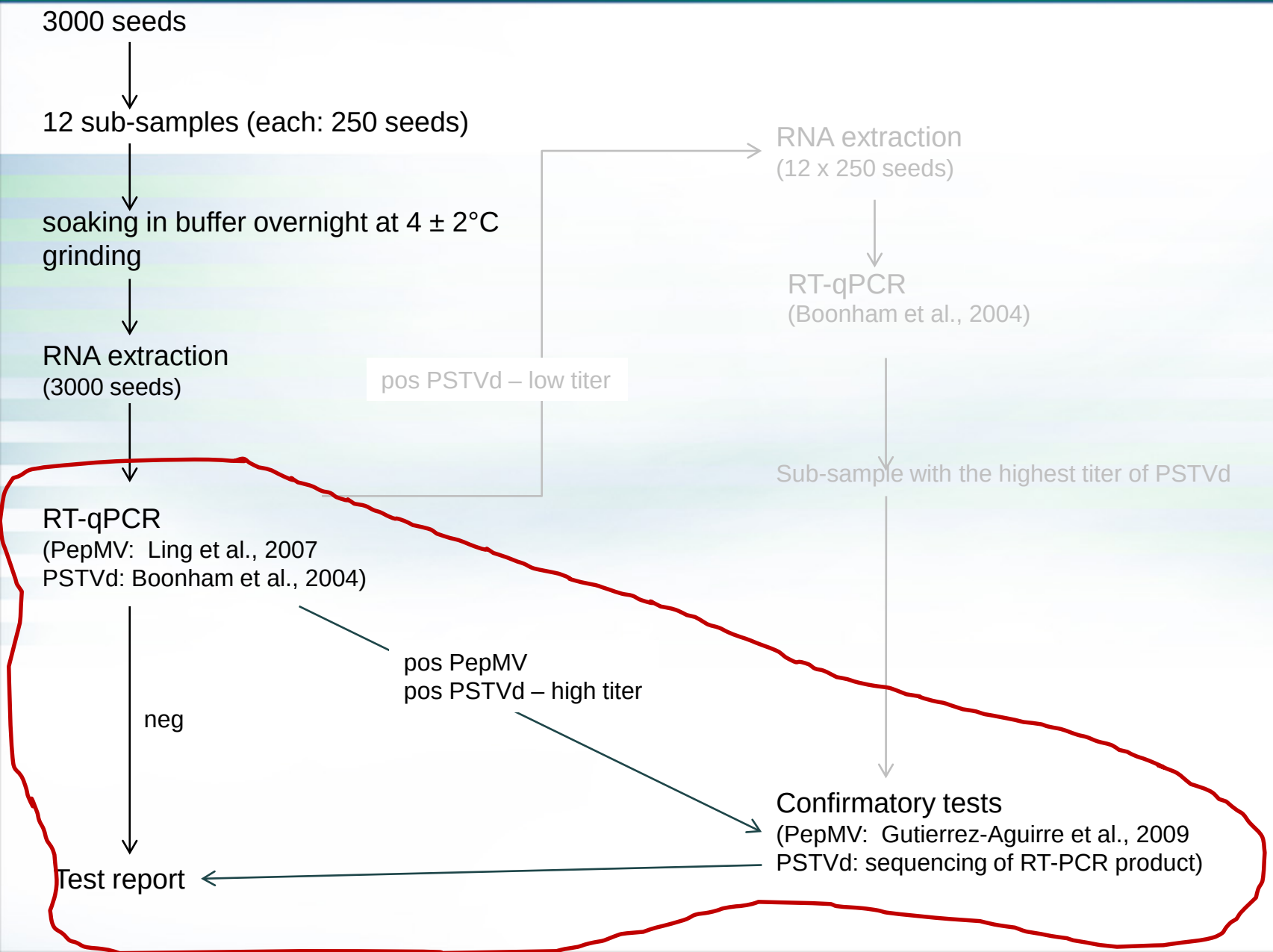
+ 600 µl of RLT buffer



The rest of the homogenate: stored for further needs at refrigerate

Qiagen RNeasy Plant kits

minor modifications:
-mercaptoethanol was omitted from the procedure -
-RNAse-free water at 65°C was added to the QIAGEN column where it was incubated for 5 min before final elution (2 x 50 µl)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Detection, identification: PepMV

Screening test | Confirmatory test

Naturally infected seed	ELISA	RT-qPCR (Ling et al., 2007)	RT-qPCR (Eur-cp)	RT-qPCR (Ch2-rep)	RT-qPCR (Ch2&US2-cp)
1:10	+	+	+	+	+
1:100	+	+	+	+	+
1:1000	+	+	+	+	+
1:5000	-	+	+	+	+
0:100	-	-	-	-	-

Gutierrez-Aguirre et al., Journal of Virological Methods, 2009: 46-55

Detection, identification: PSTVd

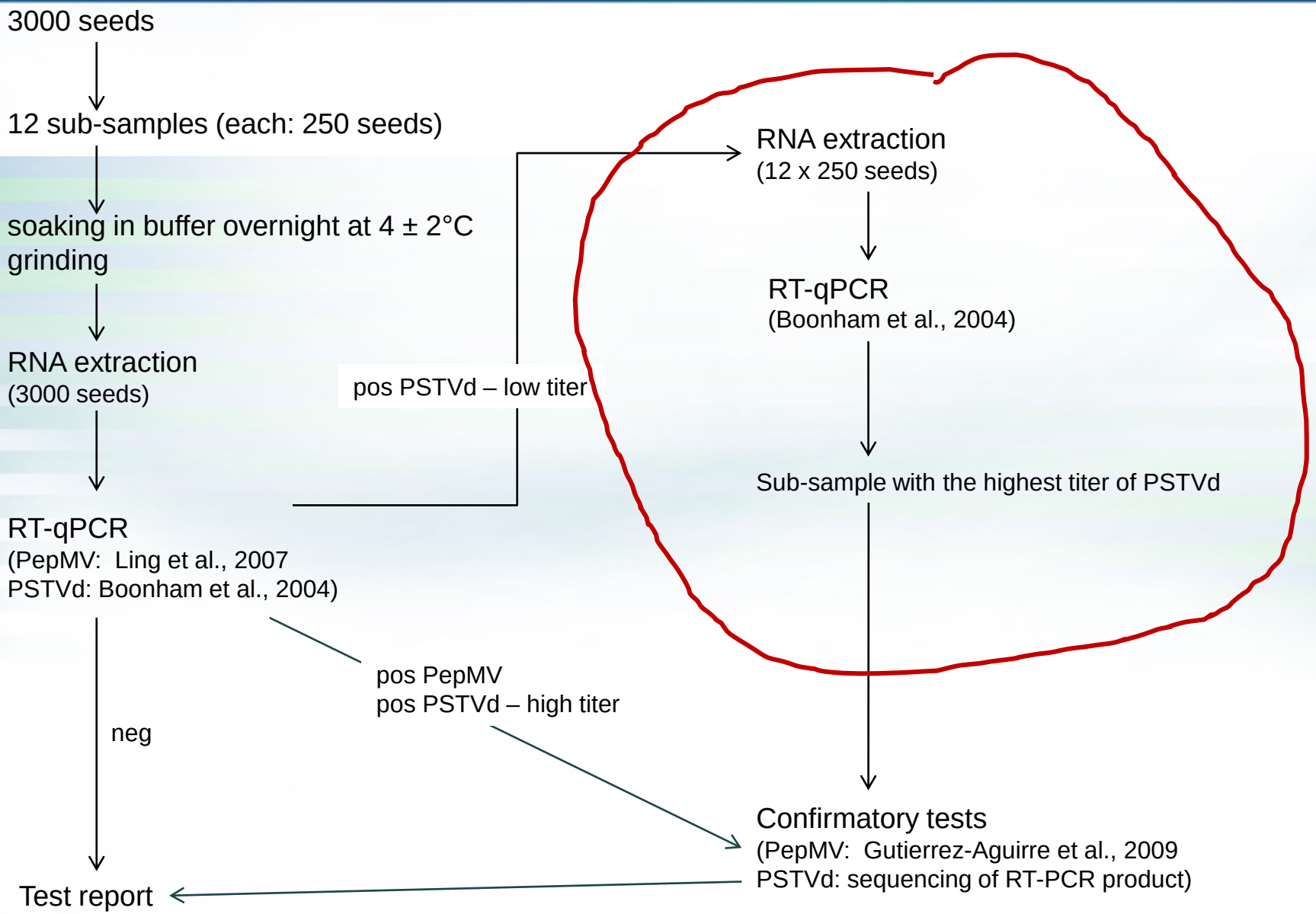
		Screening test		Confirmatory test
Artificially infected seeds (infected : uninfected)		RT-qPCR (Boonham et al., 2004)	RT-qPCR (Monger et al., 2010)	RT-PCR + sequencing
Seed infected with high conc. of PSTVd RNA (Cq 16)	1:250	+ (Cq 25)	+	+
	1:1000	+ (Cq 27)	+	+
	1:3000	+ (Cq 29)	+	-/+
Seed infected with lower conc. of PSTVd RNA (Cq 24)	1:250	+ (Cq 35)	-	-
Healthy seeds		-	-	-

Diagnostics in practice: PSTVd

2012, 2013:
2 PSTVd + / 2 seed samples (verification for foreign lab)

2013-2014:
5 positive with RT-qPCR (4 PSTVd +, 1 ?) / 29 seed samples (analysis ordered by Slovenian PARS)

All positive seeds: from import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Diagnostics in practice: PSTVd

Example D19/14:

RT-qPCR (Boonham et al., 2004)

Sub-samples

1-12	+ (Cq 36)
1	-
2	-
3	+ (Cq 39)
4	-
5	-
6	+ (Cq 39)
7	-
8	-
9	+ (Cq 39)
10	+ (Cq 33)
11	-
12	-

Example D21/14:

RT-qPCR (Boonham et al., 2004)

Sub-samples

1-12	+ (Cq 37)
1	+ (Cq 37)
2	+ (Cq 40)
3	+ (Cq 37)
4	+ (Cq 39)
5	-
6	+ (Cq 36)
7	+ (Cq 36)
8	+ (Cq 40)
9	-
10	-
11	-
12	+ (Cq 40)

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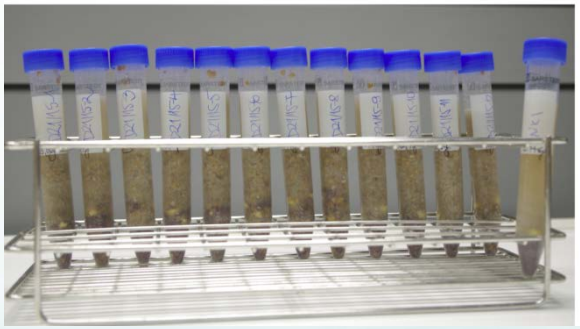
Optimisation:
seed sample preparation, RNA extraction



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RNeasy Plant Mini Kit (Qiagen)

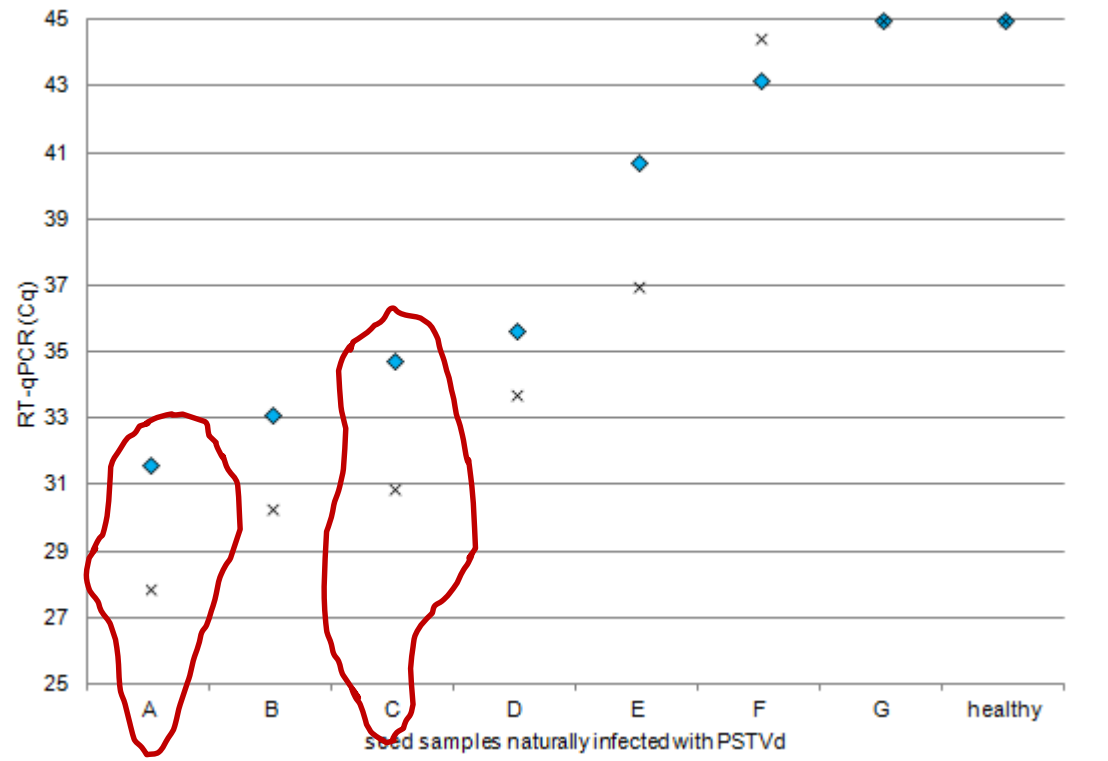
Trizol LS (Invitrogen) + MaxTract High density (Qiagen)

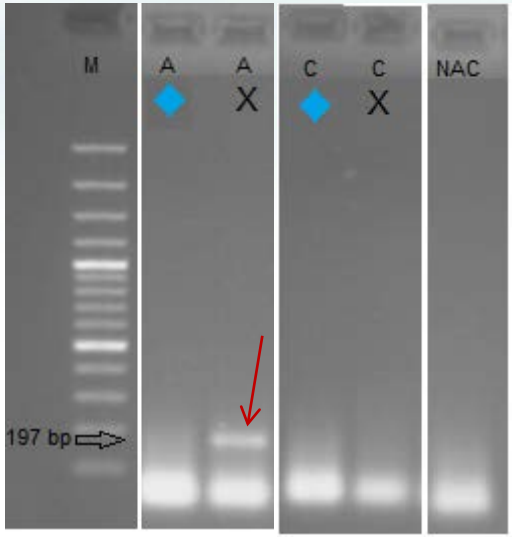
QIAmp Viral RNA Mini Kit (Qiagen)

Optimisation: RNA extraction (results)

- ◆ Rneasy Plant Mini Kit (Qiagen)
- X QIAamp viral RNA Mini Kit (Qiagen)

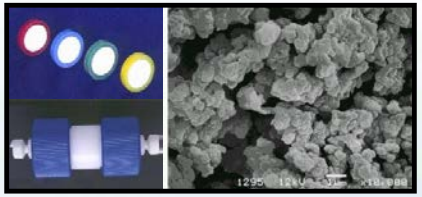
(~Trizol LS (Invitrogen) + MaxTract High density (Qiagen))





Optimisation: a method for concentrating PSTVd from the seed extract

❖ **Monolithic chromatography (Convective Interaction Media® (CIM) monoliths):**
+ fast and efficient way to concentrate highly diluted target



✓ PSTVd was concentrated from one-litre-water samples by at least two orders of magnitude
(Rušić et al., Journal of Chromatography A, 2013: 129-136)

⊗ Seed extract: polysaccharides disturb the flow through CIM

❖ **A method that is based on binding to positively charged beads**

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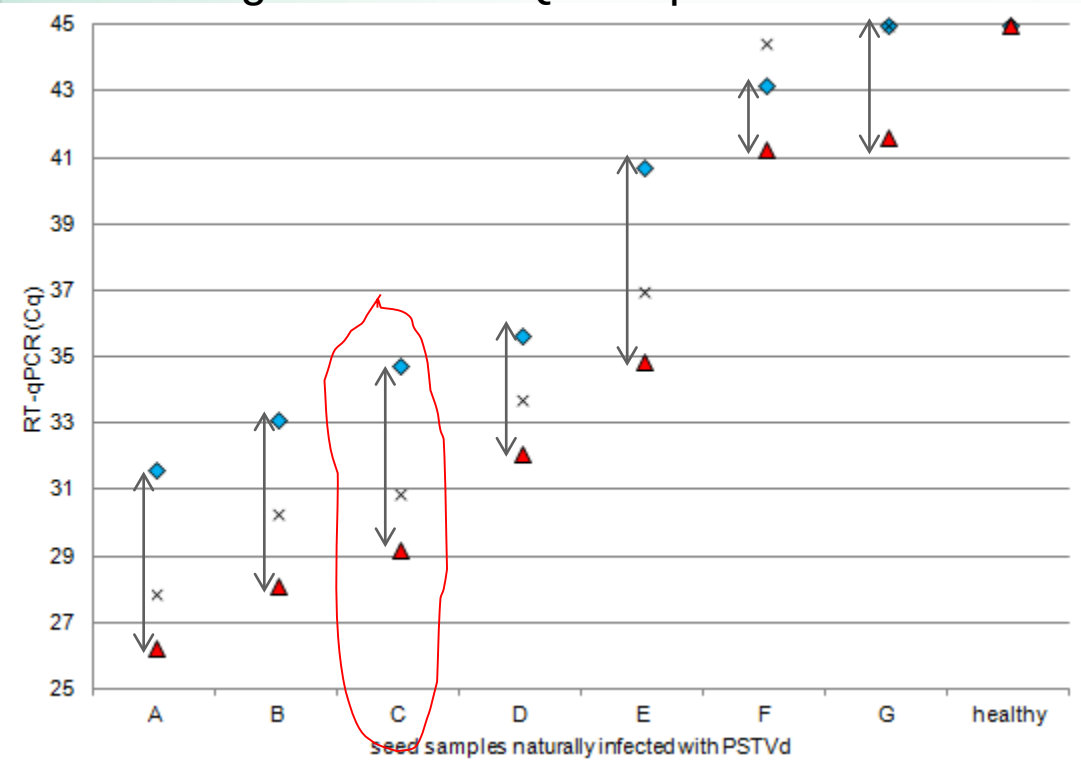
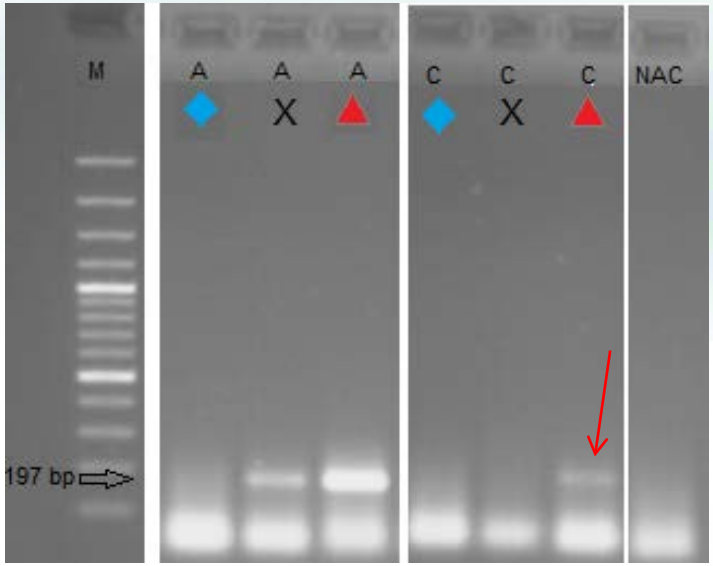
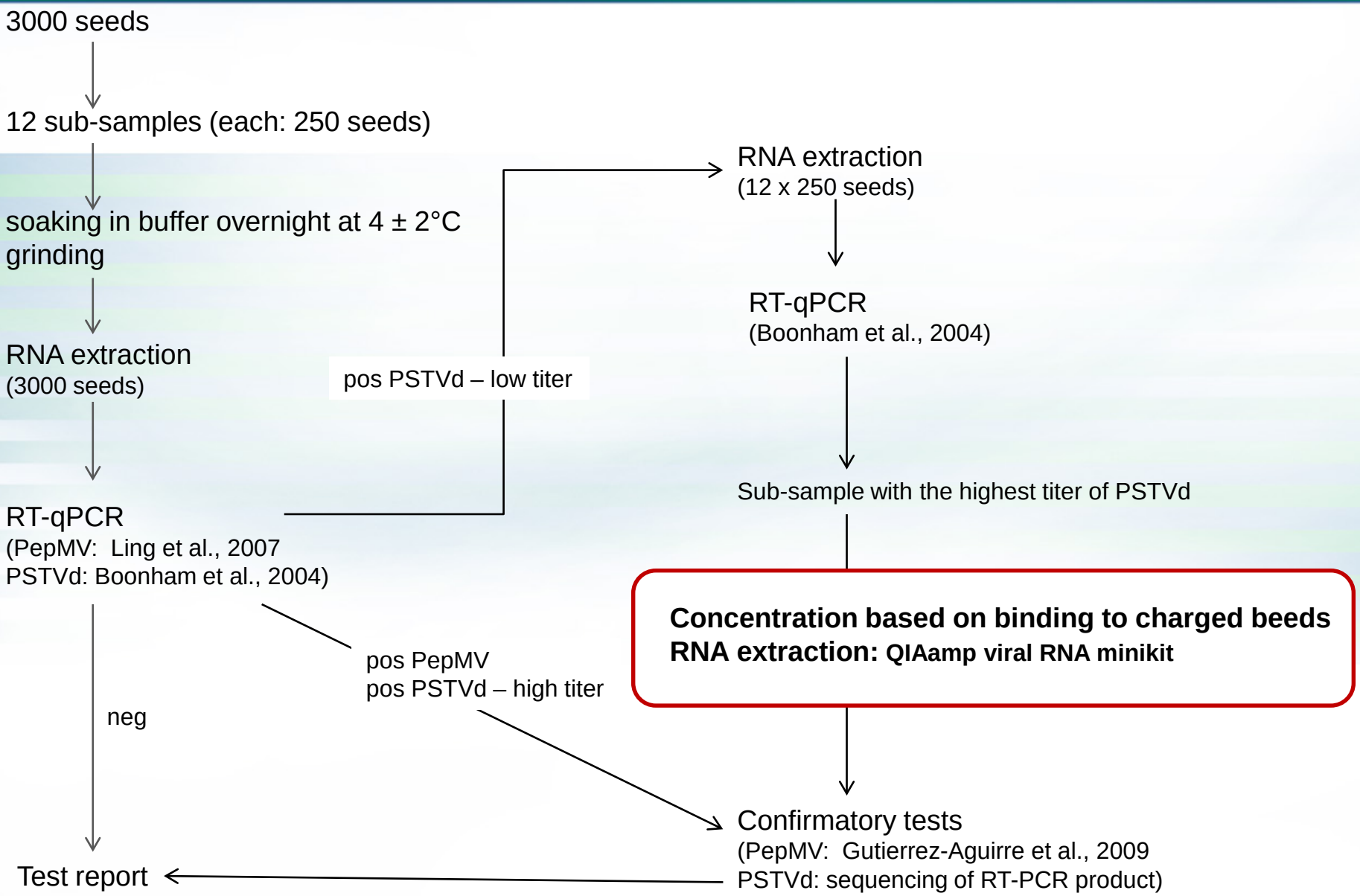
Optimisation: RNA extraction + concentration (results)

- Rneasy Plant Mini Kit (Qiagen)
- X QIAamp viral RNA Mini Kit
- concentration based on binding to positively charged beads + QIAamp viral RNA Mini Kit



2-6 Cq

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Confirmatory tests
(PepMV: Gutierrez-Aguirre et al., 2009
PSTVd: sequencing of RT-PCR product)
Test report

Conclusions

- For concentrating PSTVd from the seed extract, we have developed a new easy to use and efficient method based on binding to positively charged beads.

Acknowledgement

- Slovenian phytosanitary administration