REFERENCE PROTOCOL NAKTUINBOUW

Real-time RT-PCR (RT TaqMan PCR) for pospiviroids (Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), Pepper chat fruit viroid (PCFVd), Potato spindle tuber viroid (PSTVd), Tomato apical stunt viroid (TASVd), Tomato chlorotic dwarf viroid (TCDVd) en Tomato planta macho viroid (TPMVd) on seeds of pepper (*Capsicum annuum*), eggplant (*Solanum melongena*) and sticky nightshade (*Solanum sisymbriifolium*).

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Version:	5.0
Date:	19-03-2026
Validation:	Validated

1. Objective

To detect the absence or presence of potentially relevant pospiviroidae (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) in pepper-, eggplant- or sticky nightshade seeds by isolation of RNA followed by RT TaqMan PCR.

2. Principle

RNA from seed extract of pepper-, eggplant- or sticky nightshade seeds is isolated and purified with a kit using KingFisher. The possible presence of viroid RNA is demonstrated by means of RT TaqMan PCR using selective sets of primers and labeled TaqMan probes. Each subsample is spiked with DLVd to monitor the performance of RNA extraction and RT TaqMan PCR.

3. Abbreviations

cDNA	Complementary DNA
CEVd	Citrus exocortis viroid
CLVd	Columnea latent viroid
Ct-value	"Cycle threshold" value (number of PCR cycli till reaching the threshold)
DLVd	Dahlia latent viroid (spike viroid)
GH+ buffer	Guanidine–hydrochloride extraction buffer
KF	King Fisher
NAC	Negative amplification control (No template control)
NIC	Negative isolation control (tomato seeds process control)
PAC	Positive amplification control
PCFVd	Pepper chat fruit viroid
PIC	Positive isolation control (PSTVd contaminated tomato seed process control)
PSTVd	Potato spindle tuber viroid
RT TaqMan	Reverse transcriptase Taqman
TASVd	Tomato apical stunt viroid
TCDVd	Tomato chlorotic dwarf viroid
TPMVd	Tomato planta macho viroid

4. Materials

Equipment

Biorad CFX 96 or CFX Opus 96 PCR apparatus
Geno Grinder SPex Sample Prep. P. 2010
KingFisher Flex 96 (ThermoFisher Scientific)
Seed shaker (TeaL Agro Technologies)
Thermal mixer

Reagentia/buffers

DLVd stock (SPIKE)
DTT Sigma Aldrich
GH+ buffer (see appendix)
Primers and probes (see appendix)
Positive RNA controls (PAC)
PC/NC seed (PIC/NIC)
Sbeadex Maxi Plant Kit, 960 samples (LCG genomics, Cat. No. NAP41632)
TaqPath™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems) (or comparable, provided it is validated)

Consumables

50 ml tubes (Greiner 227261, Eppendorf 0030122178 or comparable)
Hard-Shell® Low-Profile, Thin-Wall, Skirted 96-Well PCR Plates, White Well from Biorad (or comparable, provided it is validated)
KingFisher 96 tip comb for DW magnets, (ThermoFisher Scientific, Cat. No. 97002534 or comparable, provided it is validated)

KingFisher 96 KF plate, 200µL (ThermoFisher Scientific, Cat. No. 97002540 or comparable, provided it is validated)
KingFisher deepwell 96 plate, (ThermoFisher Scientific, Cat. No. 95040450 or comparable, provided it is validated)
Microseal®'B' Adhesive Seals Optical van Biorad (or comparable)
Stainless Steel Grinding Balls (diameter 14 mm)

5. Method

5.1 Safety and warnings

Viroids can be present in very high concentrations in plant tissue and cross-contamination is a possibility. Accuracy of operations is important to reduce the chance of cross-contamination. Wear gloves and use pipets with filter tips at all times. GH+ buffer contains guanidine hydrochloride, which forms very dangerous gases when in contact with chlorine.

5.2 Execution

5.2.1a Preparation of pepper-, eggplant- or sticky nightshade seed samples (Geno Grinder method)

1. Prepare subsamples;
    a. Weigh per sample pepper or eggplant-seeds 6 subsamples of 500 seeds or the required number of subsamples of 400 seeds (conform order).
    b. Weigh per sample sticky nightshade-seeds 3 subsamples of 1000 seeds or the required number of subsamples of 400 seeds (conform order).
2. Transfer each subsample over in a 50 ml tube
3. Add 14 mm metal bead to each 50 ml tube.
4. Per series include one TASVd/CEVd/CLVd contaminated pepper seed subsample (PIC) and at least one negative seed subsample (NIC with Ct >35).
5. Position tubes upside down in Geno Grinder. Grind seeds with Geno Grinder (7 min. at 1,500 rpm).
6. Spin the 50 ml tubes in a centrifuge for 5 min. at minimum of 5,000 g.
7. Calculate the required amount of GH+ extraction buffer based on the total number of subsamples and add the right amount of a DLVd spike stock solution into the GH+ extraction buffer. DLVd is spiked to monitor inhibition during RNA extraction and RT-qPCR.
8. Open 50 ml tubes carefully and add 20 ml of GH+ buffer with DLVd spike to each tube.
9. Shake closed tubes with force (vortexing is not sufficient) during 10 seconds.
10. Centrifuge the 50 ml tubes with seed extract for 5 min. at 5,000 g.
11. Turn on the thermal mixer (setting: 65°C, 850 rpm) so that it can preheat.
12. For subsamples with 400 seeds transfer 1 ml extract (supernatant) carefully to a coded reaction tube containing 30 µl of DTT solution (5 M) and mix by vortexing.
13. Transfer for subsamples of 500 seeds 500 µl extract (supernatant) per submonster carefully to a coded reaction tube. Always pool two subsamples of 500 µl together in one coded reaction tube containing 30 µl of DTT solution (5 M).
14. Vortex the tubes thoroughly for at least 10 seconds.
15. Incubate the subsamples in for 15 minutes in the thermal mixer at 65 °C and 850 rpm.
16. Directly continue further with RNA-isolation.

5.2.1b Preparation of pepper seed samples (seed shaker method)

1. Weigh out the required number of sub-samples of 500 or 400 seeds per sample (according to the test request).
2. Transfer each sub-sample into a 50 mL tube.
3. Add one 14 mm stainless steel grinding ball to each 50 mL tube.
4. Include one positive infected control (PIC) and one negative infected control (NIC) per sample series.
5. Place the tubes upside down in the Seed Shaker. Grind the seeds using the Seed Shaker for 7 minutes at 1,300 rpm.
6. Centrifuge the 50 mL tubes containing the ground seeds for 5 minutes at a minimum of 5,000 × g.
7. Spike the total required volume of GH+ extraction buffer with concentrated DLVd stock.
8. Carefully open the lids of the 50 mL tubes and add 20 mL of GH+ buffer containing the DLVd spike to each tube.
9. Shake the tubes vigorously for 10 seconds (vortexing alone is not sufficient).
10. Centrifuge the tubes containing the seed extract for 5 minutes at a minimum of 5,000 × g.
11. Switch on the thermomixer and allow it to preheat to 65 °C and 850 rpm.
12. For sub-samples containing 300–400 seeds, carefully transfer 1 mL of seed extract (supernatant) per sub-sample into a labeled microtube containing 30 µL DTT solution (5 M).
13. For sub-samples containing 401–500 seeds, carefully transfer 500 µL of seed extract (supernatant) into a labeled microtube. Pool two 500 µL aliquots into the same labeled microtube (final volume 1 mL) containing 30 µL DTT solution (5 M).

14. Vortex the tubes thoroughly for at least 10 seconds.
15. Incubate the sub-samples in a thermomixer for 15 minutes at 65 °C and 850 rpm.
16. Proceed immediately with RNA isolation.

5.2.2 RNA isolation

1. Use the "Sbeadex Maxi Plant Kit" for RNA isolation.
2. Centrifuge the lysed seed extract containing reaction tubes for 10 minutes at 16.000 g, 4ºC.
3. Code and fill in the plates as indicated in Table 1.
4. Transfer 250 µl of the supernatant (avoid pellet!) from the subsamples, the NIC seed and PIC seed into the binding plate.
5. Store the remainder of the extract and the extract controls at -20 °C until the test is completed.
6. Place all the plates in the correct position in the KF unit.
7. Select and start the KF7 program on the KingFisher Flex 96 (Table 2).
8. When the isolation is finished cover the elution plate with foil sticker and store the elution plate on ice.
9. Continue directly with PCR or store purified RNA at -20 °C.

Table 1. Coding and composition of KF plates

Type of plate	Name	Composition
KF deep well 96 plate	Binding	600 µl binding buffer PN (green)
		50 µl Sbeadex particle suspension (white)
KF deep well 96 plate	Wash 1	600 µl Wash buffer PN1 (red)
KF deep well 96 plate	Wash 2	600 µl Wash buffer PN1 (red)
KF deep well 96 plate	Wash 3	600 µl Wash buffer PN2 (yellow)
KF 96 plate	Elution	100 µl Elution buffer PN (black)

Table 2. King Fisher Flex 96 program KF7

Step	Plate	Steps per plate
Tip 1	Tipholder	1. Remove tip comb from this plate (96 DW tip comb) 2. Release tip comb in plate 2 “Wash 1”
Binding	Plate 1 “Cell lysate”	1. Fast mixing, 10 min. 2. Collect beads, 3 x 10 sec.
Wash 1	Plate 2 “Wash 1”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 2	Plate 3 “Wash 2”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 3	Plate 4 “Wash 3”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Elution	Plate 5 “Elution”	1. Release beads, bottom mix, 1 min. 2. Preheat and fast mixing at 65ºC, 10 min. 3. Collect beads, 5 x 10 sec.

5.2.3 TaqMan RT-PCR

NB *Work on ice as much as possible and prevent prolonged exposure of probes to light. Wear clean lab coat and gloves to minimize the risk of cross-contamination.*

1. Prepare the TaqMan RT- PCR mixes (Table 3a, 3b, 3c and 3d).
2. Transfer 22 µl PCR mix into a PCR plate.
3. Pipette 3 µl of the purified RNA sample in the 22 µl TaqMan RT- PCR mixtures.

4. In each run, include a NAC (no-template control) and PAC (positive RNA control).
5. Cover the PCR plate after adding the RNA.
6. Spin the PCR plate to center the reagents.
7. Run the RT TaqMan RT- PCR (Table 4).

Tabel 3a. PCR mix PSTVd, TCDVd, TPMVd (FAM), PCFVd (VIC) and DLVd spike (Texas Red)

PCR mix	**1 reaction**	
RNase-free water	14,75	µl
TaqPath ™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems)	6,25	µl
Pospi A primer/probemix (#89)	1,0	µl
Subtotal	22,0	µl
Sample (RNA)	3,0	µl
Total	25,0	µl

Tabel 3b. PCR mix CEVd, CLVd (FAM) and DLVd (spike)(Texas Red)

PCR mix	**1 reaction**	
RNase-free water	14,75	µl
TaqPath ™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems)	6,25	µl
Pospi B primer/probemix (#90)	1,0	µl
Subtotal	22,0	µl
Sample (RNA)	3,0	µl
Total	25,0	µl

Tabel 3c. PCR mix TPMVd (FAM) and Nad5 (Texas Red)

PCR mix	**1 reaction**	
RNase-free water	14,75	µl
TaqPath ™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems)	6,25	µl
Pospi C primer/probemix (#91)	1,0	µl
Subtotal	22,0	µl
Sample (RNA)	3,0	µl
Total	25,0	µl

Tabel 3d. PCR mix TASVd (FAM)

PCR mix	**1 reaction**	
RNase-free water	14,75	µl
TaqPath ™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems)	6,25	µl
Pospi D primer/probemix (#92)	1,0	µl
Subtotal	22,0	µl
Sample (RNA)	3,0	µl
Total	25,0	µl

Tabel 4. RT-TaqMan program CFX96 (version 2 or higher) for mix A-D

	temperature	**time**
hold	50°C	10' 00"
hold	95°C	3' 00"
40 cycli	95°C	0' 10"
	60°C (+ plate read)	1' 00"

6. Evaluation and interpretation

6.1 Evaluation test result

- Turn on FAM, VIC and TR signal for all 96 wells in “view/edit plate”.
- Use single threshold setting (RFU 200).
- Check shape of curve (S-shape) for positive samples. Compare, if necessary, with PIC seed.
- Check atypical planar curves (not-S-shaped curve) and curves just above the threshold (considered as noise). In case of doubt always check with responsible person.
- Log the performance of PCs in control charts and check whether they are within the set limits. Contact the person ultimately responsible if this is not the case. Monitor PIC performance over time to maintain an overview of the assay's reliability.
- See decision matrix (Table 5a, b, c, and d) for interpretation of the results of samples. Based on the signals in different channels, an indication of the identity of a viroid can be obtained.

6.2.1 Validity of test result

- Result are valid when Ct value of spike DLVd <32, unless Ct PSTVd/TCDVd/TPMVd or CEVd/CLVd <32. Nad5 Ct >32 is acceptabel only if Ct value of spike DLVd <32. (Contact the responsible person).
- Results may only be issued if the positive controls give a clear signal (Ct PIC and PAC).
- Results may only be issued if Ct in the negative control samples > 35.
- Contact the responsible person when the results differ from the expected.

6.3 Decision matrix (subsample level)

Table 5a. Decision matrix PCR mix 3a: PSTVd, TCDVd, TPMVd (FAM), PCFVd (VIC) and DLVd (spike-TR)

Ct FAM	Ct VIC	Ct TR	
>32	>32	≤32	PSTVd, TCDVd, TPMVd (part of strains) [a] and PCFVd not detected
>32	≤32	n.a.	PSTVd, TCDVd and TPMVd not detected PCFVd detected
≤32	>32	n.a.	PSTVd/TCDVd and/or TPMVd detected PCFVd not detected
≤32	≤32	n.a.	PSTVd/TCDVd and/or TPMVd detected PCFVd detected[b]
>32	>32	>32	Not valid/repeat

Table 5b. Decision matrix PCR mix 3b: CEVd, CLVd (FAM) and DLVd (spike-TR)

Ct FAM	Ct TR	
>32	≤32	CEVd and CLVd not detected
≤32	n.a.	CEVd and/or CLVd detected
>32	>32	Not valid/repeat

Table 5c. Decision matrix PCR mix 3c: TPMVd (FAM) and Nad5 (TR)

Ct FAM	Ct TR	
>32	≤32	TPMVd (part of strains) not detected
≤32	n.a.	TPMVd detected
>32	>32	Check DLVd spike (mix A and mix B) since DLVd spike is more relevant. Contact responsible person.

Table 5d. Decision matrix PCR mix 3d: TASVd (FAM)

Ct FAM	
>32	TASVd not detected
≤32	TASVd detected

[a] TPMVd not detected when mix A and mix C are negative.
[b] Signal might leak from VIC to FAM channel when PCFVd load is high. Use singleplex Boonham to determine whether PSTVd/TCDVd and /or TPMVd are present.

7. Literature and instructions

Literature:

- Bakker, D., Bruinsma, M., Dekter, R.W., Toonen, M.A.J., Verhoeven, J. Th. J. and Koenraadt, H.M.S. 2015. Detection of PSTVd and TCDVd in seeds of tomato using real-time RT-PCR. EPPO Bulletin. Volume 45, Issue 1, pages 14–21,
- Boonham, N., L. González-Pérez, ,M.S. Mendez, E. Lilia Peralta, A. Blockley, K. Walsh, I. Barker & R.A. Mumford (2004). Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. Journal of Virological Methods 116:139-146.
- Botermans, M., van de Vossenberg, B. T. L. H., Verhoeven, J. Th. J.,Roenhorst, J. W., Hooftman, M., Dekter, R. & Meekes, E. T. M. (2013) Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. J. Virol. Methods 187, 43–50.
- Koenraadt, H., Jodlowska, A., van Vliet, A. & Verhoeven, K. (2009) Detection of TCDVd and PSTVd in seeds of tomato. Phytopathology 99, S66.
- Harrie Koenraadt, Agata Jodlowska, Maaike Bruinsma, Michel Ebskamp, Ko Verhoeven and Annelien Roenhorst. (2017) Biological relevance of the detection of pospiviroids on pepper and tomato seeds (poster at APS, San Antonio, Tx)
- Menzel, W., Jelkmann, W., and Maiss, E. (2002) Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99: 81-92.
- Monger, W., Tomlinson, J., Boonham, N., Marn, M.V., Plesko, I.M., Molinero-Demilly,V., Tassus, X., Meekes, E., Toonen, M., Papayiannis, L., Perez-Egusquiza, Z., Mehle, N., Jansen, C., Nielsen, S.L., (2010) Development and inter-laboratory evaluation of real-time PCR assays for the detection of pospiviroids. J. Virol. Methods 169, 207–210.
- Mumford, R.A., A.L. Skelton, N. Boonham, K.I. Posthuma, M.J. Kirby, & A.N. Adams (2001) The Improved Detection of Strawberry Crinkle Virus Using Real-Time RT-PCR (TaqMan®). Acta Horticulturae 656: 81-86.

Manuals:

- Sbeadex maxi plant Kit (LCG genomics)
- TaqPath™ 1-Step Multiplex Master Mix (4x) (Applied Biosystems)

8. History and revisions

- 20-12-2013 Version 1.0 - ~~Protocol based on ISO17025 accredited PSTVd /TCDVd seed assay (SPN-V003, version 3.1) for tomato~~. Overnight soaking of seeds was reduced to 30-60 minutes to limit break down of viroid. Introduction of DLVd spike to replace endogenous Nad5 target since amount of Nad5 RNA is highly variable (14-40) in the seed matrix. Use of GH+ extraction buffer instead of PN1 extraction buffer (LGC) since degradation in GH+ RNA extraction buffer is much less than in PN1 extraction buffer leading to a higher PSTVd sensitivity. Specification of method to purify RNA for NVWA. RNeasy purified RNA in general give better sequences in comparison with Sbeadex purified RNA although high Ct values samples are still problematic due to low sensitivity of sequencing method. Use of several new multiplex RT TaqMans to detect additional Pospiviroidae as CEVd, CLVD, PCFVd, TASVd and TPMVd.
- 03-02-2014 Version 1.1 - Correction numbering primer sets in appendices. 201d instead 201c, at 349 / mc instead of 249 at / c and 350a, 350b instead 250a, 250b.

- 21-04-2015 version 1.2 - 5.2.1: Specification of spike: “10 µl DLVd/100 ml GH+ buffer”.
- 15-02-2017 version 2.0 - Addition of DTT to seed extract, adaptations in Kingfisher program, replacement PCR mix, adaptation PCR program, MPVd replaced by TPMVd due to new taxonomic insights. Several textual changes. DLVd spike specification removed.
- 30-10-2017 v2.1 – Correction literature references in primer table in appendix.
- 05-03-2019 v2.2 – Several textual changes; introduction 1µl PCR mixes.
- 02-01-2020 v2.3 - In description of objective "possible" was removed due to change in Quarantine status. Table 5a: PSTVd/TCDVd[b] and/or TPMVd: “suspected” replaced with “detected”.
- 06-03-2020 v3.0 – Enlargement of scope: eggplant- and sticky nightshade seeds added. Differentiated between subsample size per matrix. Tabel 5a: “suspected” replaced with “detected”. “b PSTVd suspected extracts are send to the Dutch NPPO for sequence analysis” removed. Correction 5.2.1-3 limit NIC >35.
- 15-05-2020 v3.1 – Added 5.2.1-7: “10 ml (pepper and eggplant) or 20 ml (sticky nightshade seed) GH+ buffer”. 6.2 “Contact the responsible person when a seed sample is suspect” removed.
- 10-11-2020 v3.2 – Correction in History and Revision version 1.0; first sentence crossed out.
- 31-3-2022 v3.3 – Textual changes.
- 01-07-2025 v4.0 – Centrifugation step moved to RNA isolation; point 5.1 adjusted. Ultraplex replaced by TaqPath in materials, Tables 3a to 3d and in manuals. RNA input decreased from 6µl to 3µl and increased water volume Table 3 and section 5.2.3. Textual changes to harmonize with other virus SE-PCR protocols.
- 5-9-2025 v4.1 - Textual changes.
- 19-03-2026 v5.0 – Introduction of the Seed Shaker as a grinding method for pepper seed samples; reintroduction of the vortex step after DTT addition; further specification of PIC performance criteria in the evaluation of test results; further specification of the DLVd spike signal in positive samples; various editorial and textual revisions.

9. Appendices

GH+ extraction buffer (6M)	Adjust to 1 liter demiwater	
guanidine-hydrochloride (harmful)	573	g
NaAC-buffer (4M)	50	ml
EDTA (di-natrium)	9.3	g
PVP-10	25	g

NaAc buffer (4M)	Adjust to 1 liter with demiwater	
NaAC (CH_3COONa)	328 g	g
Check/adjust pH to 5.2		

Primer	**No. Naktuinbouw primer collection**	**Sequence**	**Lit. ref.**	**End concentration (nM)**
PSTV-231F1	201a	GCCCCCTTTGCGCTGT	1	300
PSTV-296R	201b	AAGCGGTTCTCGGGAGCTT	1	300
PSTV-251T-	201d	6FAM-CAGTTGTTTCCACCGGGTAGTAGCCGA-BHQ1	1	200
DaVd1-FT	320a	GCTCCGCTCCTTGTAGCTTT	2	300
DaVd1-RT	320b	AGGAGGTGGAGACCTCTTGG	2	300
DaVd1-P	320c	Texas red-CTGACTCGAGGACGCGACCG-BHQ2	2	200
TPMVd-F1	350a	AAAAAAGAATTGCGGCCAAA	3	300
TPMVd-R	350b	GCGACTCCTTCGCCAGTTC	3	300
pUCCR2	307i	6FAM-CCGGGGAAACCTGGA-NFQ-MGB	4	200
CLVd-F	279a	GGTTCACACCTGACCCTGCAG	5	300
CLVd-F2	279b	AAACTCGTGGTTCCTGTGGTT	5	300
CLVd-R	279c	CGCTCGGTCTGAGTTGCC	5	300
CLVd-P	279d	6FAM-AGCGGTCTCAGGAGCCCCGG-BHQ1	5	200
CEVd-F2-	280a	CTCCACATCCGRTCGTCGCTGA	5	300
CEVd-R2-	280b	TGGGGTTGAAGCTTCAGTTGT	5	300
CEVd-P2-	280c	6FAM-CCCTCGCCCGGAGCTTCTCTCTG-BHQ1	5	200
TASVd-F2-	281a	CKGGTTTCCWTCCTCTCGC	5	300
TASVd-R2-	281b	CGGGTAGTCTCCAGAGAGAAG	5	300
TASVd-P2-	281c	6FAM-TCTTCGGCCCTCGCCCGR-BHQ	5	200
PCFVd-F	349a	TCTTCTAAGGGTGCCTGTGG	6	300
PCFVd-R	349b	GCTTGCTTCCCCTTTCTTTT	6	300
PCFVd-	349c	VIC-CTCCCCCGAAGCCCGCTTAG-BHQ1	6	200
nad5 F	151a	GATGCTTCTTGGGGCTTCTTGTT	7	300
nad5 R	151b	CTCCAGTCACCAACATTGGCATAA	7	300
nad5-Probe	151d	Texas red-AGGATCCGCATAGCCCTCGATTTATGTG-	4	200

1: Boonham et al. (2004), 2: Naktuinbouw, 3: Naktuinbouw, 4: Botermans et al. (2013), 5: Monger et al. (2010), 6: Naktuinbouw, 7: Menzel et al (2002) and Monger et al. (2010).

Composition primer and probe mixes A, B, C and D		
Mix 89	Pospi primer/probe mix A	201a, 201b, 349a, 349b, 320a, 320b, 201d, 349c, 320c
Mix 90	Pospi primer/probe mix B	280a, 280b, 279a, 279b, 279c, 320a, 320b 280c, 279d, 320c
Mix 91	Pospi primer/probe mix C	350a, 350b, 151a, 151b, 307i, 151d
Mix 92	Pospi primer/probe mix D	281a, 281b, 281c