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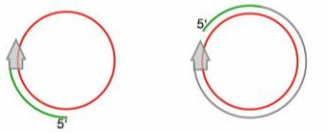
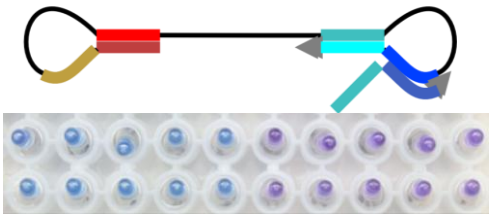
Fachgebiet für Obstbau

Development of an isothermal nucleic acid amplification protocol for high-throughput monitoring of *Plum pox virus* infection in stone fruit production





Johannes Hadersdorfer

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Development of an isothermal nucleic acid amplification protocol for high-throughput monitoring of *Plum pox virus* infection in stone fruit production

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Agrarwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. W. Schwab
Prüfer der Dissertation:	1. Univ.-Prof. Dr. D. R. Treutter
	2. Univ.-Prof. Dr. T. Fischer (Ludwig-Maximilians-Universität München)

Die Dissertation wurde am 02.08.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 05.12.2012 angenommen.

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Abbreviations

℃	degree Celsius, centigrade
µl	microlitre
3SR	self-sustained sequence replication
3WJ	three way junction
Å	Ångström
ACLSV	*Apple chlorotic leaf spot virus*
AMV	avian myeloblastosis virus
APLPV	*American plum line pattern virus*
ApLV	*Apricot latent virus*
ApMV	*Apple mosaic virus*
b	base
BART	bioluminescent assay in real-time
BIP	backward inner primer
bn	billion
bp	basepair
BSA	bovine serum albumin
Bst	*Bacillus stearothermophilus*
C2CA	circle to circle amplification
cDNA	complementary DNA
CI	cylindrical inclusion protein
CLP	circularizable probe
CP	coat protein
CRCA	cascade rolling circle amplification
C_Q	quantification cycle
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
ELISA	enzyme linked immuno sorbent assay
ELOSA	enzyme linked oligosorbent assay
ERCA	exponential rolling circle amplification
ET SSB	extreme thermostable single stranded binding pro
FIP	forward inner primer
FITC	fluorescein isothiocyanate
FWD	forward
GFP	green fluorescent protein
h	hour
HCPro	helper component proteinase
HDA	helicase dependent amplification
HNB	Hydroxynaphthol blue
HP	hairpin
HPLC	High-performance liquid chromatography
HRCA	hyperbranched rolling circle amplification
IGSS	immunogold silver staining
IMDA	isothermal multiple displacement amplification
kb	kilobase
kDa	kiloDalton
LAMP	loop-mediated isothermal amolification
LFD	lateral flow device
LIMA	Linear target isothermal multimerization and amplif
LNA	locked nucleic acid
M	molar
MAb	monoclonal antibody
MDA	multiple displacement amplification
mg	miligram
mill.	million
min	minute
M-MLV	Moloney murine leucemia virus
nad5	NADH dehydrogenase subunit 5
NASBA	nucleic acid sequence based amplification
NI	nuclear inclusion
nt	nucleotides
NTC	no template control
NTP	nucleotide triphosphat
ORF	open reading frame
PAGE	polyacrylamid gel electrophoresis
PBNSPaV	*Plum bark necrosis stem pitting associated virus*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	*Prune dwarf virus*
PLP	Padlock probe
PNA	peptide nucleic acid
PPV	*Plum pox virus*
PNRSV	*Prunus necrotic ringspot virus*
PVP	polyvinyl pyrrolidone
RAM	ramification amplification
Rbcl	ribulose 1,5-bisphosphate carboxylase
RCA	rolling circle amplification
RCR	rolling circle replication
REV	reverse
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPA	recombinase polymerase amplification
rpm	rounds per minute
RT	reverse transcription
SDA	strand displacement amplification
sec	second
SMAP2	smart amplification process 2
SMART	signal mediated amplification of RNA technology
SmartAmp2	smart amplification process 2
SNP	single nucleotide polymorphisms
SPIA	single primer isothermal amplification
SSB	single stranded binding protein
ssDNA	single stranded DNA
Taq	*Thermus aquaticus*
TAS	transcription based amplification system
tHDA	thermophilic helicase dependent amplification
T_m	melting temperature
TMA	transcription mediated amplification
T_T	time treshold
UTR	untranslated region
VPg	potyviral virus genome-linked protein
WGA	whole genome amplification

I Introduction

Orchardists have to consider numerous aspects in fruit production to harvest high quality fruits. An important crop cultivation measure is pest management to maintain a high phytosanitary status of an orchard. Knowledge of the causal organism is necessary to fight the pests or to avoid the spread of the pests. Damages and diseases of trees are caused by mammals, mites, insects, nematodes, fungi, bacteria, phytoplasmas, viruses or viroids.

The fungus *Venturia inaequalis* and the bacterium *Erwinia amylovora* cause serious diseases in pome fruits known as apple scab and fire blight, respectively. The *Plum pox virus* (PPV) affects all important stone fruit varieties, especially European and Japanese plum, apricot and peach. The disease pattern which arose from PPV infection is called Sharka. It causes severe economic impacts in nearly all regions of the world where stone fruits are cultivated due to crop loss and eradication measures.

In the following, the Sharka disease is characterized with special emphasis on detection systems developed for the specific detection of PPV infection of plants. These systems can be clustered to three groups according to their underlying techniques, which are either biological, immunological or molecular biological. Each method has intrinsic advantages and drawbacks. In most cases, specialized laboratories have to perform these tests due to the complex techniques used for detection and the need for sophisticated equipment. As a consequence, the diagnosis is costly and time consuming. For high-throughput monitoring of orchards and nurseries to recognize PPV infection as early as possible, a fast, reliable and low cost detection system is needed, which can be used by the orchardists and nurserymen themselves.

A new detection system based on the isothermal amplification of nucleic acids has been developed, which allows for a fast, reliable and inexpensive analysis of samples derived from different plants as well as from different tissues, such isothermal approaches are presented in comparison to PCR analysis.

II Literature survey

II-1. The Sharka disease

II-1.1 Symptoms indicative for the Sharka disease

A symptom which is indicative for the Sharka disease is mottling of the leaves. The blotches can be lightly green coloured but also chlorotic. The shape of the blotches varies from single spots, streaks along the veins and ribbons across the leaf to rings (Fig. II-1-1 top). Vein clearing or deformation of the leaf blade can also be observed. The mottling is particularly visible in spring, whereas these symptoms are concealed in summer due to high temperatures (Atanasoff, 1935, EPPO, 2004).

Mosaic like mottling emerges on the fruits as well. The colour of the ring, arch or streak shaped blotches changes prematurely to blue. The mottling is accompanied by malformations like immersions and sinkings in most cases (Fig. II-1-1 middle). The fruit flesh can be depressed and discoloured (Fig. II-1-1 bottom). Affected fruits tend to drop before ripening (Atanasoff, 1935, EPPO, 2004).

Infected trees have a reduced number of leaves, have more shoots which die off and are more susceptible to winter frost (Atanasoff, 1935).

Each of these symptoms results in economic losses due to reduced photosynthesis rate (Neumüller, 2005) and reduced growth rate (Nemeth, 1994). However, it depends on the genotype, which symptoms arise upon PPV infection and how severe they are. Tolerant cultivars exhibit no or just few symptoms, although the crop yield is also reduced (Neumüller, 2005).

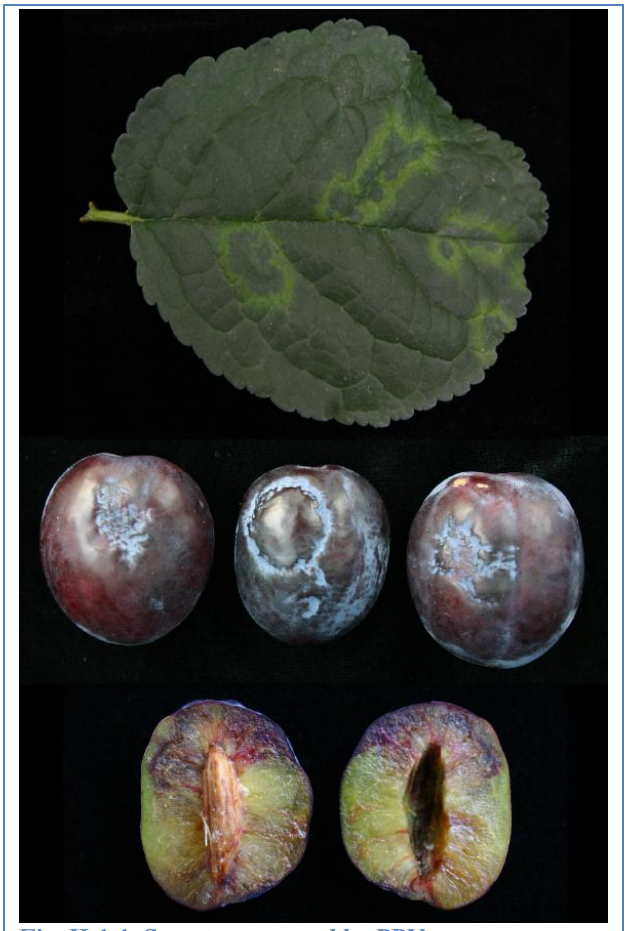


Fig. II-1-1: Symptoms caused by PPV
Ring shaped chlorosis on a leaf of the plum cultivar 'Elena' (top), mottling and immersions on fruits of 'Zwinbachers Frühe' (middle) and depressed and discoloured fruit flesh in a fruit of 'Harbella' (bottom)

II-1.2 Costs associated with the Sharka disease

During the 30 years prior to 2006 the estimated loss due to PPV infection leading to unmarketable fruits worldwide amounted to about 3.6 bn €, 5.4 bn €, 126 mill. € and 576 mill. € for apricot, European and Japanese plum and peach, respectively. Eradication programs caused costs of about 175 mill. € in Spain, in the USA, in Canada and in the EU. About 24.5 mill. € were granted for research on the Sharka disease and its causal agent (Cambra et al., 2006b).

II-1.3 *Plum pox virus* (PPV) - the causal agent of Sharka

The pythopathogen, which causes the Sharka disease, is the *Plum pox virus* (PPV) and is listed in the "Top 10 plant viruses in molecular plant pathology" (Scholthof et al., 2011). PPV is - among about 100 known viruses - a member of the genus *Potyvirus* (López-Moya et al., 2009). This genus along with the genera *Rymovirus*, *Macluravirus*, *Ipomovirus*, *Tritimovirus* and *Bymovirus* are united to the family *Potyviridae*.

Apart from Sharka, viral diseases of essentially all agricultural and horticultural crops can be attributed to a member of *Potyviridae*. Partially, these diseases are the most severe ones of a crop as the Sharka disease is the most important viral disease of stone fruits. *Potato virus Y* (PVY) is a threat to potato and *Wheat streak mosaic virus* (WSMV) to cereals (López-Moya et al., 2009).

The genome of *Potyviridae* consists of positive sense single stranded RNA. All genera have a single RNA molecule with the exception of *Bymovirus* which has two discrete molecules of RNA. The viral RNA is encapsulated by numerous units of the coat protein (CP). Natural vectors are aphids (*Potyvirus*, *Macluravirus*), mites (*Rymovirus*, *Tritimovirus*), whiteflies (*Ipomovirus*) and plasmodiophorida (*Bymovirus*) (López-Moya et al., 2009).

II-1.4 Occurrence of PPV hosts susceptible to PPV infection

The presence of Sharka has been recorded for most European and Mediterranean countries as well as for countries of the Middle East, Asia and the Americas (EPPO, 2006).

Important stone fruit species of the genus *Prunus* are natural host of PPV: *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina*. *P. avium*, *P. cerasus* and *P. dulcis* are casually infected (Atanasoff, 1935, Nemeth, 1994, EPPO, 2004). PPV also affects *P. cerasifera*, *P. glandulosa*, *P. insititia*, *P. spinosa* and *P. tomentosa* which represent wild and ornamental species as well as species used for rootstocks (Nemeth, 1994, EPPO, 2004).

Susceptibility was shown for further species of *Prunus* and for *Sorbus domestica* by artificial inoculation (EPPO, 2004). *Nicotiana benthamiana*, *N. glutinosa*, *Pisum sativum* and *Chenopodium foetidum* are herbaceous plants which could be artificially infected for research on PPV (EPPO, 2004, Nemeth, 1994).

Natural distribution of PPV is carried out in a non-persistent manner by aphids to nearby trees. Species known to be vectors are *Aphis craccivora*, *Aphis fabae*, *Aphis spiraecola*, *Brachycaudus cardui*, *Brachycaudus helichrysi*, *Hyalopterus pruni*, *Myzus persicae*, *Myzus varians* and *Phorodon humuli* (Nemeth, 1994, EPPO, 2004, Šubr and Glasa, 2008).

Long distance spread occurs by trading and propagation via grafting of infected plant material (EPPO, 2004).

II-1.5 Virion composition and genome structure

A PPV virion is rod shaped with 660-750 nm in length and 12-20 nm in width (Salvador et al., 2006).

The genomic RNA contains about 9,800 nt. It is attached to potyviral virus genome linked protein (VPg) at the 5'-end followed by

poly (A) tail at the 3'-end of the RNA (Salvador et al., 2006). The RNA encodes an open reading frame (ORF, Fig. II-1-2) which is translated into a 355.5 kDa polyprotein. This polyprotein is processed to ten proteins by three proteinases encoded by the virus itself. Two of these enzymes cleave at their respective C termini autocatalytically: P1 proteinase which depends on a plant cofactor and the helper component proteinase (HCPro). The cleavage of the remaining proteins is carried out by the C-terminal proteinase domain of the nuclear inclusion a (NIa) protein. In recent years a second ORF located in P3 coding sequence was found resulting in the protein P3N + PIPO which may be involved in pathogenicity (Fig. II-1-2; López-Moya et al., 2009).

The ORFs are enclosed by two untranslated regions (UTR). The UTR at the 5'-end has a length of about 150 nt and is involved in viral replication, viral fitness and pathogenesis. A function of the 3' UTR (~220 nt) is not known, yet (Salvador et al., 2006).

The first protein of the polyprotein contains a serine proteinase domain. Due to the RNA binding activity of P1 an involvement in RNA replication, translation or translocation is assumed. Another hypothesis states for a possible role in the specificity to the host because of the high variability of the sequence coding for P1 in analysed isolates (Salvador et al., 2006).

Besides the cysteine proteinase activity, the HCPro interacts with the CP, virions and aphid stylets indicating a role in the aphid transmission of the virus. It is also known that HCPro hinders the protective RNA silencing of plants (Salvador et al., 2006).

The function of P3 in PPV is not known. However, factors affecting the pathogenicity of PPV for different hosts are located in the P3 and 6K1 region. The latter one could take part in membrane integration due to a hydrophobic region. Complete cleavage of these two proteins seems not to be necessary for the virus viability (Salvador et al., 2006).

The cylindrical inclusion protein (CI) contains a nucleotide triphosphat (NTP) binding domain. It has NTPase and RNA helicase activity. Both of these activities are mandatory for RNA replication. In addition, CI participates in the movement of PPV via plasmodesmata (Salvador et al., 2006).

Similar to 6K1, 6K2 has a hydrophobic domain which links the RNA replication to the endoplasmic reticulum membranes (Salvador et al., 2006).

The NIa protein catalyses the cleavage of the polyprotein to mature proteins except P1 and HCPro. It accumulates in the nucleus of infected cells forming crystalline inclusions without a

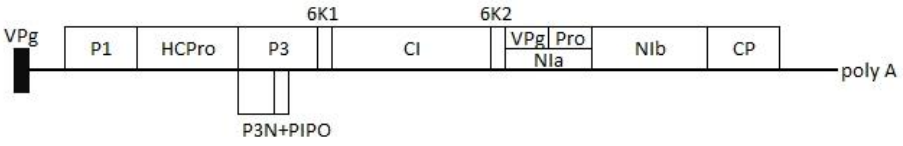


Fig. II-1-2: Genomic map of PPV
The genomic RNA contains two ORFs. The polyprotein by one ORF is cleaved to ten functional proteins (P1, HCPro, P3, 6K1, CI, 6K2, NIa which is further processed to VPg and Pro, NIb and CP). The second ORF is translated into the protein P3N + PIPO. The RNA is linked to the VPg protein at the 5'-end and to a poly A tail at the 3'-end (according to López-Moya et al. (2009)).

known function. The VPg protein which is linked to 5'-end of the RNA strand is derived from NIa. This protein eventually initiates the RNA replication and translation. It is also involved in long distance movement (Salvador et al., 2006).

The nuclear inclusion protein b (NIb) constitutes the RNA dependent RNA polymerase which catalyses the replication of the viral genome (Salvador et al., 2006).

The capsid is formed by numerous units of the coat protein (CP). The N- and C-terminal regions of the protein are exposed on the virion surface. A third domain is necessary for virus assembly and movement from cell to cell. The N-terminal domain is essential for aphid transmission by interacting with HCPro (Salvador et al., 2006). The capsid is made of 2,000 units of the CP and encloses the virus genome (EPPO, 2004).

P3N + PIPO, the protein encoded by the second ORF, is probably involved in the pathogenicity of the virus (López-Moya et al., 2009).

II-1.6 Breeding for PPV resistance

In general, PPV infected *P. domestica* trees do not recover from this disease. Exceptions are hypersensitive genotypes with a PPV infection being restricted to few cells (Neumüller, 2005). In apricots, recovery was recently reported (Karayiannis et al., 2010). Therefore, most measures to fight PPV are restricted to the prevention of infection and to minimizing economic losses. Useful measures are the application of insecticides against aphid vectors, the efficient and forceful eradication of infected trees in regions where PPV is not endemic, use of virus free plant material, and an optimal orchard management (Neumüller, 2005). The use of tolerant and quantitative resistant genotypes allows the cultivation of plums in regions with area-wide distribution of PPV. But these genotypes are sources of infection as symptoms can hardly be observed. Resistance based on hypersensitivity is the single reliable way to protect orchards against economic losses from Sharka (Neumüller, 2005). In hypersensitive genotypes which can both be used as scions or rootstocks infected cells die off and prevent the systemic spread of the virus. As a consequence, the major aim of the Weihenstephan plum breeding program is to obtain genotypes exhibiting hypersensitivity resistance accompanied by inheritance analysis for relevant traits. For detailed information concerning the hypersensitivity resistance the reader may be referred to Neumüller (2005).

II-2. PPV detection

The disease specific symptoms (Fig. II-1-1) are very good indicators for the presence of PPV. However, the symptom expression varies in the course of the year with clearly visible symptoms in spring. Due to increasing temperatures symptoms may become masked and hardly visible (Atanasoff, 1935, Kegler et al., 1998). Symptoms are better visible on young developing leaves than on mature leaves. Mixed infections with PPV and other pythopathogens can lead to atypical symptoms. Another problem constitutes for the uneven distribution of PPV within a plant (Morvan and Castelain, 1976, Wetzel et al., 1991b, Knapp et al., 1995, EPPO, 2004) or even within a single leaf (Paskaš, personal communication). Infected leaves can be found next to healthy ones (Adams et al., 1999). The expression of symptoms depends on the genotype (Atanasoff, 1935, Hamdorf, 1976, Kegler et al., 1985, Kegler et al., 1998b, Grüntzig et al., 2002). Sensible genotypes exhibit distinct symptoms on leaves

and fruits. In contrast, tolerant genotypes do not show symptoms either on fruits, leaves or both.

Because of the inconsistent symptom expression depending on the genotype, the virus distribution, the time for evaluation and mixed infections, diverse techniques for the precise detection of PPV were established. These techniques are based on biological, immunological or molecular biological approaches. In the following, general as well as strain specific detection of PPV is described including major advantages and disadvantages of each method.

II-2.1 Universal detection of PPV

II-2.1.1 Indicator plants

Plants suitable for the indication of the presence of PPV show clear symptoms upon infection. As already shown by Atanasoff (1935), PPV is transmissible by grafting. Therefore, scions of cultivars and genotypes to be tested are grafted onto the indicator plant. Already in the 1950s woody indicator plants for the detection of PPV were proposed (Nemeth, 1994). The indicator plants which are recommended by the EPPO (2004) are seedlings of *Prunus persica* 'GF 305', *P. persica* 'Nemaguard' or *P. tomentosa*. According to Damsteegt et al. (1997) *P. tomentosa* is more advantageously than 'GF 305' seedlings as it is easier to cultivate and easier to propagate. On the whole, grafting represents a simple and reliable way of testing but it is a very time consuming method (Tab. II-2-1), because the plant used for PPV indication has to be cultivated for months including a dormant phase. However, this method still exhibits the highest sensitivity.

Tab. II-2-1: Course of biological diagnosis of PPV by indicator plants

Working step	Use of	Duration
grafting	virusfree indicator plants	5 min
symptome expression	insectproof greenhous	6 weeks
	common cultivation measures	
evaluation		10 min
chilling period	climate chamber	6 weeks
new growth + symptome	insectproof greenhous	6 weeks
	common cultivation measures	
evaluation		10 min
	total time: 18 weeks 25 min	

II-2.1.2 Immunological approaches

The demonstration of mechanical transmission of PPV to an herbaceous host plant in the 1960s allowed for the purification of the virus leading to the production of antisera (Nemeth, 1994). The invention of radial immunodiffusion 1975 permitted the analysis of large numbers of samples (Nemeth, 1994, Cambra et al., 2006a).

A cornerstone in PPV detectability was the adaption of the enzyme-linked immunosorbent assay (ELISA) to PPV and other plant viruses in the double antibody sandwich (DAS) format. The application of DAS-ELISA enabled detection as well as quantification of the virus (Clark et al., 1976, Clark and Adams, 1977). Usually, polyclonal antibodies targeting the CP are used. Their quality varies from batch to batch. As a consequence, specificity and sensitivity of DAS-ELISA alternates with, in parts, inadequate results (Cambra et al., 1994). There is also evidence for cross reactivity of polyclonal PPV antibodies with other viruses (Cambra et al., 2006a). PPV can be detected in leaves, dormant and developing buds, unripe and ripe fruits, flowers and roots (Clark et al., 1976) and in bark (Adams et al., 1999).

To overcome the problems associated to polyclonal antibodies, several working groups developed monoclonal antibodies (MAb) which bind to the CP of isolates of all PPV strains

known so far. Hilgert et al. (1993) developed the MAb 05. The MAb 5B established by Cambra et al. (1994) was shown to recognize isolates of PPV-D and -M as well as isolates of PPV-W, -EA, -C (James et al., 2003) and PPV-T (Serçe et al., 2009). As PPV-Rec is a recombination of PPV-D and PPV-M, this strain is also detected. The European Plant Protection Organization (EPPO) recommends the MAb 5B for general detection of PPV by ELISA (Tab. II-2-4, EPPO, 2004).

The MAbs described above target the CP of PPV. However, there are also reports on the development of MAb 11E5H and MAb 11F against the CI (Cambra et al., 1994) and MAb 2A targeting NIb (Esteban et al., 2003) applicable in Western blots and tissue-print ELISA as well as in conventional ELISA.

Compared to the use of indicator plants for detection of PPV, an ELISA is completed within one day (Tab. II-2-2). Due to commercial availability of ELISA reagents and due to the simple setup it is commonly used for PPV testing. It can also be used for quantification of the virus titre in plants. However, the low sensitivity of ELISA is disadvantageous.

Tab. II-2-2: Detection of PPV by DAS-ELISA

	Working step	Use of	Duration
sample preparation	grinding	PBS buffer	5 min
	dilution		5 min
ELISA preparation	coating of the wells		1 h
	washing		10 min
	incubation with samples		6 h
	washing		10 min
detection	incubation with 2nd antibody		1 h
	washing		10 min
	incubation with substrate		2 h
	evaluation		10 min
			total time: 10 h 50 min

II-2.1.3 Molecular biological methods

First attempts to detect PPV via its genome were based on dot-blot hybridization of cDNA probes (Varveri et al., 1987) or cRNA probes (Varveri et al., 1988, Wetzel et al., 1990). However, the use of radioactive labels impeded the application of these techniques for routine diagnostic. This can be circumvented by using a biotinylated capture RNA and a digoxigenin labelled RNA probe. Both RNA molecules hybridize to PPV RNA enabling the binding to streptavidin coated ELISA plates for stringent washing steps and visualisation via an antidigoxigenin/ alkaline phosphatase conjugate (Palkovics et al., 1994). Multiplex detection based on molecular hybridization using a riboprobe containing sequences specific to PPV, *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *American plum line pattern virus* (APLPV), and *Apple chlorotic leaf spot virus* (ACLSV) is possible as well (Herranz et al., 2005).

A more convenient method to detect the PPV genome specifically is the reverse transcription of PPV RNA prior to polymerase chain reaction (RT-PCR) with primers targeting the coding sequence of the CP. Korschineck et al. (1991) developed the primers A/B resulting in a 210 bp fragment. The primer pair P1 and P2 established by Wetzel et al. (1991b) spans a 243 bp amplicon. This protocol is recommended by EPPO (2004) for reliable detection of PPV by PCR (Tab. II-2-4). To avoid the cross reactivity of the primers with other members of the genus *Potyvirus*, Levy and Hadidi (1994) developed primers specific to the 3' UTR which yields in a 220 bp amplicon. Co-operational PCR (Co-PCR) utilizes two pairs of primer with one pair (P1/P2) amplifying a smaller fragment (243 bp) within

the amplicon (359 bp) determined by the external pair of primer (P10/P20) similar to nested PCR to enhance sensitivity. By contrast, all primers are added to one reaction to simplify the work flow resulting in four amplicons different in length: a large amplicon derived from P10/P20, a small fragment enclosed by P1/P2 and two intermediate amplicons each determined by one outer primer and one inner primer. In the course of the reaction the large fragments are accumulated as the shorter ones prime the large ones as well (Olmos et al., 2002).

With the invention of real-time PCR it was possible to quantify PPV by SYBR® Green I (Olmos et al., 2004) as well as by the TaqMan® technology (Olmos et al., 2004, Schneider et al., 2004) with TaqMan® chemistry being more sensitive (Olmos et al., 2004). Real-time PCR also allows for the quantification of the viral load in single aphids (Olmos et al., 2005).

For routine diagnostics one-step multiplex RT-PCR protocols were developed to detect simultaneously several viruses affecting stone fruit trees. One protocol targets up to eight viruses (ApMV, PNRSV, PDV, APLPV, PPV, ACLSV, *Apricot latent virus* (ApLV) and *Plum bark necrosis stem pitting associated virus* (PBNSPaV)) and ribulose 1,5-bisphosphate carboxylase (*Rbcl*) as an internal control (Sánchez-Navarro et al., 2005). Another one was established to detect three viruses, PPV, PNRSV, PDV, and *nad5* (NADH dehydrogenase subunit 5, internal control) in one reaction (Jarošová and Kundu, 2010).

Two isothermal nucleic acid amplification techniques were established for the detection of PPV to avoid the need for expensive technical equipment. One method is called nucleic acid sequence based amplification (NASBA; Olmos et al., 2007); the other one loop-mediated isothermal amplification (LAMP; Varga and James, 2006b). For detailed information on these techniques see chapter II-3.2.1 (p. 14) and chapter II-3.2.7 (pp. 25-31), respectively.

The detection of PPV by RT-PCR is fast and highly sensitive. However, molecular biological equipment is needed. The RNA has to be extracted prior to the PCR analysis and, for visualisation, gel electrophoreses has to be carried out. Both procedures require the handling of toxic reagents (Tab. II-2-3). The overall costs are high, especially in the case of real-time PCR.

Tab. II-2-3: RT-PCR based detection of PPV (according to Wetzel et al. (1991b) and Bühler (2007))

	Working step	Use of	Duration
sample preparation	grinding	N_2	5 min
	RNA extraction	β-Mercaptoethanol	2 h
		chloroforme	
amplification	cDNA synthesis		1 h
	PCR	Thermal cycler	2 h
visualization	gel electrophoresis	ethidium bromide	1 h
		gel documentation	
		total time: 6 h 5 min	

II-2.2 Strain specific detection of PPV

Up to now, seven subgroups of PPV have been discovered. As early as in 1979 two major strains, PPV-D and PPV-M, were differentiated (Kerlan and Dunez, 1979). PPV-Rec arose from the recombination of a PPV-D and a -M isolate and represents the third major strain (Glasa et al., 2002a, Glasa et al., 2004). PPV-C which predominantly infects cherry trees was found in 1994 (Kalashyan et al., 1994). The strain PPV-EA is locally distributed in Egypt (Wetzel et al., 1991a). PPV-W was first observed in Canada (James et al., 2003). However, its origin is located in the Baltic countries (Glasa et al., 2011). The strain recently discovered in Turkey is PPV-T (Serçe et al., 2009). Below, the character-

istics of these subgroups of PPV are described including techniques for strain typing and problems arising thereof.

First differentiation of PPV subgroups was achieved by Kerlan and Dunez (1979), who were able to differ between PPV-D and PPV-M by immunodiffusion analysis. PPV-D naturally affects plums, apricots and rarely peach (EPPO, 2004). Isolates found in peach are predominantly members of PPV-M. These isolates cause more severe symptoms than PPV-D and are spread faster by aphids (EPPO, 2004). Dallot et al. (2011) found two subclusters within PPV-M by sequencing. The isolates belonging to the subcluster PPV-Ma were collected in Mediterranean countries, whereas isolates grouped to PPV-Mb originated from eastern European countries. Restriction fragment length polyphorism (RFLP) applied to the PCR amplicon derived from the protocol of Wetzel et al. (1991b) allowed for the discrimination of strain D and M as well. The 243 bp amplicon of PPV-D contains both *AluI* and *RsaI* restriction sites, whereas the latter one is missing in the amplicon derived from PPV-M (Tab. II-2-4; Wetzel et al., 1991b). However, the *RsaI* restriction site cannot be found in the amplicons of PPV-Rec, -EA, -C, -W and -T as well. The restriction site of *AluI* is present in all strains except in PPV-C and PPV-W.

With growing number of available PPV sequences a heminested PCR protocol was developed. PCR products derived from primers P1 and P2 were subjected to a second round of amplification. The primer pairs P1/PD and P1/PM were used to differentiate PPV-D and PPV-M, respectively (Olmos et al., 1997). Unfortunately, the primer PM detects not only PPV-M but also the strains Rec, T and EA (Tab. II-2-4). First sequences of PPV-D were obtained by Lain et al. (1989), Maiss et al. (1989) and Teycheney et al. (1989) and of PPV-M by Cervera et al. (1993) and Palkovics et al. (1993) (Fig. II-2-1). ELISA based strain typing of PPV-D using specific MAb 4DG5 and MAb 4DG11 (Cambra et al., 1994) and of PPV-M by MAb AL (Boscia et al., 1997) is possible as well (Tab. II-2-4).

Cervera et al. (1993), Glasa et al. (2001) and Glasa et al. (2002b) found evidence for recombination in the C-terminal region (around nt 8440) of the NIb gene of PPV-D and PPV-M. As a consequence, a new strain, PPV-Rec, was described by Glasa et al. (2002a) and Glasa et al. (2004) (Fig. II-2-1). Isolates of this subgroup are found in central and in southeast Europe preferentially on plums and apricots (Glasa et al., 2004). An ancient recombination was found in all three major strains at nucleotide position 2813 with high homology in the upstream sequence (Glasa et al., 2004). By RFLP analysis (Wetzel et al., 1991b) PPV-Rec would be misleadingly assigned as PPV-M. Šubr et al. (2004) afforded the discrimination of PPV-D, PPV-M and PPV-Rec by primers which enclose the recombination break-point. PPV-D is detected by the primers mD5 and mD3 resulting in a 664 bp amplicon, PPV-M by primers mM5 and mM3 enclosing a fragment of 459 bp. The combination of mD5 and mM3 produces a 605 bp fragment when recognizing PPV-Rec. However, the primers mM5 and mM3 are also specific to PPV-T (Tab. II-2-4). Sequence analyses support the hypothesis of PPV-Rec originating from former Yugoslavia (Glasa et al., 2005). Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) can also be used for discrimination of PPV-D, -M and -Rec. The CP of PPV-D has a lower mobility than PPV-M whereas the CP of PPV-Rec exhibits a double band with one band moving faster and one

migrating more slowly than the CP of PPV-M (Šubr and Glasa, 2008, Šubr et al., 2010) However, due to atypical results other techniques should be used to validate the results (Šubr and Glasa, 2008). Isolates belonging to PPV-Rec would be falsely assigned to PPV-M by immunological techniques based on MAb 4DG5, MAb 4DG11 and MAb AL (Tab. II-2-4; Glasa et al., 2004).

In Egypt, a PPV isolate was detected which showed a low level of nucleic as well as amino acid sequence homology compared to PPV-D. This isolate was dedicated to strain PPV-EA (Fig. II-2-1; Wetzel et al., 1991a). In PPV-EA as in PPV-M the *RsaI* restriction site within the P1/P2 amplicon is missing (Wetzel et al., 1991a). Glasa et al. (2006) and Myrta et al. (2006) provided whole genome sequences of this strain. Differentiation of PPV-EA is possible by ELISA using MAb EA24 (Tab. II-2-4; Myrta et al., 1998).

Contrary to the hypothesis of PPV not infecting cherry trees, the Sharka disease was found on cherry (Kalashyan et al., 1994). This divergent isolate was assigned to strain PPV-C due to missing restriction sites in RFLP analysis (Nemchinov and Hadidi, 1996, Crescenzi et al., 1997a), due to the analysis of the CP coding sequence as well as of whole genome sequence (Fig. II-2-1; Maiss et al., 1995, Nemchinov et al., 1996, Crescenzi et al., 1997b, Fanigliulo et al., 2003) and due to the reactivity in ELISA with MAbs specific to PPV in general (MAb 5B) but not with monoclonal antibodies recognizing PPV-D (MAb 4DG5, MAb 4DG11) and PPV-M (MAb AL). Strain typing can be conducted by ELISA using polyclonal antibodies targeting the CP of PPV-C (Crescenzi et al., 1997b) and MAb

Tab. II-2-4: PPV strain differentiation based on ELISA and PCR
blue – MAb or primers for the general detection of PPV, green – MAb or primers developed for the detection of the indicated strain, red – MAb or primers resulting in a positive signal but were originally developed for the detection of another strain

	ELISA		PCR						
			Wetzel et al. (1991b)		Olmos et al. (1997)	Szemes et al. (2001)		Šubr et al. (2004)	
	general	strain specific	general	RFLP		general	strain specific		
PPV-D	MAb 5B[1]	MAb 4DG5[1]; MAb 4DG11[1]	P1/P2	*AluI* + *RsaI*	P1/PD	M3-5' + M4-5'/ M2-3'	M1-5'/M5-3'	mD5/mD3	
PPV-Rec		MAb AL		*AluI*	P1/PM		M6-5'/M7-3'	mD5/mM3	
PPV-M		MAb AL[2]		*AluI*	P1/PM		M6-5'/M7-3'	mM5/mM3	
PPV-T				*AluI*	P1/PM		M6-5'/M7-3'	mM5/mM3	
PPV-C		MAb TUV[3]; MAb AC[3]		neither			M10-5'/ M11-3'		HSoC-2/ CSoC-2[5]
PPV-EA		MAb EA24[4]		*AluI*	P1/PM		M8-5'/M9-3'		
PPV-W				neither					W8328F/ W8711R[6]

[1]Cambra et al. (1994)
[2]Boscia et al. (1997)
[3]Myrta et al. (2000)
[4]Myrta et al. (1998)
[5]Nemchinov and Hadidi (1998)
[6]Glasa et al. (2011)

TUV and MAb AC (Myrta et al., 1998, Myrta et al., 2000). Nemchinov et al. (1996) established a cRNA probe for specific detection of PPV-C via dot-blot hybridization. A more convenient way to differentiate PPV-C and the other subgroups of PPV is based on a PCR utilizing the primers HSoC-2/CSoC-2 (Tab. II-2-4; Nemchinov and Hadidi, 1998).

In Canada, an isolate was found which could not be assigned to a subgroup known so far neither by ELISA using MAbs specific to PPV-D (MAb 4D), -M (MAb AL), -EA (MAb EA24) and -C (MAb AC) nor by strain typing PCRs and RFLP analysis (James et al., 2003). Sequencing and subsequent phylogenetic analysis evidenced that this isolate is a representative of a new PPV strain called PPV-W (Fig. II-2-1; James and Varga, 2005). Glasa et al. (2011) designed the primers W8328F and W8711R recognizing the C-terminal region of NIb and N-terminal of CP for specific detection of PPV-W (Tab. II-2-4). Sequence analysis of Latvian isolates confirmed the origin of this strain being Latvia or nearby countries but not Canada (Glasa et al., 2011).

Sequence analysis of Turkish isolates derived from plums and apricots revealed a seventh strain PPV-T (Fig. II-2-1; Glasa and Candresse, 2005, Serçe et al., 2009). At the moment, sequencing is the only way to discriminate PPV-T. The genomic RNA exhibits a recombination event at nucleotide position 1478 - 1568 compared to PPV-M (Glasa and Candresse, 2005). In the evolutionary history of PPV, there is a second recombination located in the P3 gene (Glasa et al., 2004). Applying the P1/P2 PCR product to restriction digestions according to Wetzel et al. (1991b), PPV-T would be classified as PPV-M. PPV-T isolates show an indifferent pattern upon analysis by ELISA using PPV-D and -M specific monoclonal antibodies (Tab. II-2-4; Serçe et al., 2009).

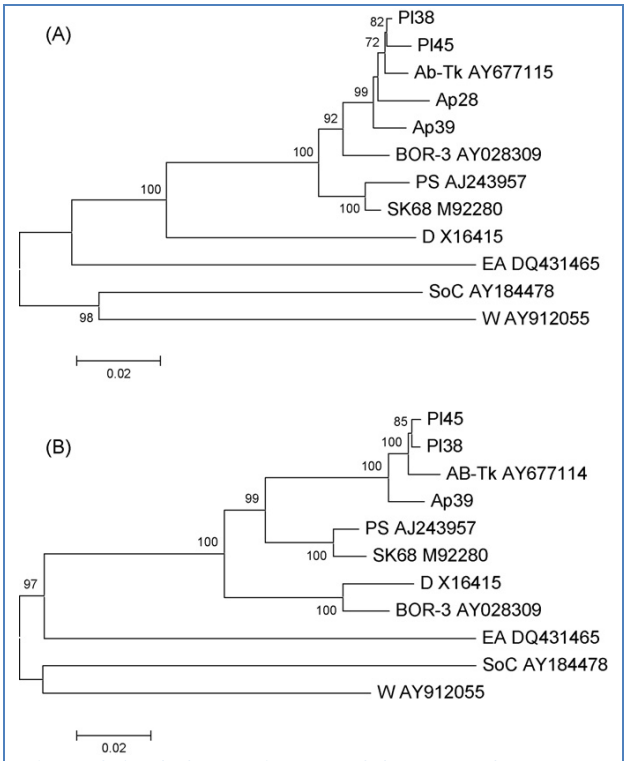


Fig. II-2-1: Phylogenetic trees of the PPV subgroups
Phylogenetic tree of either 3' (A) or 5' (B) genomic regions of isolates each representing one of the seven PPV subgroups known so far
PPV-D - D; PPV-M - PS, SK68; PPV-Rec - Bor-3; PPV-C - SoC; PPV-EA - EA; PPV-W - W; PPV-T - PI38, PI45, Ab-Tk, Ap28, Ap39
Reprinted from Serçe et al. (2009) with permission from Elsevier

Aside from the sequences mentioned above, numerous further sequences either covering single regions or the whole genome are available in sequence databases as deposited at http://www.ncbi.nlm.nih.gov/.

An integrated RT-PCR/nested PCR technique was established for specific detection of PPV-D, PPV-M, PPV-C and PPV-EA with the outer primers recognizing PPV in general and with the nested primers detecting the PPV subgroups (Szemes et al., 2001). However, the primers specific to PPV-M also detect PPV-Rec and

-T (Tab. II-2-4). Real-time RT-PCR can be used for strain typing of PPV-D, -M, -C, -EA and -W as well in a multiplex or single reaction format via melting curve analysis (Varga and James, 2005, Varga and James, 2006a). An oligonucleotide microarray can also be used for detection and strain typing of PPV-D, PPV-M, PPV-C and PPV-EA (Pasquini et al., 2008).

Other protocols to differentiate PPV subgroups were developed by Deborré et al. (1995, Hammond et al. (1998) and Glasa et al. (2002b). However, none of the assays is suitable to distinguish between all known strains.

II-3. Nucleic acid detection techniques

To date, the most sensitive methods for detecting pathogens rely on the partial amplification of the pathogen's genome (target amplification) or on the amplification of a probe specific to the target genome (signal amplification). The predominant method for the amplification of nucleic acids *in vitro* is the polymerase chain reaction (PCR). However, there exist numerous techniques for which temperature conditions are isothermal. In contrast, the temperature during PCR needs to be risen and lowered in a cyclic manner. For this, costly technical equipment is required.

II-3.1 Polymerase chain reaction (PCR)

Since its introduction in the 1980s (Saiki et al., 1985, Mullis and Faloona, 1987) the PCR has become one of the mostly applied techniques in molecular biology. The simple experimental design as well as the various adaptations of the basic technique for specific requirements such as real-time PCR for quantification of DNA account for this. The fundamental approach relies on the cyclic repetition of denaturation of dsDNA, annealing of the primers and elongation of the primers by heat stable DNA polymerase. As these steps are carried out at different temperatures sophisticated technical equipment is necessary.

II-3.2 Isothermal techniques for the amplification of nucleic acids *in vitro*

The ease of experimental design, the diversity of possible applications as well as the wide-ranging availability of reagents and the improvement of thermostable DNA polymerases led to the PCR becoming the standard in nucleic acid amplification *in vitro*.

However, numerous approaches were developed to circumvent the use of a thermal cycler needed for PCR. Isothermal amplification techniques are nucleic acid sequence-based amplification (NASBA; Compton, 1991), helicase dependent amplification (HDA; Vincent et al., 2004), isothermal multiple displacement amplification (IMDA; Dean et al., 2002), loop-mediated isothermal amplification (LAMP; Notomi et al., 2000), rolling circle amplification (RCA; Fire and Xu, 1995, Lizardi et al., 1998), recombinase polymerase amplification (RPA; Piepenburg et al., 2006), strand displacement amplification (SDA; Walker et al., 1992b), signal mediated amplification of RNA technology (SMART; Wharam et al., 2001), smart amplification process 2 (SmartAmp 2 or SMAP 2; Mitani et al., 2007) and single primer isothermal amplification (SPIA; Kurn et al., 2005).

In the following, a short description of the more important and more common techniques is given. CRCA and LAMP are characterized in detail.

II-3.2.1 Nucleic acid sequence-based amplification (NASBA)

Three enzymes and two primers are required for the course of action of NASBA (Fig. II-3-1). In the non-cyclic phase, the first primer, which contains a target specific sequence at the 3'-end as well as a T7 RNA polymerase promoter region at its 5'-end, binds to the RNA template. An AMV (avian myeloblastosis virus) reverse transcriptase synthesizes cDNA. The RNA in the heteroduplex is degraded by RNase H. Therefore, the second primer and the reverse transcriptase can bind to the ssDNA yielding dsDNA. By implication, the promoter is activated and the T7 RNA polymerase produces numerous copies of the template. The cyclic phase starts with the annealing of the second primer to the RNA. After cDNA synthesis and RNA degradation the T7 RNA polymerase is activated and synthesises RNA copies starting a second cycle (Compton, 1991).

This method is more suitable for the analysis of RNA than of DNA, because dsDNA has to be denatured before adding the reaction mix. Therefore, native genomic DNA is not amplified (Compton, 1991).

NASBA is also known as self-sustained sequence replication (3SR; Guatelli et al., 1990). Both methods are an improvement of the transcription based amplification system (TAS; Kwoh et al., 1989). In TAS the RNA in the RNA-DNA-hybrids is not degraded by the action of RNase H, but the hybrids are denatured at high temperatures. For this reason the thermolabile enzymes have to be added after each denaturing step again.

As with PCR it is possible to view the amplification real-time with NASBA. For this purpose molecular beacons are added to the reaction which is called AmpliDet RNA (Leone et al., 1998, Niesters, 2001).

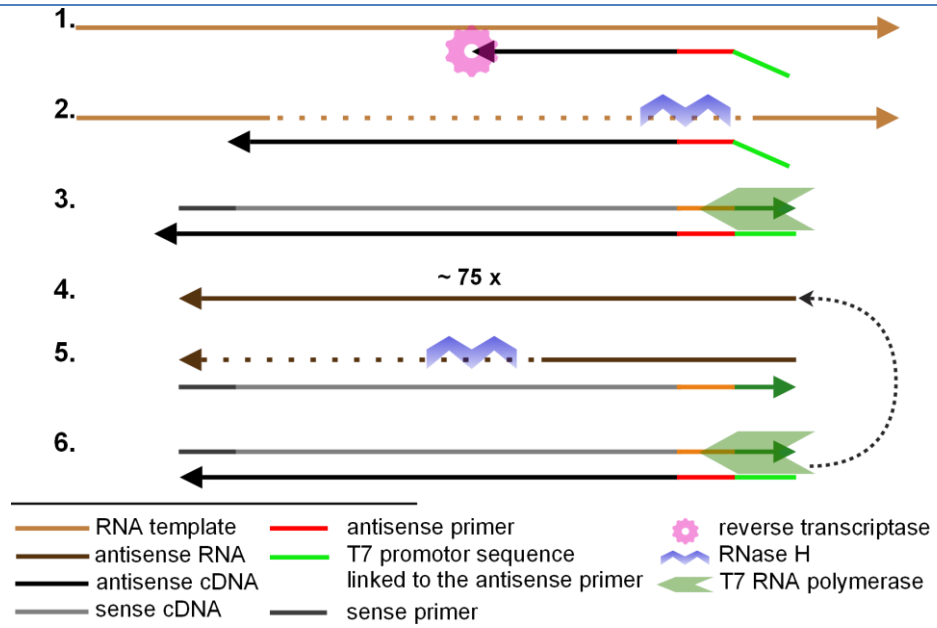


Fig. II-3-1: Nucleic acid sequence-based amplification (NASBA)
RNA is reversely transcribed to cDNA and, afterwards, degraded. Upon synthesis of dsDNA, the T7 RNA polymerase produces multiple RNA transcripts which serve as template for cDNA syntheses starting the cyclic phase of the amplification (according to Compton (1991)).

II-3.2.2 Strand displacement amplification (SDA)

Walker et al. (1992b) developed the SDA which uses two primers, the endonuclease *Hinc*II and the *E.coli* DNA polymerase I (exo- Klenow). The primers contain the *Hinc*II recognition site at the 5'-end in addition to their 3'-ends which are complementary to the target sequence (Fig. II-3-2). The target DNA is restriction digested to obtain defined 5'- and 3'-ends. After heat denaturation of dsDNA, the primers hybridize to the DNA and are elongated by the exo- Klenow fragment of E.coli DNA polymerase I by incorporating dGTP, dCTP, dTTP, and deoxy-adenosine 5'-[α-thio] triphosphate (Fig. II-3-2B). *HincII* just introduces a nick in the DNA strand containing the primer as the complementary strand is protected by phosphothioate. At the 3'-end of the nick, exo- Klenow can start DNA synthesis displacing the down-stream strands (Fig. II-3-2C). In turn, the primers bind to these strands starting a new cycle

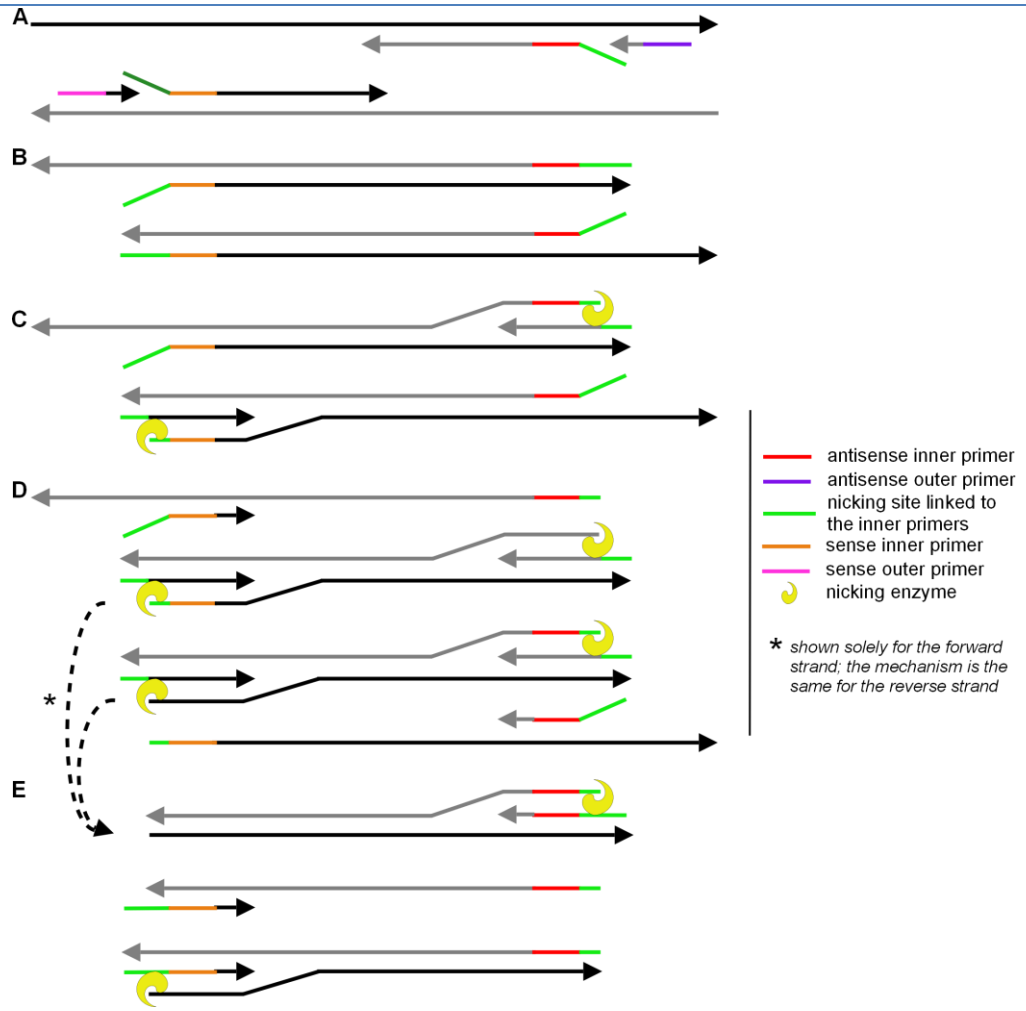


Fig. II-3-2: Strand displacement amplification (SDA)
Outer primers and primers containing a *Hinc*II restriction site at the 5'-end are elongated by a strand displacing DNA polymerase using dGTP, dCTP, TTP, and dATP[αS] (A). The SDA primers anneal to the displaced ssDNA starting the amplification (B). Upon the action of the endonuclease at the hemiphosphothiate restriction sites, DNA synthesis is initiated at the nick releasing ssDNA (C). Primers can bind to the resulting ssDNA for further amplification (D+E) (according to Walker et al. (1992a)).

(Fig. II-3-2D). Released strands which contain the restriction site are amplified in the same way. After annealing of the reverse primer, both 3'-ends are elongated creating a new restriction site (Fig. II-3-2E).

To avoid the need for restriction digestion at the beginning, Walker et al. (1992a) introduced a second pair of primers. These so called outer primers anneal to the template upstream to the respective inner primer, which contain the *Hinc*II recognition site. The strands, elongated from inner primers, are displaced by the polymerase elongating the outer primers. The ssDNA serve as template for a second dsDNA synthesis (Fig. II-3-2A). The substitution of the enzymes by thermophilic enzymes increased the rate of amplification and the specificity of the reaction (Spargo et al., 1996). Meanwhile, nicking enzymes are commercially available which cleave only one strand of dsDNA per se without the need for modified nucleotides to prevent cleavage of the both strands.

II-3.2.3 Signal mediated amplification of RNA technology (SMART)

Unlike NASBA and SDA, SMART does not amplify the target itself, which can be either DNA or RNA. The signal generated upon specific detection of the template is multiplicated by *Bst* DNA polymerase and T7 RNA polymerase (Wharam et al., 2001).

Two oligonucleotides called extension probe and template probe are designed to hybridize at adjacent positions on the template (Fig. II-3-3A). Furthermore, the template probe contains a transcription template, the promotor sequence of T7 RNA polymerase and an 8 nt sequence which is complementary to the 3'-end of the extension probe (Fig. II-3-3A). Just in the case of annealing of the probes to the target, the short complementary overlap of one probe binds to the other one forming a three-way junction (3WJ). The *Bst* DNA polymerase elongates the extension probe by what the promotor is activated. The T7

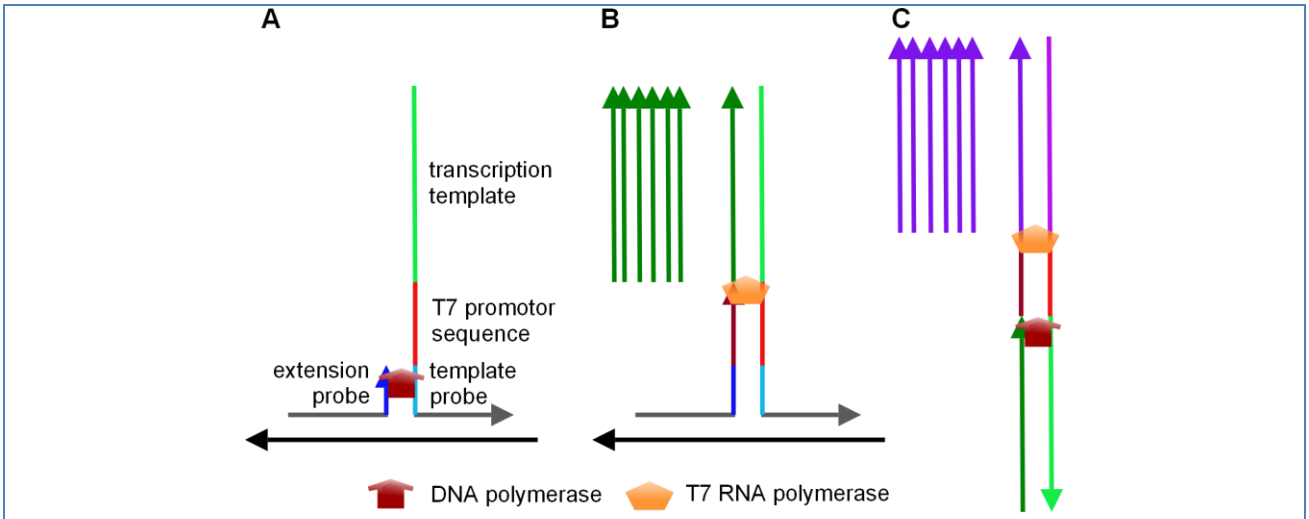


Fig. II-3-3: Signal mediated amplification of RNA technology (SMART)
Target DNA is detected by an extension probe and by a template probe which bind side-by-side to the target as well as to each other (A). The extension probe is elongated by a DNA polymerase with the template probe as template. Now a RNA polymerase transcribes RNA molecules (B) which initiate a second transcription (C) (according to Wharam et al. (2001)).

RNA polymerase releases several copies of the transcription template (Fig. II-3-3B). In turn, these copies bind to the 3'-end of a third probe. It bears the same sequence at the 5'-end as the template probe at its 3'-end for the further amplification (Fig. II-3-3C). The signal is detected via ELOSA (enzyme-linked oligosorbent assay).

II-3.2.4 Helicase-dependent amplification (HDA)

The amplification of nucleic acids by HDA is similar to PCR: two primers define the target region and are elongated by a DNA polymerase. However, the mode of denaturation of dsDNA is different. In HDA, a helicase is used to unwind and to separate the two DNA strands mimicking the natural DNA replication (Fig. II-3-4; Vincent et al., 2004).

In the beginning, *E.coli* UvrD helicase and exo- Klenow were used supplemented by the protein MutL and the single-strand binding protein (SSB) bacteriophage T4 gene 32 protein. The first protein is essential for the activity of the *E.coli* UvrD helicase; the latter one prevents the rehybridization of ssDNA. The reaction temperature was 37 °C (Vincent et al., 2004).

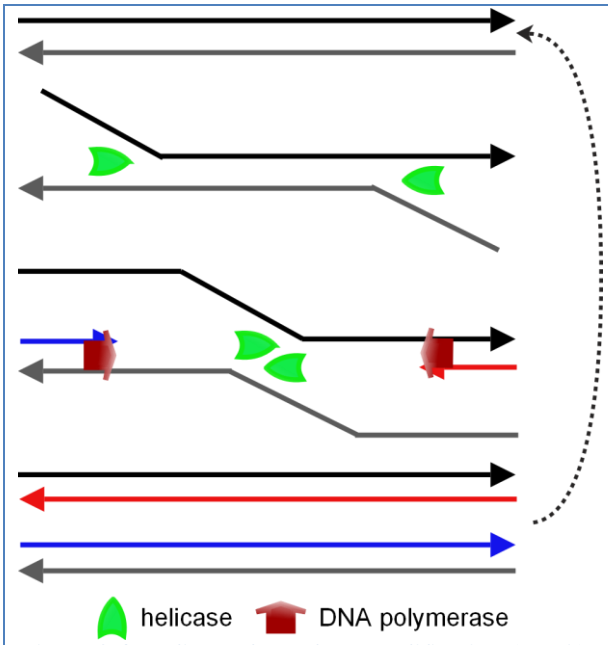


Fig. II-3-4: Helicase-dependent amplification (HDA) A helicase unwinds and separates dsDNA allowing primer annealing and extension similar to PCR (according to Vincent et al. (2004)).

An et al. (2005) replaced *E.coli* UvrD helicase by Tte-UvrD helicase derived from the thermophilic bacterium *Thermoanaerobacter tengcongensis* and exo- Klenow by *Bst* DNA polymerase. Thus, the reaction can be conducted at elevated temperatures of 60 - 65 °C. The use of the accessory proteins is not necessary. The reaction is termed tHDA (thermophilic HDA).

The tHDA reaction was further improved by the protein fusion of Tte-UvrD helicase and *Bst* DNA polymerase creating a helimerase. The fusion protein is able to amplify significant longer fragments up to 2.3 kb compared to less than 200 b in tHDA (Motré et al., 2008).

The detection of RNA targets by tHDA is able by the use of a thermophilic reverse transcriptase in combination with Tte-UvrD helicase and *Bst* DNA polymerase. The RT-tHDA can be conducted in a one-tube one-step format (Goldmeyer et al., 2007).

II-3.2.5 Recombinase polymerase amplification (RPA)

As HDA, RPA is similar to PCR in terms of utilizing two primers and a DNA polymerase. In contrast to HDA and PCR, annealing of the primers is facilitated by the use of a recombinase. The primer-recombinase-complexes scan the target DNA for homology and replace the homologous sites with the primers (Fig. II-3-5). The exchanged DNA strand is stabilized by SSBs to

avoid the dislodging of the primers. *Bsu* DNA polymerase I (large fragment) derived from *Bacillus subtilis* and exhibiting strand displacement activity is employed for the elongation of the primers. Cyclic repetition of these steps allow for exponential amplification at a constant temperature (Fig. II-3-5; Piepenburg et al., 2006).

T4 uvsX the recombinase used by Piepenburg et al. (2006) assembles with the primer in the presence of ATP. The ATP, in turn, is hydrolysed to ADP by the complex. As a consequence, the recombinase can be replaced by the SSB T4 gp32. For the correct action of RPA, the recombinase loading factor T4 uvsY and Carbowax20M should be added.

The RPA can be monitored in real-time by the addition of SYBR® Green I or fluorophore/quencher probes (Piepenburg et al., 2006).

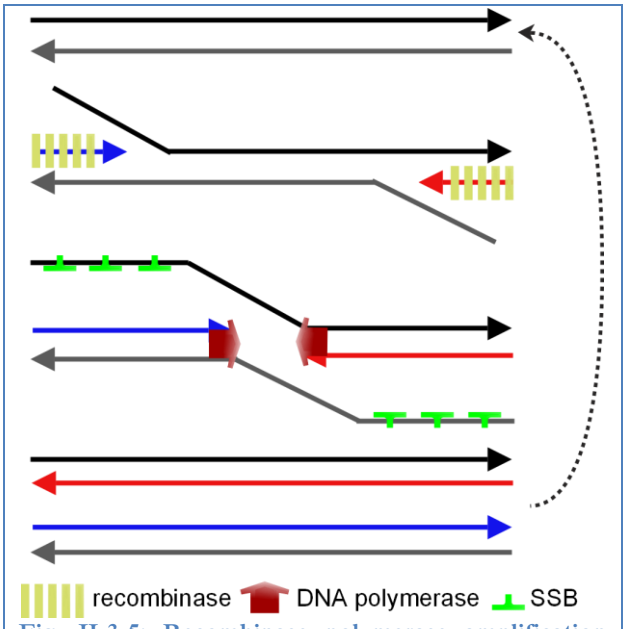


Fig. II-3-5: Recombinase polymerase amplification (RPA)
Complexes of recombinases and primers search for homologous sites in dsDNA. Upon recombination, the SSBs bind to exchanged strand and the primers are elongated by a strand displacing polymerase. The resulting dsDNAs enter into the next round of RPA (according to Piepenburg et al. (2006)).

II-3.2.6 Cascade rolling circle amplification (CRCA)

In nature, phages and some plasmids replicate their circular genome via a rolling circle mechanism. Initiator proteins bind specifically to the dsDNA and introduce a nick in the leading strand. A replisome composed of SSB, polymerase, helicase and initiator protein performs the polymerization starting at the 3' side of the nick. After one round the displaced leading strand is recircularized. In phages the replication proceeds for about 20 rounds. In contrast, the plasmid replication is inhibited after the second cycle due to an inactivated initiator protein (Novick, 1998). Viruses belonging to the family *Geminiviridae* exhibit also the rolling circle replication (RCR). Many of these viruses can infect plants causing high economically losses. Since their genome is small, they utilize host cellular proteins for replication (Gutierrez, 1999). The circular RNA of viroids is amplified by RCR as well (Góra-Sochacka, 2004).

The size of replicated circular genomes range from 300 nt (viroids; Góra-Sochacka, 2004) to 4,500 bp (plasmids; del Solar et al., 1993). In contrast, Fire and Xu (1995) and Liu et al. (1996) showed that *E. coli* DNA polymerase I, the Klenow fragment of *E. coli* DNA polymerase I, T7 as well as T4 DNA polymerase are capable to amplify DNA circles as small as 34 nt and 26 nt, respectively, resulting in a single DNA strand with up to 12,000 nt in length containing tandem repeats of the template circle. Compared to the natural RCR, no additional proteins were required for these *in vitro* experiments. A new *in vitro* nucleic acid based amplification technology has been evolved from these results called rolling circle amplification (RCA, Fig. II-3-6C).

Such small single stranded DNA circles can also be transcribed to concatameric RNA using T7 RNA polymerase or *E. coli* RNA polymerase via the rolling circle mechanism. Typically, the transcription by RNA polymerases depends on a specific double stranded promoter sequence. However, in the event of rolling circle transcription neither a promoter nor a primer is needed for initiation of RNA synthesis (Daubendiek et al., 1995, Daubendiek and Kool, 1997).

Two important characteristics have to be considered regarding RCA: First, the polymerase is not able to pass through small DNA circles following its helical turns since the dimensions of the enzymes are bigger than the diameter of the oligonucleotide circle (Fire and Xu, 1995, Daubendiek and Kool, 1997, Banér et al., 1998). For example, the diameter of a 26 nt circle is about 40 Å and the Klenow fragment has a size of about 65 * 65 * 85 Å (Liu et al., 1996). Therefore, the DNA circle rotates to arrange the bases at the outer side while the polymerase moves around the circular template (Fire and Xu, 1995, Banér et al., 1998). Second, dsDNA is rather rigid compared to ssDNA, wherefore dsDNA circles contain at least 150 bp. For small circles, it is implausible for ssDNA to become totally double stranded during the elongation of the template strand and strand displacement is carried out by the polymerase. More likely, the newly synthesized strand is displaced by topological forces a number of nucleotides behind the polymerization site (Fire and Xu, 1995, Liu et al., 1996, Banér et al., 1998).

A DNA circle suitable as template for RCA arises from enzymatic (Fire and Xu, 1995, Daubendiek and Kool, 1997) or chemical (Liu et al., 1996) ligation of a linear precursor oligonucleotide. Nilsson et al. (1994) designed a linear probe which consisted of two target complementary segments at either end of the probe linked by

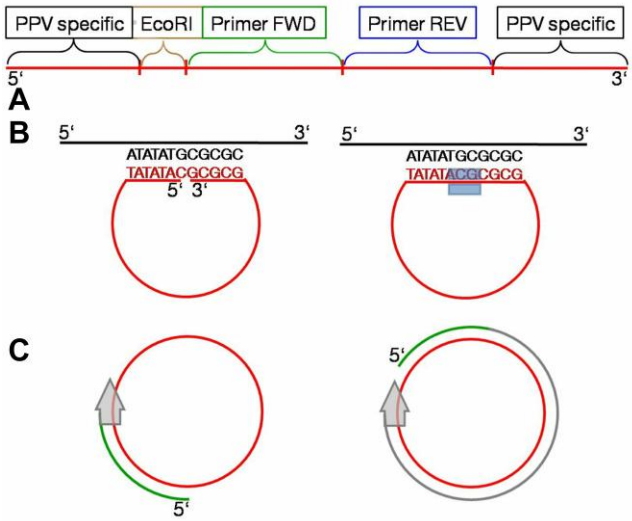


Fig. II-3-6: Cascade rolling circle amplification (CRCA)
A circularizable probe (CLP) contains target specific sequences at each end linked by restriction site for control purposes and two sites for the later amplification, one complementary to primer FWD and one homologous to primer REV (A). Upon the hybridization of the CLP ends to the target, the nick is closed by ligase (B). The actual CRCA is initiated by annealing of primer FWD to the circle and elongation by a strand displacing polymerase (C). After each circumnavigation, primer REV hybridizes to the released ssDNA. DNA synthesis starting at primer REV results in displacing of downstream primer REV strands which, in turn, are recognized by primer FWD (D) (according to Zhang et al. (1998)).

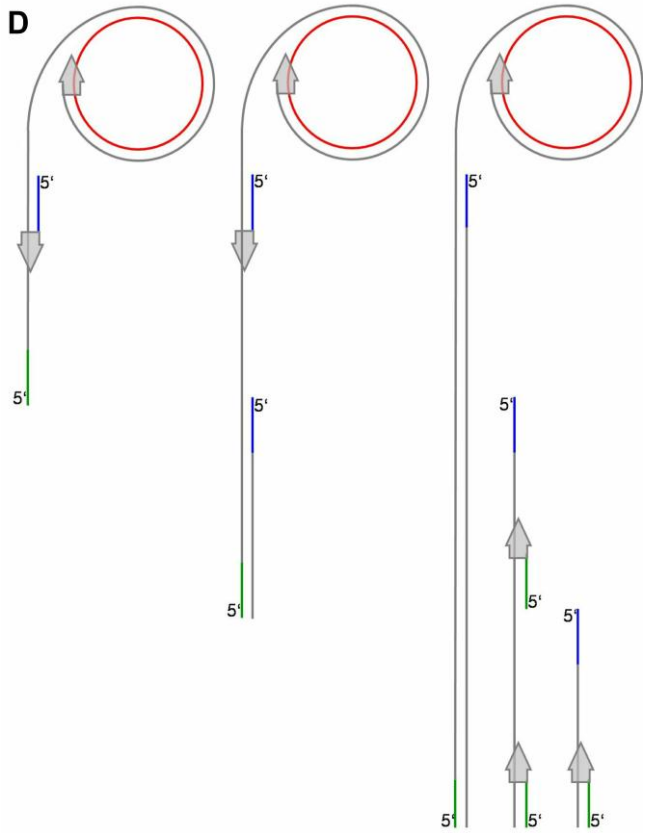


Fig. II-3-6 continued

a sequence of detection purposes (Fig. II-3-6A). The target specific regions hybridize to the target side-by-side. Since the 5'-end is phosphorylated a circularizable probe (CLP), also called padlock probe (PLP) if localization of the probe is intended, can be circularized by a ligase (Fig. II-3-6B). The complementary regions and the target strand are twisted in the helical structure resulting in a topological catenation of the probe and the target. CLPs are suitable tools for diagnostic purposes and RCA (Szemes et al., 2005, Wang et al., 2005).

By applying a second primer to RCA which is complementary to the released strand, exponential accumulation is achieved (Fig. II-3-6D). This is called hyperbranched rolling circle amplification (HRCA; Lizardi et al., 1998), cascade rolling circle amplification (CRCA; Thomas et al., 1999), ramification amplification (RAM; Zhang et al., 1998, Zhang et al., 2001a) or exponential rolling circle amplification (ERCA; Faruqi et al., 2001). In the following, the term CRCA refers to CRCA as well as to HRCA, RAM and ERCA. The amplification rate of RCA can also be enhanced by digesting the multimers obtained by RCA to monomers. These linear monomers are self ligated to form a circular DNA strand which initiates a second RCA. This circle to circle amplification (C2CA) can be repeated several times (Dahl et al., 2004).

The need for primers can be circumvented by the help of a nicking enzyme. This is an endonuclease, which cleaves specifically just one strand of dsDNA. After hybridization of a circular oligonucleotide to the target, the nicking enzyme cleaves the target strand. The RCA is

started at the 3' side of the nick. With every turn of the polymerase new primer sites are generated (Murakami et al., 2009). RCA and CRCA amplification products are visualized by gel electrophoresis, fluorescent labels (Lizardi et al., 1998) or can be monitored in real-time (Faruqi et al., 2001, Hafner et al., 2001).

Oligonucleotides, which are immobilized either on a glass surface (Lizardi et al., 1998) or on magnetic beads (Hatch et al., 1999), can be used to initiate RCA upon ligation of the CLP to the target. By reason of immobilization of the concatameric strand this technique is applicable for microarray analysis (Nallur et al., 2001). CLPs can be ligated in cytological samples followed by RCA detection to enhance visualisation via fluorescence labels (Lizardi et al., 1998, Christian et al., 2001, Zhang et al., 2001a, Stougaard et al., 2007).

The high specificity of CLPs due to two target complementary sequences combined with the prominent mismatch discrimination of thermostable ligases allows for the differentiation of single nucleotide polymorphisms (SNP). Ligated CLPs can be detected by fluorescence labels either before (Nilsson et al., 1997) or after RCA (Faruqi et al., 2001, Qi et al., 2001). It is possible to discriminate both alleles of a SNP simultaneously by using two CLPs. The CLPs are amplified after specific ligation by CRCA with the forward primer being universal to both CLPs and two reverse primers. Each of these primers is specific to one of the CLPs and contains an Ampliflour structure to fluoresce upon incorporation in dsDNA (Faruqi et al., 2001).

The possibility to join a nick in a DNA strand which is hybridized to a RNA strand is given (Nilsson et al., 2000). Thereby, miRNAs can be detected by the specific ligation of a CLP. The circularized oligonucleotide is then amplified via RCA initiated by the miRNA itself (Jonstrup et al., 2006).

RCA can also be utilized for the detection of specific sequences in dsDNA. So called PNA openers (peptide nucleic acid) dissolve the two strands of the DNA at the site of interest. An oligonucleotide hybridizes to one of the separated strands enabling restriction enzymes to digest this dsDNA. Therefore, a nick is introduced to the target DNA. A DNA circle binds to the 3'-end of the nick. The 3'-end primes the RCA. A reverse primer enhances the amplification rate (Kuhn et al., 2003).

RCA is suited for the detection of proteins as well. An oligonucleotide is linked to an antibody. After recognition of the antigen, the oligonucleotide is elongated via a DNA circle and a DNA polymerase with strand displacement activity. The result is visualised by fluorescence. This reaction is called immunoRCA (Schweitzer et al., 2000) which is also adaptable to a microarray format. Up to 51 different proteins can be detected simultaneously by specific antibodies, which are linked to the RCA initiating oligonucleotide. This primer is universal to all antibodies (Schweitzer et al., 2002).

Using the strand displacing Phi29 DNA polymerase and random hexamers a multiple-primed RCA can be performed on circular templates with the size of more than 1,000 nt like plasmids and the circular genome of viruses. The amplification products can directly be applied to restriction digestion, sequencing, *in vitro* cloning and other molecular biological protocols (Dean et al., 2001, Ding et al., 2003, Mizuta et al., 2003, Inoue-Nagata et al., 2004). The mechanism of

multiple primed DNA and strand displacement synthesis is not limited to circular genomes but can also be used for whole genome amplification (WGA; Dean et al., 2002, Detter et al., 2002, Lage et al., 2003).

The ligase mediated generation of CLPs combined with rolling circle replication represents a powerful system for the detection of DNA with both high specificity and sensitivity (Banér et al., 1998, Lizardi et al., 1998).

II-3.2.6a) Design of circularizable probes

The design of CLPs was an improvement of the probes for a ligase-mediated gene detection technique. This protocol utilized two oligonucleotides probes and a ligase. The probes were 20 nt in length and hybridized side-by-side to the target. Ligation events were detected by autoradiography or fluorescence (Landegren et al., 1988). Nilsson et al. (1994) assembled these two oligonucleotide probes to one CLP. The target complementary regions are located at the 5'- and the 3'-end of the probe linked by a spacer with 50 nt in length. The spacer segment can be used for detection (Nilsson et al., 1994, Nilsson et al., 1997) and for amplification either by RCA (Banér et al., 1998, Lizardi et al., 1998, Thomas et al., 1999), by PCR (Zhang et al., 1998, Thomas et al., 1999) or by LAMP (Marciniak et al., 2008). The amplification of a CLP via RCA or PCR is grouped to signal amplification tech-niques, whereas PCR, in common use, belongs to the group of target amplification techniques.

CLPs exhibit a high specificity due to the two target complementary sequences and the joining of these segments by a ligase. The specificity of CLPs can be further increased by a difference in the length of the terminal regions. The 5'-end should have a length of 20 - 30 nt, whereas the 3'-end a length of 12 - 20 nt. Thus, the melting temperature is about 5 °C above and 10 °C below the ligation temperature, respectively. The 5'-end hybridises stable to the target. In the case of the 3'-end, there is equilibrium of bound and free ends for enhanced discriminatory power of the CLP (Thomas et al., 1999, Faruqi et al., 2001, Szemes et al., 2005). Enhancing the ligation temperature has a similar effect. Furthermore, discriminating bases should be located at the end of the 3' complementary sequence because an intrinsic characteristic of ligases is to detect mismatches on the 3' side of the nick more efficiently than on the 5' side (Luo et al., 1996).

II-3.2.6b) Ligases suitable for specific ring closure upon probe hybridization to the target

The correct joining of the 5'- and 3'-ends of the CLP is crucial for the detection of specific DNA sequences via CLPs followed by CRCA. Besides enzymatic ligation, there exist various protocols to join two DNA molecules chemically. Phosphorothioate linked to the 3'-end and *N*-Succinimidyl iodoacetat linked to the 5'-end provide a very fast chemical ligation within 60 sec. This method can be used for ligation on a RNA template. However, recognition of mismatches is lower than by using ligases (Abe et al., 2008).

T4 DNA ligase is the enzyme of choice for many molecular biological tasks. This ligase as well as *E. coli* DNA ligase are very discriminatory regarding mismatches (Zhang et al., 2009) and are active at room temperature. In contrast, Ampligase® Thermostable DNA Ligase, which is a mutant form of *Tth* DNA ligase derived from *Thermus thermophilus*, *Taq* DNA ligase and 9°N™ DNA Ligase (*Thermococcus* sp. strain 9°N) withstand high temperatures enabling strin-

gent hybridization conditions. By introducing an artificial mismatch at position 3 of the 3' side, the fidelity of the *Tth* DNA ligase and *Taq* DNA ligase is enhanced (Luo et al., 1996, Szemes et al., 2005). Similar melting temperatures of the oligonucleotides and ligation temperature as well as mutant *Tth* DNA ligase increase specificity (Luo et al., 1996). Ampligase® Thermostable DNA Ligase promotes the specific ligation of CLPs which are 60 nt in length, but not CLPs with 44 nt in length at a temperature of 37 °C (Qi et al., 2001) as well as at elevated temperatures of more than 60 °C (Thomas et al., 1999).

II-3.2.6c) Influence of thermal cycling of the ligation on the detection limit of CRCA

Ampligase® Thermostable DNA Ligase, *Taq* DNA ligase and *Pfu* DNA Ligase are thermostable enzymes, which are capable to withstand several hours at 95 °C. Therefore, it is possible to ligate CLPs in target DNA in a cyclic manner similar to PCR amplifying DNA (Banér et al., 2003, Szemes et al., 2005, Wang et al., 2009). However, a thermal cycler is needed which is contrary to the aim of technical simplification of PPV detection.

II-3.2.6d) Comparisons of DNA polymerases regarding strand displacement during CRCA

Beside the ligase which catalyses the specific circularization of the CLP after hybridization to the target a polymerase is needed for the rolling circle amplification of the CLP as well as for the cascade like amplification of the released strand. The most important characteristic of a polymerase suitable for CRCA is its displacement activity since the two strands of the DNA are not separated by heating as in PCR but by the polymerase itself at isothermal conditions. Using small circular templates like CLPs, the newly synthesized strand is rather released because of topological restraints than because of the polymerase (Fire and Xu, 1995, Liu et al., 1996, Banér et al., 1998). However, the hyperbranched amplification initiated on the released strand requires a polymerase with strand displacement activity.

Phi29 DNA polymerase, derived from bacteriophage Phi29 of *Bacillus subtilis*, exhibits a very high processivity of 50 nt/sec with its optimum at 37 °C (Nelson et al., 2002) resulting in a strand with a length of up to 70 kB. It possesses strand displacement activity and 3'-5' exonuclease activity (Blanco et al., 1989). Mutant Phi29 DNA polymerases, which lack the exonuclease activity, show a considerably reduced strand displacement activity (Esteban et al., 1994). The exonuclease activity affects not only ssDNA but also ssRNA. Phi29 DNA polymerase degrades the RNA in 3'-5' direction up to where the RNA is hybridized to a DNA circle. As a consequence, the RNA serves as primer for RCA (Lagunavicius et al., 2008, Lagunavicius et al., 2009).

The full length DNA polymerase of *Bacillus stearothermophilus* (*Bst*) has 5'-3' exonuclease activity, but not the large fragment of the enzyme. Both variants do not exhibit 3'-5' exonuclease activity (Aliotta et al., 1996).

Vent DNA polymerase (*Thermococcus litoralis*) has a low processivity of 7 nt/initiation event (*Taq* DNA polymerase: 40 nt/initiation event), a temperature depend strand displacement activity and 3'-5' exonuclease activity. This enzyme is thermostable (Kong et al., 1993).

Polymerases exhibiting a very high strand displacement activity like Phi29 DNA polymerase, *Bst* DNA polymerase and *Tfu* DNA polymerase (*Thermococcus fumicolans*) do not slip off the template strand during replication. In contrast, polymerases without strand displacement activity (*Pfu* DNA polymerase (*Pyrococcus furiosus*), *E. coli* DNA polymerase II and T4 DNA polymerase) can easily slip off. The strand displacement activity of *Taq* DNA polymerase, Vent DNA polymerase, *E. coli* DNA polymerases I and III and T7 DNA polymerase is influenced by the reaction conditions. The addition of SSB to the reaction can result in an increased strand displacement activity and reduced rate of slippage. The influence of SSB varies for each polyerase (Canceill et al., 1999, Viguera et al., 2001).

II-3.2.6e) Improvement of CRCA by the use of additives

The specificity of an *in vitro* amplification technique can be enhanced by varying the reaction conditions like Mg-concentration, denaturation and annealing temperature. In some cases this is still insufficient. Additives for suppressing the amplification of nonspecific fragments on PCR can be glycerol, nonyl phenoxy-polyethoxy-ethanol (NP-40), dimethyl sulfoxide (DMSO), formamide, Tween-20, Triton X-100, ethanol, urea and *N*-Methyl-2-pyrolidone (NMP). However, their functionality regarding inhibition and yield of PCR has to be determined for each experiment (Varadaraj and Skinner, 1994). Bovine serum albumin (BSA), dithiothreitol (DTT) and glycerol are able to support the PCR amplification either single or in combination (Nagai et al., 1998). Betaine, DMSO and a combination of both chemicals, respectively, can enhance the PCR efficiency (Kang et al., 2005) especially on GC-rich sequences (Baskaran et al., 1996, Jensen et al., 2010). Betaine decreases the melting temperature of DNA depending on the base composition rather by stabilizing AT-pairs than by destabilizing GC-pairs resulting in a destabilized DNA double helix (Rees et al., 1993). Besides, betaine increases the thermostability of proteins (Santoro et al., 1992). DMSO decreases the inter- and intra-strand reannealing (Varadaraj and Skinner, 1994). Another additive used in PCR is trehalose. This non-reducing disaccharide reduces the melting temperature of DNA to a similar extent as betaine (Spiess et al., 2004, Hart et al., 2010). Another characteristic of trehalose is its capability of thermoprotection, -stabilization and -activation of enzymes (Carninci et al., 1998).

Betaine, BSA, T4 gene 32 protein and proteinase inhibitors can overcome the inhibitory effect of blood, faeces and meat samples on PCR (Al-Soud and Rådström, 2000).

An increased activity of the polymerase during RCA can be observed by adding supporting proteins like *E. coli* SSB and T4 gene 32 protein (Lizardi et al., 1998). The addition of T4 gene 32 protein and DMSO increases the amplification rate of CRCA driven by Phi29 DNA polymerase (Zhang et al., 2001a) as well as of CRCA driven by *Bst* DNA polymerase (Zhang et al., 2001b). The mutant SSB-225 of *Thermus thermophilus* reduces unspecific background amplification of RCA in contrast to the wild-type protein (Inoue et al., 2006, Mikawa et al., 2009).

II-3.2.6f) Techniques to improve background amplification to signal ratio

Background amplification besides specific amplification is reported to be a common problem associated to CRCA. This is caused by the amplification of CLPs which are not ligated.

A procedure to avoid background amplification is the exonucleolysis of all linear CLPs (Nilsson et al., 1994, Zhang et al., 1998, Faruqi et al., 2001, Banér et al., 2003, Dahl et al., 2004, Millard et al., 2006, Wang et al., 2009, Wang et al., 2010). A similar approach is to remove non circularized CLPs by stringent washes upon the ligation reaction either applicable to *in situ* experiments (Nilsson et al., 1994, Nilsson et al., 1997, Lizardi et al., 1998, Larsson et al., 2004, Li and Zhong, 2007) or target directed nucleic acid extraction by magnetic beads (Hsuih et al., 1996, Zhang et al., 2001a).

II-3.2.7 Loop-mediated isothermal amplification (LAMP)

The LAMP technique invented by Notomi et al. (2000) utilizes two pairs of target specific primers and *Bst* DNA polymerase (large fragment) at elevated temperatures of 60 - 65 °C. Two primers, forward inner primer (FIP) and backward inner primer (BIP), are designed to contain a sequence, F2 and B2, at the 3'-end complementary to the target strand region, F2c and B2c, and a homologous sequence at the 5'-end, F1c and B1c (Fig. II-3-7A). The F1c and B1c regions are located downstream the F2c and B2c sequences on the respective target strand. The remaining primers F3 and B3, equal to PCR primers in design, target the regions F3c and B3c which are located upstream of F2c and B2c (Fig. II-3-7A). For initiation of the reaction, primer B3 as well as primer BIP hybridize to the complementary sequence (Fig. II-3-7B). Upon primer B3 elongation the strand beginning at BIP is released due to the strand displacement activity of the polymerase. Since the B1c region is now complementary to the region B1 located on the same strand, a loop is formed (Fig. II-3-7C). This strand serves as template for F3 and FIP resulting in a loop-stem-loop structure (Fig. II-3-7D+E) which is the starting point of the actual amplification. On the one hand, the loop exhibiting the free 3'-end is elongated by the polymerase. On the other hand, primer BIP anneals to the same loop leading to DNA synthesis and displacement of the recently synthesized strand (Fig. II-3-7F+G). At the 3'-end of this strand, a loop is formed due to self complementary sequences. The 3'-end is elongated by *Bst* DNA polymerase (large fragment) and primer FIP binds to the loop which is also elongated (Fig. II-3-7H). The combination of loop forming, inner primer annealing,

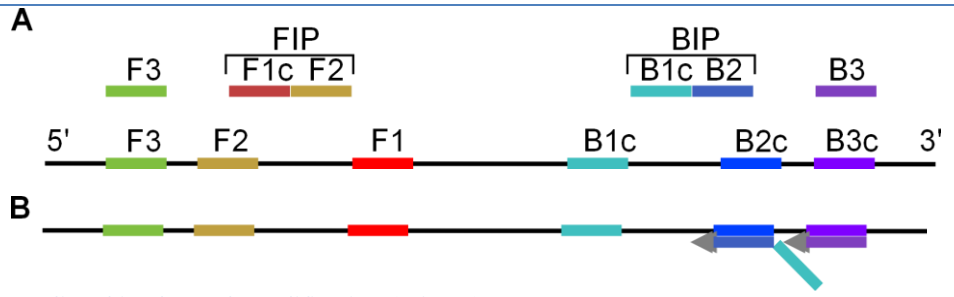


Fig. II-3-7: Loop-mediated isothermal amplification (LAMP)
The basic design of LAMP utilizes four primers: two outer primers F3 and B3 and two inner primers FIP and BIP which contain a target complementary and a target homologous sequence (A). BIP and B3 hybridize to the target strand (B) and are elongated by *Bst* DNA polymerase, by what the BIP strand is released and a loop is formed due to the self complementary sequences (C). This also happens in the opposite direction (D) resulting in ssDNA with loops at both ends (E). The polymerase elongates the 3'-end of the loop as well as BIP which has annealed to the same loop (F+G). At the resulting loop DNA synthesis is initiated at its 3'-end and at FIP (H). The DNA molecule is extended by each elongation step. Displaced strands are available for amplification as well (I) (according to Notomi et al. (2000)).

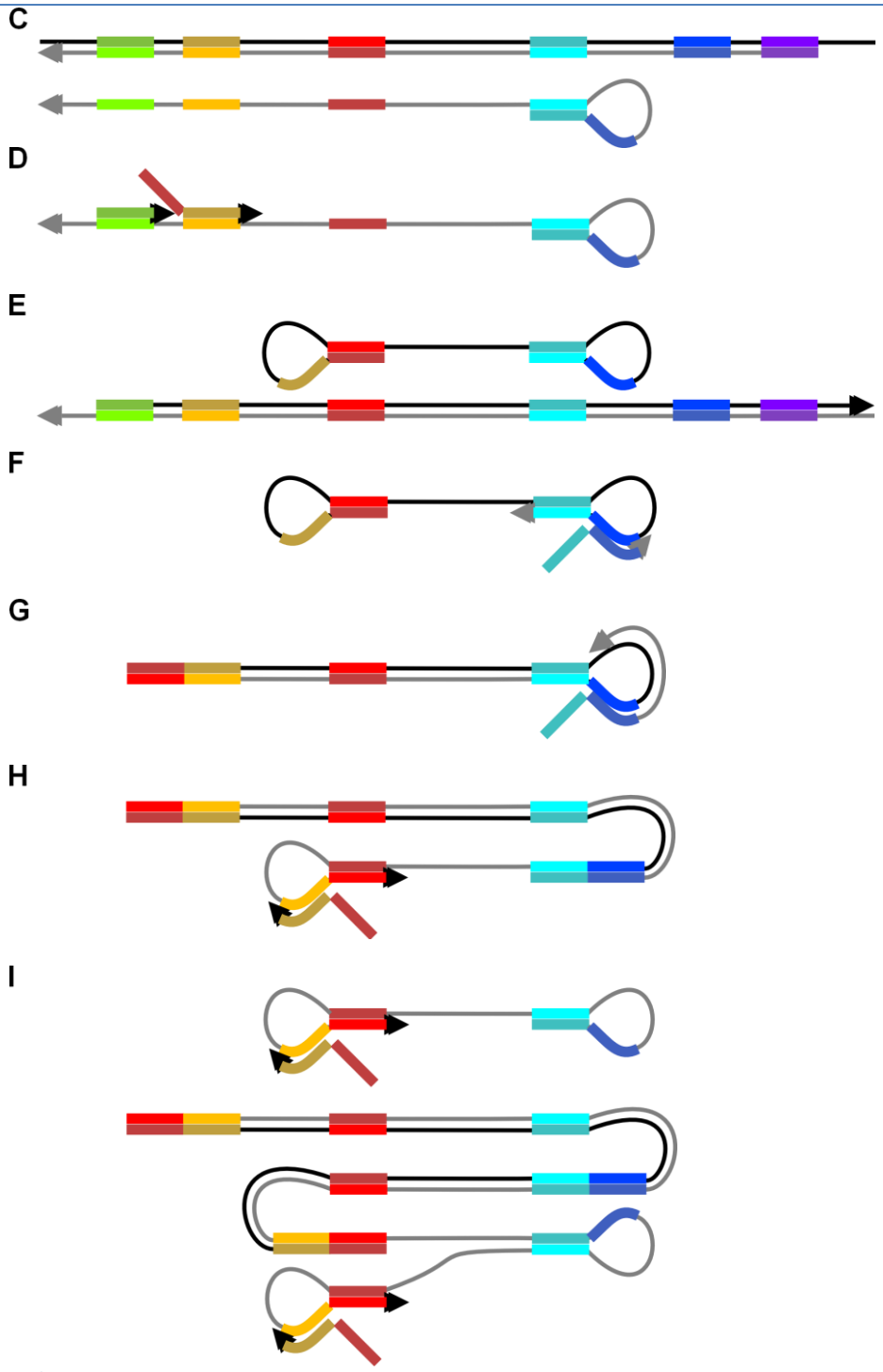


Fig. II-3-7 continued

elongation and strand displacement results in a self-employed growing of the DNA molecule and in releasing of DNA strands which, in turn, are subjected to the amplification (Fig. II-3-7I; Notomi et al., 2000). Addition of primers F-loop and B-loop accelerates the reaction and enhances the amplification process. These primers bind to the loops which are not targeted by the inner

primer and initiate the DNA synthesis as well (Nagamine et al., 2002).

The possibility of direct RNA detection by adding a reverse transcriptase to the LAMP reaction enables a one-step RT-LAMP avoiding the need for a time consuming separate cDNA synthesis prior to the amplification (Fukuta et al., 2003a).

To maintain the temperature which is optimal for the LAMP reaction, no sophisticated laboratory equipment is needed as for PCR. A heating block or water bath is sufficient for conducting the reaction (Notomi et al., 2000), but also disposable pocket warmers can be used (Hatano et al., 2010).

Important human pathogens like *Salmonella enterica*, verotoxin producing *Escherichia coli*, severe acute respiratory syndrome corona virus, influenza viruses (including avian influenza virus subtype H5N1), *Mycobacterium tuberculosis*, *Plasmodium falciparum* and human immunodeficiency virus facilitating the diagnosis especially in developing countries (Parida et al., 2008, Mori and Notomi, 2009). In veterinary medicine, LAMP based pathogen detection gain weight (Fall et al., 2008, Liu et al., 2008, Mekata et al., 2008, Saleh, 2009, Nemoto et al., 2010). For diagnosis of phytopathogens LAMP is used as well. Especially in recent years the number of published LAMP assays increased considerably (Tab. II-3-1). Authentication of plant species is also possible by LAMP (Chaudhary et al., 2011). The LAMP technique can be used for the detection of genetically modified organisms (Ahmed et al., 2009, Lee et al., 2009, Liu et al., 2009a) and for the identification of embryo sex (Zhang et al., 2008). Li et al. (2011a) established a LAMP assay for the ultrasensitive detection of miRNAs. The application of LAMP for *in situ* analysis is possible as well (Liu et al., 2009b). LAMP can be used for the detection of SNPs (Fukuta et al., 2006).

Tab. II-3-1: List of phytopathogens for which a LAMP based detection protocol has been published

Phytopathogen	Protocol established by
Japanese yam mosaic potyvirus (JYMV)	Fukuta et al. (2003a)
Tomato yellow leaf curl virus (TYLCV)	Fukuta et al. (2003b)
Tomato spotted wilt virus (TSWV)	Fukuta et al. (2004)
Potato virus Y (PVY)	Nie (2005)
Plum pox virus (PPV)	Varga and James ()
Phytophthora ramorum	Tomlinson et al. (2007) Tomlinson et al. (2010b)
Ralstonia solanacearum	Kubota et al. (2008)
Peach latent mosaic viroid (PLMVd)	Boubourakas et al. (2009)
Bursaphelenchus xylophilus	Kikuchi et al. (2009)
Pseudomonas syringae pv. phaseolicola	Li et al. (2009b)
Botrytis cinerea	Tomlinson et al. (2010c)
Squash leaf curl virus (SLCV)	Kuan et al. (2010)
rice viruses	Le et al. (2010)
Tobacco mosaic virus (TMV)	Liu et al. (2010)
Fusarium graminearum	Niessen and Vogel (2010)
Citrus bacterial canker (CBC)	Rigano et al. (2010)
phytoplasmas	Tomlinson et al. (2010a) Obura et al. (2011)
Potato spindle tuber viroid (PSTVd)	Tsutsumi et al. (2010)
Xylella fastidiosa	Harper et al. (2010)
Barley yellow dwarf viruses (BYDV)	Zhao et al. (2010)
Cymbidium mosaic virus (CymMV)	Lee et al. (2011)

II-3.2.7a) Homogenous visualisation of the LAMP product

Gel electrophoresis is eliminated by using real-time PCR, which utilizes intercalating fluorescence dyes or primer and probe designs based on the FRET mechanism. Intercalating fluorescence dyes such as SYBR® Green I (Iwamoto et al., 2003, Sun et al., 2006, Zhang et al., 2008, Li et al., 2009b, Liu et al., 2009a, Saleh, 2009, Koide et al., 2010, Yin et al., 2010, Zhao et al., 2010), PicoGreen® (Dukes et al., 2006, Tomlinson et al., 2007, En et al., 2008, Liu et al., 2008) and GeneFinder™ (Liu et al., 2010) can be used to monitor the LAMP reaction as well either in real-time or as end point observation by real-time thermal cyclers or hand held UV-lights. Naked eye inspection is also possible but the dyes have to be added after reaction completion (Tomlinson et al., 2007, Liu et al., 2008, Li et al., 2009b, Liu et al., 2009a, Saleh, 2009, Zhao et al., 2010) since high concentrations inhibiting the amplification of such dyes are required for this purpose (Tomlinson et al., 2007). However, primer dimers could also lead to the intercalation of this dye and could hinder the interpretation of the results (Varga and James, 2006b). The opening of tubes upon amplification increases the risk of cross contaminations. The colour change from orange to green may be observed difficultly (En et al., 2008, Goto et al., 2009).

The visualisation of amplification can be achieved by the use of lateral flow devices (LFD). FIP has to be 5'-biotinylated. Following the LAMP reaction, a fluorescein isothiocyanate (FITC) labelled DNA probe which is specific to amplified sequence is added. Afterwards, the LFD is placed in the reaction tube. The FITC probes move chromatographically along the LFD due to gold-labelled anti-FITC antibodies. A streptavidin coated area recognizes the biotinylated amplicons resulting in a colouration of this area. FITC probe which did not hybridize to an amplicon due to a negative result or saturation of amplicons are visualized separately for means of control (Jaroenram et al., 2008, Kiatpathomchai et al., 2008, Nimitphak et al., 2008, Kikuchi et al., 2009, Ding et al., 2010, Tomlinson et al., 2010d). Kiatpathomchai et al. (2008) and Ding et al. (2010) observed a higher sensitivity applying this approach than by conventional gel electrophoresis. As for the use of SYBR® Green I or PicoGreen® for naked eye inspection the use of LFDs requires post amplification handling of the LAMP reaction including the reopening of the reaction tube. Additional costs are incurred by reason of biotinylated FIPs, FITC labelled DNA probes and the LFDs themselves.

For direct visualisation of the LAMP product to avoid the gel electrophoresis, magnesium pyrophosphate precipitation can be used. Pyrophosphate is produced in large excess during the LAMP reaction as by-product of DNA synthesis. Magnesium and pyrophosphate precipitate. In LAMP, more than 10 µg DNA are synthesized in a reaction volume of 25 µl within 60 min. In contrast, DNA synthesis of PCR using the outer primers of the LAMP reaction yields about 0.2 µg DNA in an equal reaction volume. The low pyrophosphate concentration of approximately 0.02 mM in PCR is insufficient to precipitate with magnesium. Compared to PCR, LAMP produces at least 0.5 mM pyrophosphate. The pyrophosphate can be hydrolysed in the denaturation step of PCR due to heat but not at the temperature used in LAMP (Mori et al., 2001).

The turbidity caused by the accumulation of magnesium-pyrophosphate-precipitates can be observed with the naked eye. Upon centrifugation

of the LAMP reaction, a pellet can be seen more clearly (Mori et al., 2001). This is insufficient in the case of pathogen detection because slightly positive samples can be declared as negative ones (Varga and James, 2006b). Sun et al. (2006) showed that the detection limit based on naked eye observation of turbidity was 500 copies of plasmid, whereas gel electrophoresis and addition of SYBR® Green I revealed a detection limit of 5 copies. In comparison, the tubes containing a positive and a negative LAMP reaction depicted by Tsutsumi et al. (2010) can both be declared as positive because there is turbidity visible in the negative reaction as well. Several studies report on the use of turbidity to replace gel electrophoresis for visualisation of LAMP products (Fukuta et al., 2003a, Nie, 2005, En et al., 2008, Kubota et al., 2008, Zhang et al., 2008). Technical equipment (spectrophotometer) to measure the turbidity is available (Nie, 2005, Kubota et al., 2008) but this is contrary to the aim of technical simplification.

Upon exceeding the threshold of pyrophosphate solubility, the amount of synthesized DNA and the accumulation of magnesium-pyrophosphate-precipitates correlate linearly. As a consequence, the time course of increasing turbidity can be used to quantify the initial amount of DNA using a real-time turbidimeter. Similar to quantification by real-time PCR, which is achieved by determining the quantification cycle (C_Q) at which cycle a certain value is exceeded by the fluorescence measured, the DNA is quantified by identifying the time threshold (T_T) indicating the time at which the turbidity surmounts the threshold value (Mori et al., 2001, Mori et al., 2004). The application of real-time LAMP as well as of real-time RT-LAMP for the quantification of DNA and RNA, respectively, is emerging (Parida et al., 2004, Fukuta et al., 2006, Mekata et al., 2008, Perera et al., 2008, Kubo et al., 2010, Nemoto et al., 2010, Saitou et al., 2010, Tsutsumi et al., 2010).

Due to the magnesium pyrophosphate precipitation, the concentration of magnesium ions declines. Thus, the DNA synthesis can be indirectly visualized by the addition of a metal ion indicator suitable for the detection of decreasing magnesium concentration to the solution prior to the amplification. Such metal ion indicator can be either calcein (Yoda et al., 2007, Tomita et al., 2008) or hydroxy naphthol blue (HNB; Goto et al., 2009). Calcein emits strong fluorescence upon forming complexes with divalent metal ions. However, addition of sole calcein does not result in a visible colour change. Additionally supplemented manganese ions quench the fluorescence of calcein. In the course of the amplification the pyrophosphate precipitates with these manganese ions. As a consequence, calcein complexes with magnesium ions result in increasing fluorescence. In visible light the colour change from light orange to light green is difficult to observe, but this can be circumvented by the use of UV-light (Yoda et al., 2007, Tomita et al., 2008, Goto et al., 2009). Several LAMP protocols utilizing calcein have been established (Saleh, 2009, Hatano et al., 2010, Nemoto et al., 2010, Le et al., 2010, Niessen and Vogel, 2010).

If the reaction mix is supplemented with HNB, the colour of the mix will change from purple to blue in positive samples and will remain unchanged in negative samples (Goto et al., 2009). The absorbance values measured at 650 nm can be used for differentiating positive and negative results of LAMP amplification of DNA as well (Goto et al., 2009, Ma et al., 2010). HNB is superior for visualisation of LAMP

products since the colour change can be assessed by the naked eye, sole HNB has to be added prior to the reaction and tubes do not have to be opened upon amplification. Due to these advantages HNB is increasingly applied for visualisation of a positive LAMP reaction (Goto et al., 2010, Harper et al., 2010, Ma et al., 2010, Tomlinson et al., 2010a).

Addition of $CuSO_4$ after completion of the amplification results in ring-shaped deposit in the middle of the reaction volume in negative samples but not in positive samples. This is caused by consumption of dNTPs during DNA synthesis in positive LAMP reactions, whereas the concentration of dNTPs remains stable in negative reactions (Zhang et al., 2008).

Coupling a bioluminescent assay in real-time (BART) to LAMP allows for qualitative as well as for quantitative detection of target DNA. Beside the reagents needed for amplification, a LAMP-BART reaction contains adenosine- 5'-O-phosphosulfate (APS), ATP sulfurylase, luciferin and firefly luciferase. Pyrophosphate derived from the DNA synthesis and APS are converted to ATP by ATP sulfurylase. The firefly luciferase utilizes ATP and luciferin to produce oxyluciferin and light which can be measured (Gandelman et al., 2010). However, a costly CCD-camera within a light box or diode based device is needed for analysis.

The above mentioned approaches for visualisation of LAMP amplicons share a basic characteristic: They all can be observed in any case of DNA amplification either specific or non specific. Mori et al. (2006) added DNA probes specific to the single stranded loops occurring during the LAMP reaction. These probes are similar to the loop primers used for enhancing the amplification rate. The DNA probes are labelled with fluorescent dyes such as FITC or Rox. They specifically hybridize to the LAMP products. Following the amplification the cationic polymer polyethylenimine (PEI) is added to the reaction mixture. Due to its positive charge it forms insoluble complexes with the negatively charged DNA. By centrifugation the complexes are pelletized. As the molecular weight of PEI is low, it does not complex with unreacted DNA probes. The fluorescence of bound probes is visible under UV-light. This technique allows for multiplex detection of different targets, i.e. gene of interest or pathogen and an internal control. However, like SYBR® Green I it requires the post amplification opening of tubes.

II-3.2.7b) Simplified sample preparation

High quality RNA and DNA, respectively, as template are crucial for molecular biological applications such as PCR and qPCR. Diverse commercial kits and reagents are available to obtain nucleic acids highly purified and in high yields. They are adapted to miscellaneous sample material derived either from humans and animals or plants. However, the kits and reagents are costly and often require the use of toxic chemicals like β-mercaptoethanol and chloroform. Laboratory equipment is needed as well. In parts, the protocols using these kits and reagents are time consuming and include numerous working steps.

A second aim of this study is to simplify the handling and the preparation of samples to obtain nucleic acids suitable to serve as template for a nucleic acid based detection system but to avoid the drawbacks of kits and reagents mentioned above.

For sampling and sample storage nitrocellulose membranes, nylon membranes or so called FTA cards® can be used. Leaves used for analysis are pressed on the membrane until plant sap is soaked by the membrane. The membrane is subjected to downstream applications such as DNA extraction and PCR (Owor et al., 2007, Capote et al., 2009). For the detection of phytopathogens whose genomes are made of DNA the membrane based sampling may be sufficient. RNA might be degraded by RNases.

Immunocapture of PPV prior to RT-PCR lowers the detection limit significantly due to a higher reaction volume and partial elimination of inhibitory substances present in the plant suspension (Wetzel et al., 1992). This procedure requires the use of PPV specific antibodies.

Wetzel et al. (1991b) ground plant samples in the presence to sterile water in plastic bags containing a membrane used for ELISA. The plant extract was centrifuged. The supernatant was tenfold diluted and then used as template for RT-PCR. This is also applicable at a 1.5 ml reaction tube scale (Lee et al., 2009). Similar to this approach Capote et al. (2009) used phosphate buffered saline (PBS) supplemented with 2 % polyvinyl pyrrolidone (PVP) and 0.2% sodium diethyl dithiocarbamate for homogenizing plant material in ELISA bags.

Biotinylated DNA probes specific to the target can be used for nucleic acid extraction. The probes hybridized to the target are bound to paramagnetic beads coated with streptavidin. Due to magnetic separation the target nucleic acid is purified and can be used as template for PCR. For release and purification of the viral genome guanidinium thiocyanate is used (Hsuih et al., 1996, Zhang et al., 1998). This procedure is rather expensive because of biotinylated probes and paramagnetic beads. Guanidinium thiocyanate is toxic.

Nucleic acids can be released from tissues without homogenization. Therefore, the tissue is infiltrated and incubated in a solution containing potassium ethyl xanthogenate (PEX) followed by precipitation of nucleic acids. If necessary, this pellet can subjected to the removal of polysaccharides and phenols by treatment with 2-butoxyethanol and HCl. The resolved nucleic acid can serve as template for RT-PCR (Nakahara et al., 1999). Due to several working steps and the use of toxic chemicals this preparation is not suitable for the simplification of sample preparation.

III Aim of this study

The ELISA technology is widespread used for the detection of proteins in general and of pathogens in particular. Its advantages compared to other detection systems are the reliability, the possibility to process numerous samples in parallel, low costs and easy set up. The duration of the protocols and the low sensitivity, however, are disadvantageous. The detection of specific nucleic acid sequences by PCR has been established since the invention of this technology. High sensitivity and high specificity as well as short reaction times also account for the distribution of this nucleic acid amplification technique. However, drawbacks are high costs for technical equipment and fluorescent chemistry needed for qPCR, increased risk of cross contaminations of samples and the need for highly purified DNA and RNA to serve as template.

The aim of this work is to develop a nucleic acid based detection method compromising the advantages of PCR while avoiding its drawbacks resulting in a PPV detection method, which is reliable, fast, inexpensive, highly sensitive and highly specific.

Isothermal nucleic acid amplifications techniques exhibit detection limits comparable to PCR without the need for costly technical devices for thermal cycling. Due to numerous approaches to enable specific synthesis of DNA or RNA at first the CRCA technique was chosen to develop a new PPV detection system for following reasons: i) High specificity based on the ligase mediated ring closure of an oligonucleotide probe hybridized to PPV cDNA or RNA. ii) Simple design of CRCA primers and DNA probe. iii) Use of universal CRCA primers for general detection of PPV as well as for strain typing. iv) Possibility to create a multiplex reaction for strain typing. v) *in situ* detection of PPV. vi) High sensitivity. The CRCA has to be combined with a homogenous visualisation and a simplified sample preparation for rapid diagnostic of PPV.

It is compared to a previously published protocol for detection of PPV based on the LAMP technology. LAMP exhibits a very high sensitivity due to the enormous rate of amplification and a very high specificity because of up to eight specific sequences which have to be recognized for amplification. However, the design of LAMP primers is more complex compared to primers used in CRCA or PCR. A big advantage of LAMP is the grouping of several working steps: reverse transcription of RNA, detection and amplification can be conducted at once. Due to the high amount of DNA synthesised during LAMP, a homogenous visualisation of amplification is possible based on the accumulating pyrophosphate-magnesium-complexes. Additionally, preparation of samples has to be simplified. Thereby, the time needed for diagnosis as well as the risk of cross contamination is greatly reduced.

IV Material and Methods

IV-1. Conventional RNA extraction

Plant material (100 mg) grinded in liquid nitrogen was applied to the RNA extraction using the Concert™ RNA Plant Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's manual: After adding 0.5 ml Concert™ RNA Plant Reagent the mixture was incubated for 5 min and centrifuged for 2 min at 12,000 rpm at room temperature. The supernatant was transferred to a 1.5 ml reaction tube and supplemented with 0.1 ml of 5 M NaCl and 0.3 ml of chloroform followed by centrifugation for 10 min at 4 °C. The aqueous phase was transferred to a 1.5 ml reaction tube and incubated with an equal volume of isopropyl alcohol for 10 min at room temperature. The RNA was obtained by centrifugation for 10 min at 12,000 rpm and 4 °C. One ml of 75 % ethanol was added to the pellet and centrifuged at room temperature for 1 min at 12,000 rpm. After removing the ethanol the pellet was resolved in 100 µl RNase free water.

The RNA solution was applied to RT-LAMP, to cDNA synthesis as well as to one-step multiplex RT-PCR or stored at -80 °C until use.

IV-2. Controls

Positive controls refer to a sample derived from a PPV infected plant and negative controls to a sample derived from a plant not infected by PPV as proven by RT-PCR. For the no template control (NTC), an appropriate volume of water was applied to the diverse reactions instead of template RNA or cDNA.

IV-3. cDNA synthesis

In case of RT-PCR and CRCA, the RNA was first reversely transcribed to cDNA prior to DNA amplification. One µl of oligo(dT)-primer or primer cDNA 1 (25 µM, Tab. IV-4-1) and 1 µg of RNA were mixed and incubated at 75 °C for 5 min and then cooled down on ice. The final reaction volume was 25 µl with 50 mM Tris-HCl (pH 8.3 at 25 °C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs (New England Biolabs, Frankfurt, Germany) and 200 U of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Mannheim, Germany). The cDNA synthesis was carried out at two temperatures: 40 °C for 5 minutes for optimal annealing of the primer followed by 48 °C for 35 minutes for optimal elongation. The cDNA was stored at -20 °C or placed on ice for direct use.

IV-4. PCR

IV-4.1 PPV detection in general

The PCR detection of PPV was based on the protocol of Wetzel et al. (1991b), which was slightly modified (Bühler, 2007). The reaction conditions were 12.5 mM Tris-HCl (pH 8.8 at 25 °C), 62.5 mM KCl, 0.1 % Nonidet P40, 1.875 mM $MgCl_2$, 0.3 mM dNTPs (New England Biolabs, Frankfurt, Germany), 1 µM primers P1 and P2 and 0.5 U of *Taq* DNA Polymerase (Fermentas, St. Leon-Rot, Germany). The primers (Tab. IV-4-1) were HPLC purified and purchased from Microsynth (Balgach, Switzerland) or Eurofins MWG Operon (Ebersberg, Germany), respectively. One µl of cDNA was added to the reaction.

The PCR schedule included five steps: 94 °C for 2 min, then 40 times 94 °C for 30 sec, 61.5 °C for 30 sec and 72 °C for 1 min. The reaction was completed with a final elongation for 5 min at 72 °C. It was run on a C1000 cycler (Bio-Rad, Munich, Germany).

IV-4.2 Strain typing

The 20 µl PCR reaction mixture contained 1 µl cDNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08 % Nonidet P40, 1.875 mM $MgCl_2$, 0.3 mM dNTPs, 1.25 µM sense and antisense primer respectively and 0.5 U *Taq* DNA Polymerase (Fermentas, St. Leon-Rot, Germany). PPV in general was detected by an equimolar mixture of 5' primers M3-5' and M4-5' in combination with the 3' primer M2-3' (Tab. IV-4-1). Subsequently, strain specific primers assigning PPV-D (M1-5', M5-3'), PPV-M (M6-5', M7-3'), PPV-EA (M8-5', M9-3') and PPV-C (M10-5', M11-3') (Tab. IV-4-1) were used in a nested PCR in which 2 µl 1:500 diluted PCR product from the

Tab. IV-4-1: Primers used for cDNA synthesis and PCR

Name	Sequence	Target	Amplicon size	Origin
P1	ACC GAG ACC ACT ACA CTC CC	PPV	243 bp	Wetzel et al. (1991b)
P2	CAG ACT ACA GCC TCG CCA GA			
M3-5'	TCC AAC RTT GTT RTR CAC CA	PPV	~ 500 bp	Szemes et al. (2001)
M4-5'	TCC AAY RTA GTK GTS CAT CA			
M2-3'	CGY YTR ACT CCT TCA TAC CA			
M1-5'	GCA GCA ACT AGC CCA ATA MT	PPV-D	159 bp	
M5-3'	TGT TCC AAA AGT TTG CRR TTG AGG T			
M6-5'	GYG GCA ACR ACT CAA CCA G	PPV-M/Rec	207 bp	
M7-3'	CCT TCC TGY RTT CAC CAA AGT			
M8-5'	TAG TCA CCA CTA CAC AGC AG	PPV-EA	167 bp	
M9-3'	AGG AGG TGT AGT AGT TGT TG			
M10-5'	GGG AAA TGA TGA CGA CGT AAC TCT	PPV-C	224 bp	
M11-3'	CAA TTA CCC CAT ACG AGA AT			
M14-5'	GGA AGA TGA CGA TCC AAC TAT	PPV-W	185 bp	this study
M15-3'	ATG TAG TTG GCT TCA CGC T			
mD5	TAT GTC ACA TAA AGG CGT TCT C	PPV-D	664 bp	Šubr et al. (2004)
mD3	GAC GTC CCT GTC TCT GTT TG			
mM5	GCT ACA AAG AAC TGC TGA GAG	PPV-M	459 bp	
mM3	CAT TTC CAT AAA CTC CAA AAG AC			
mD5	TAT GTC ACA TAA AGG CGT TCT C	PPV-Rec	605 bp	
mM3	CAT TTC CAT AAA CTC CAA AAG AC			
PPV-RR	CTC TTC TTG TGT TCC GAC GTT TC	PPV	345 bp	Jarošová and Kundu (2010)
F3	GGA ATG TGG GTG ATG ATG G			
PDVdpR	CCT TTA ATG AGT CCG T	PDV	220 bp	
PDVdpuF	CCG AGT GGA TGC TTC ACG			
PNcpR	CTT TCC ATT CGG AGA AAT TCG	PNRSV	425 bp	
PNcpinF	GAG TAT TGA CTT CAC GAC CAC			
Nad5-R	CTC CAG TCA CCA ACA TTG GCA TAA	Plant (*nad5*)	181 bp	
Nad5-F	GAT GCT TCT TGG GGC TTC TTG TT			
oligo(dT)	ACC CGT CGA TAG TAC TTT TTT TTT TTT TTT TT	oligo A RNA		Bühler (2007)
cDNA 1	TAC CTT GGC ATG TAT GCT	PPV		this study

first reaction served as template (Szemes et al., 2001). PCR conditions for all reactions mentioned above were as follows: after an initial denaturation at 94 °C for 2 min 35 rounds of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 1 min elongation at 72 °C were performed. The reaction was completed by an elongation at 72 °C for 5 min.

Since the primers of the nested PCR hybridise to a region situated in the coding sequence of the coat protein (Szemes et al., 2001) and the breakpoint of recombination is located in the C terminus of the NIb gene (Glasa et al., 2004) the primers M6-5' and M7-3' do not only detect isolates of the PPV-M strain but also the isolates grouped to PPV-Rec. Šubr et al. (2004) created primers to differentiate between strain PPV-D (mD5, mD3), PPV-M (mM5, mM3) and PPV-Rec (mD5, mM3) (Tab. IV-4-1). The PCR conditions were the same except that the amplification was done in 40 cycles with 61.5 °C annealing temperature.

IV-4.3 One-step multiplex RT-PCR

Another PCR based PPV detection protocol was developed by Jarošová and Kundu (2010). The one-step multiplex RT-PCR is able to detect the RNA of PPV, PDV, PNRSV and the RNA transcript of *nad5* gene as internal control in parallel. The SuperScript® III One-Step RT-PCR System with Platinum *Taq* (Invitrogen, Darmstadt, Germany) was used with the 25 µl reaction mixture containing 12.5 µl 2x reaction mix, 0.24 µM of each primer (PPV: PPV-RR and F3, PDV: PDVdpR and PDVdpuF, PNRSV: PNcpR and PNcpinF, *nad5*: Nad5-R and Nad5-F, Tab. IV-4-1), 2 µl RNA and 0.6 µl enzyme mix. The primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). Using a C1000 cycler (Bio-Rad, Munich, Germany) the parameters were: reverse transcription at 51 °C for 30 min and for PCR 94 °C for 2 min, 33 cycles 94 °C for 30 sec, 51 °C for 45 sec and 68 °C for 80 sec and a final incubation at 68 °C for 7 min.

IV-5. CRCA

For the detection of PPV by CRCA, PPV RNA was first reversely transcribed to cDNA. Ligation and amplification parameters were adapted from Thomas et al. (1999) for initial experiments. These reaction conditions were used throughout this work otherwise stated.

IV-5.1 Ligation conditions

A 15 µl ligation reaction contained 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, 0.01% Triton® X-100, 0.12 µM CLP, 2 µl cDNA and 0.5 U of Ampligase® Thermostable DNA Ligase (Epicentre Biotechnologies, Madison, USA). After denaturation at 95 °C for 5 min the ligation was incubated at 63 °C for 15 min. Two µl of the ligation reaction were added to the CRCA mixture.

Numerous different ligases are available. To test which DNA joining enzyme provides the optimal basis for the following amplification, *Taq* DNA Ligase, 9°N™ DNA Ligase and T4 DNA Ligase (New England Biolabs, Frankfurt, Germany) were compared to Ampligase® Thermostable DNA Ligase. The reaction conditions of each ligase are listed in Tab. IV-5-1.

For increased sensitivity the cyclic repetition of ligation using Ampligase® Thermostable DNA Ligase was evaluated. Each ligation cycle comprised denaturation at 95 °C for 5 min and li-

Tab. IV-5-1: Ligases used for circularizing of CLPs upon hybridisation to PPV cDNA
Each ligation reaction had a volume of 15 µl containing appropriate buffer provided by the supplier, 2 µl cDNA and enzyme in the concentration (U) as listed below. *Taq* - *Taq* DNA Ligase; 9°N - 9°N™ DNA Ligase; T4 - T4 DNA Ligase; Ampligase - Ampligase® Thermostable DNA Ligase

Ligase	U	Denaturation	Ligation
Taq	12	3 min at 95 °C	15 min at 64 °C
9°N	20	3 min at 95 °C	15 min at 64 °C
T4	200	3 min at 95 °C (without enzyme)	15 min at 37 °C (followed by 10 min at 65 °C for inactivation)
Ampligase	0.5	3 min at 95 °C	15 min at 64 °C

gation at 65 °C for 15 min. Up to six cycles were conducted in a thermal cycler (PCR-Express, Hybaid, Ashford, UK).

For the design of the CLPs predominantly whole genome sequences covering isolates of all known PPV strains derived from the NCBI nucleotide database were aligned using the Clustal W2 software (Larkin et al., 2007). The aligned sequences were handled using the BioEdit software especially for visualisation purposes. CLPs were tested by BLAST (http://blast.ncbi.nlm.nih.gov/) to be specific to PPV. Primer parameters were calculated by using the OligoAnalyzer located at the IDT website in default settings (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/).

Several CLPs varying in target specific regions, annealing sites for primers and design were tested for their suitability for CRCA based detection of PPV (Tab. V-2-1). The CLPs were synthesised, 5'-phosphorylated and HPLC purified by Microsynth (Balgach, Switzerland) or Eurofins MWG (Ebersberg, Germany). All CLPs were able to recognise all strains of PPV known so far (Fig. V-2-2).

IV-5.2 Exonucleolysis of unreacted CLPs

To reduce background amplification, the exonucleolysis of CLPs which were not circularized during ligation was tested. Therefor, a 6 µl exonucleolysis reaction contained 3.5 µl of the ligation product and either 25, 35 or 45 U of Exonuclease I (Fermentas, St. Leon-Rot, Germany). The reaction was incubated at 37 °C for 30 min and terminated at 85 °C for 15 min. The whole volume of this reaction was applied to the following CRCA.

IV-5.3 Reaction conditions for the amplification of the ligated CLP

The 25 µl amplification reaction contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 200 µM dNTPs, 1 µM of each primer FWD and primer REV, 1.6 U of *Bst* DNA polymerase (large fragment) (New England Biolabs, Frankfurt, Germany) and 2 µl of the ligation reaction. Incubation lasted 1 h at 65 °C.

The range of polymerases that meet the demand of high strand displacement activity is limited. *Bst* DNA polymerase (large fragment) was tested against IsoTherm™ DNA and DisplaceAce™ DNA Polymerase (Epicentre Biotechnologies, Madison, USA), both active at 65 °C, and Phi29 DNA Polymerase (New England Biolabs, Frankfurt, Germany). The last one exhibits a very high processivity with its optimum at 37 °C (Tab. IV-5-2).

For optimisation of the CRCA diverse additives were tested: betaine, DMSO, DTT (Sigma-Aldrich, Munich, Germany) and ET SSB (New England Biolabs, Frankfurt, Germany).

Tab. IV-5-2: DNA polymerases tested for their potential use for CRCA of circularized CLPs
These polymerases exhibit strong strand displacement activity, which is necessary for CRCA. A 25 µl CRCA reaction contained the appropriate buffer, 200 µM dNTPs, 1 µM of each primer FWD and primer REV, 2 µl of ligation reaction and the polymerase at a concentration (U) as indicated. IsoTherm - IsoTherm™ DNA Polymerase; DisplaceAce - DisplaceAce™ DNA Polymerase; Phi29 - Phi29 DNA Polymerase; *Bst* - *Bst* DNA Polymerase (large fragment); Vent - Vent (exo-) DNA Polymerase

Polymerase	U/25 µl reaction	Incubation
IsoTherm	5	1 h at 65 °C
DisplaceAce	10	1 h at 65 °C
Phi29	5	1. 3 min at 95 °C (without enzyme) 2. 1 h at 37 °C
Bst	1.6	1 h at 65 °C
Vent	min 2 max 8	2 h at 65 °C

The primers for the amplification of the CLPs were chosen to be specific to the respective CLP but not to PPV and *P. domestica* nucleic acid as proven by BLAST analysis. The primers varied in length, sequence and annealing site on the CLP (Tab. V-2-2 and Tab. V-2-3). They were purchased from Microsynth (Balgach, Switzerland) or Eurofins MWG Operon (Ebersberg, Germany).

IV-5.4 Restriction digestion

An aliquot (4 µl) of the amplification reaction was subjected to restriction digestion using *EcoRI* (Fermentas, St. Leon-Rot, Germany) for 2 h at 37 °C. Products of CRCA as well as of the restriction digestion were resolved by gel electrophoresis.

IV-5.5 Sequencing

For sequencing, 1 U *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) was added to the CRCA product. The mixture was incubated for 1 min at 72 °C to extend all fragments by a single 3' adenine overhang. One µl of these products were applied to a 10 µl ligation reaction containing 40 mM Tris-HCl, 10 mM MgCl2, 10 mM DTT, 0.5 mM ATP, 5 U T4 DNA Ligase (Fermentas, St. Leon-Rot, Germany) and 50 ng plasmid pCR2.1 (Invitrogen, Darmstadt, Germany). The reaction was incubated for 4 h at 14 °C. Two µl of this reaction and TOP10 One-Shot competent cells (Invitrogen, Darmstadt, Germany) were gently mixed and incubated for 30 min on ice followed by a heat shock for 30 sec at 41 °C. SOC media (250 µl) was added. The mixture was incubated at 37 °C and 400 rpm for 1 h. Afterwards, it was transferred at volumes of 80 µl and 200 µl to LB Agar plates containing 200 µl X-Gal, 200 µl IPTG and 200 µl kanamycin. Overnight incubation at 37 °C was followed by storage of the plates at 4 °C for 2 h to enhance the colour intensity for white/blue screening. White colonies were transferred to a LB master-plate and cultivated overnight at 37 °C. Upon colony PCR (10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08 % Nonidet P40, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.375 µM primer TOPO FWD as well as primer TOPO REV, 0.2 U *Taq* DNA Polymerase (Fermentas, St. Leon-Rot, Germany)), selected clones were transferred to 5 ml LB media and cultivated overnight at 37 °C and 140 rpm. Plasmids were isolated by using the Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Munich, Germany) according to the manufacturer's manual. Plasmids were sequenced by Eurofins MWG Operon (Ebersberg, Germany).

IV-6. RT-LAMP

The RT-LAMP assay for the detection of PPV was carried out according to the protocol developed by Varga and James (2006b). A 25 µl RT-LAMP reaction contained 20 mM Tris-HCl

(pH 8.8 at 25 °C), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 8 mM $MgSO_4$, 0.1 % Triton X-100, 0.8 M betaine (Sigma-Aldrich, Munich, Germany), 1.4 mM dNTPs (New England Biolabs, Frankfurt, Germany), 0.2 µM primers F3 and B3, 1.6 µM primers FIP and BIP, 0.8 µM primers F-loop and B-loop, 1.6 U of RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen, Darmstadt, Germany), 20 U of SuperScript® III Reverse Transcriptase (Invitrogen, Darmstadt, Germany) and 8 U of *Bst* DNA polymerase (large fragment) (New England Biolabs, Frankfurt, Germany). The sequences of the primers are listed in Tab. IV-6-1 and are depicted in Suppl. Fig. 4. Primers FIP and BIP were HPLC purified as recommended by Varga and James (2006b). The primers were purchased from Microsynth (Balgach, Switzerland) or Eurofins MWG Operon (Ebersberg, Germany). Target DNA or RNA, respectively, had a volume of 2 µl. The reaction was incubated at 63 °C for 1 h and terminated at 80 °C for 10 min.

IV-6.1 Visualisation of DNA synthesis by RT-LAMP without gel electrophoresis

For the visualisation of DNA amplification during RT-LAMP without gel electrophoresis, either the pyrophosphate turbidity accompanying the LAMP reaction or the alkaline earth metal ion indicators calcein and HNB or LFDs were tested. Tubes and LFDs were photographed or, in most cases, scanned with an office scanner Epson GT-1500 for colour documentation.

IV-6.1.1 Turbidity

To observe the turbidity upon DNA synthesis, no additional reagents or working steps were required. For the ease of differentiation, centrifugation of the reaction tubes resulted in a pellet of pyrophosphate at the bottom of the tube, which can be seen more clearly than the turbidity.

IV-6.1.2 Calcein

Calcein (Sigma-Aldrich, Munich, Germany) as well as $MnCl_2$ (AppliChem, Darmstadt, Germany) were added to the reaction mix at a concentration of 25 µM and 0.5 mM, respectively. Upon termination of RT-LAMP, the colour of the reaction mix can be observed either under daylight or under UV light. Reactions without DNA amplification show a light orange colour (daylight) or light green colour (UV light). In contrast, the DNA synthesis results in a colour change to light green and green fluorescing, respectively.

Tab. IV-6-1: RT-LAMP primer sequences for the detection of PPV in general
The primers were developed by Varga and James (2006b) and were proved to recognize all PPV subgroups. Inner primers were HPLC purified. Bold - spacer sequence

Name	Sequence (5'-3')	Length (nt)
F3 PPV	GGA ATG TGG GTG ATG ATG G	19
B3 PPV	AGG CTG TAG TCT GTC AGG	18
FIP PPV	TTG TCT AAA AGT GGG TTT CGC A**TT TT**A AAC ACA AGT GGA GTA TCC AAT	48
BIP PPV	ATG GCA CAT TTC AGT AAC GTG G**TT TT**G AAT CCC ATA CCT TGG CAT G	46
F-Loop PPV	GAT CCA ACA ATG GC	14
B-Loop PPV	CTG AAG CGT ATA TTG	15

IV-6.1.3 Hydroxy naphthol blue

For illustration of the decreasing Mg concentration during LAMP, calcein and $MnCl_2$ could also be replaced by 120 µM HNB (Sigma-Aldrich, Munich, Germany). The colour of negative reactions remains purple, whereas the colour of positive reactions changes to bright blue. The colour is visible at daylight.

To improve the colouration of the reactions, the concentrations of the metal ion indicator, of magnesium and of dNTPs as well as the incubation time were adjusted.

IV-6.1.4 Lateral flow device

For visualisation of the amplicons by LFDs, the same reaction conditions were used for RT-LAMP as described above (IV-6). Additionally, primer FIP was biotinylated at the 5'-end. Upon termination, the reaction was supplemented with 20 µM probe labelled with FITC (5'-FITC-CAG TAA CGT GGC TGA AGC G-3') and incubated at 94 °C for 1 min followed by 5 min at 65 °C for hybridizing of the probe to the amplicons. Eight µl of the hybridization reaction were transferred to a reaction tube containing 150 µl HybriDetect Assay Buffer (Milenia Biotec, Giessen, Germany). Finally, the Hybri Detect Dipstick (Milenia Biotec, Gießen, Germany) was place in the tube, incubated for 15 min and evaluated.

IV-6.1.5 Detection limit of HNB-RT-LAMP

The detection limits of the modified RT-LAMP and of the RT-PCR according to Wetzel et al. (1991b) and Bühler (2007) were tested using the plasmid pICPPV-NK-GFP (EF569215; Salvador et al., 2008) that contains the complete cDNA of PPV. The stock solution of the plasmid had a concentration of 100 ng/µl. It was 10 fold serially diluted down to 10 ag/µl (ag = 10^{-18} g) which is equivalent to 1.85 copies of plasmid per µl. The influence of background nucleic acid on the detection limit was also tested. Background RNA or cDNA were derived from healthy plants, as shown by RT-PCR, and were applied with the same volume as the template was. The reactions were run in triplicate.

IV-6.2 Preparation of a virus suspension by a fast plant extraction procedure to serve as template

As the extraction procedures to obtain nucleic acid suspension suitable for *in vitro* amplification proposed by Wetzel et al. (1991b), Lee et al. (2009) and Capote et al. (2009) were shown to be fast and to avoid the use of toxic chemicals and sophisticated equipment they were tested for their applicability to gain a virus suspension feasible for detection of PPV by RT-LAMP.

Leaf samples were placed in filter extraction bags "Universal" (Bioreba, Reinach, Switzerland). After adding 9 ml of deionised water the leaves were homogenised with the Homex-6 machine (Bioreba, Reinach, Switzerland). For lesser sample weights the volume of water was adjusted. An aliquot of the homogenates (1.5 ml) was transferred to a 2 ml reaction tube and centrifuged for 2 min at 10,000 rpm. The supernatant was diluted 1:10. The dilution obtained by the fast plant extraction procedure was used directly for RT-LAMP combined with HNB based visualisation, for one-step multiplex RT-PCR (Jarošová and Kundu, 2010) and for cDNA synthesis followed by PCR (Wetzel et al., 1991b, Bühler, 2007), respectively, or stored at -20 °C.

IV-6.2.1 Comparison of the fast plant extraction procedure and conventional RNA extraction

Twenty four genotypes derived from the Weihenstephan plum breeding program of *Prunus domestica* were tested for the presence of PPV. One tree per genotype has been artificially inoculated with a PPV-D isolate using the double grafting method described by Kegler et al. (1994). The infection was assessed by visual monitoring for symptoms on leaves as well as by RT-PCR according to the EPPO standards for verification of an infection by two different methods (EPPO, 2004). One tree per genotype remained PPV free as monitored by RT-PCR. Six leaves per tree were taken and put in two stacks with three leaves per stack. Each stack had a weight of about 1.5 g. Using a razor blade, each stack was dissected laterally to four stripes. Stripes 1 and 3 of the first stack and stripes 2 and 4 of the second stack were pooled to obtain a mixed sample. A second sample was produced with the remaining stripes.

The first sample was subjected to the fast plant extraction procedure to obtain the virus suspension. The second sample was ground to fine powder in the presence of liquid nitrogen. A 1 ml aliquot of the virus suspension as well as the second sample were applied to conventional RNA extraction. For comparison, the diluted virus suspension, the RNA derived from the virus suspension and from the second sample, respectively, were applied to the nucleic acid amplification protocols described above.

The results of the extraction trial were used to calculate the sensitivity and specificity of each detection method tested based on the definition of Altman and Bland (1994): Sensitivity is the ratio of the number of truly positive samples detected by a particular method to the number of all truly positive samples. Specificity is the ratio of the number of detected true negative samples to the number of all true negative samples.

IV-6.2.2 Influence of the virus suspension on RT-LAMP performance

The suitability of virus suspensions for the nucleic acid amplification by RT-LAMP was evaluated. Therefore, samples composed of different ratios of plant material from a healthy tree and an artificially PPV infected tree were used (virus dilutions). Samples of 0.5 g derived either from a healthy tree, a PPV inoculated tree or consisting of a mixture of both kinds of samples with ratios of 1:2, 1:10 and 1:50 (positive: negative) were applied to the fast plant extraction procedure. One aliquot of each of the resulting mixed virus suspensions was serially diluted tenfold down to 10^{-6} with RNase free water (sample dilution). The dilutions were used for RT-LAMP as described above. Another aliquot was subjected to conventional RNA extraction followed by dilution of RNA with the same ratios as the virus suspension. Blue LAMP and cDNA synthesis prior to PCR were performed with these dilutions of RNA as templates. All amplification reactions were run in triplicate.

IV-6.2.3 Determination of the reliability by an orchard survey

Reliability of the modified RT-LAMP protocol was tested with orchard samples. For this, 102 samples were collected in an orchard with natural infection rate. Four leaves per sample were placed in a filter extraction bag. The fast plant extraction procedure was set up as described above and the virus suspension was ap-

plied directly to HNB supplemented RT-LAMP and one-step multiplex RT-PCR for comparison.

IV-6.3 Assigning of isolates to the PPV subgroups

The sequences of isolates grouped to all known strains of PPV (Tab. IV-6-2) were aligned using the Clustal W2 software avaible online at http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Larkin et al., 2007) and searched for differences characteristic to each subgroup. For the generation of strain specific LAMP primer sets the software PrimerExplorer V4 was used (http://primerexplorer.jp/e/). For each strain an appropriate target region was selected. As PrimerExplorer V4 is capable to process sequences with a maximal length of 2,000 nt, the selected sequences were shortened to the regions containing the most prominent differences to the other strains to fit this limitation. Upon opening the sequences by PrimerExplorer V4, the nucleotides which are not identical to the other subgroups were marked. The software was set to detail settings and to the specific design option. The generated primer sets were evaluated regarding their specificity to the designated strain by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The selected primer sets were reloaded to PrimerExplorer V4 to design loop primers.

Tab. IV-6-2: Sequences used for alignment to develop strain specific LAMP primers
The sequences were derived from the nucleotide database on http://www.ncbi.nlm.nih.gov/nuccore or from the SharCo database (*). The sequences with the accession number written in red were used for the primer design.

Strain	Accessionnumber
PPV-D	DQ465243; EF611248; AF401295; AF401296; EF611244; DQ465242; AY953267; AY912056; AY912057; AY953261; AY912058; X16415; X81083; D13751; D-147-SK-300pl*
PPV-Rec	AY028309; Rec-146-BG-300pl*; Rec-148-RS-300pe*
PPV-M	M92280; AJ243957; M-245-IT-19pe*
PPV-T	EU734794
PPV-C	Y09851; AY184478
PPV-EA	DQ431465; AM157175
PPV-W	AY912055

The strain specific primer sets were purchased from Eurofins MWG Operon (Ebersberg, Germany) with the internal primers being HPLC purified. They were tested in the same RT-LAMP conditions as described for the general detection of PPV except that betaine was replaced by 3 mM trehalose (Sigma-Aldrich, Munich, Germany) and incubation time was shortened to 40 min.

Twenty three samples were used to evaluate the strain specific HNB-RT-LAMP. All samples were subjected to RNA extraction. The RNA was applied to HNB-RT-LAMP and to cDNA synthesis. cDNA was used as template for RT-PCR for the general detection of PPV (Wetzel et al., 1991b) as well as for the RT-PCRs for strain differentiation (Szemes et al., 2001, Šubr et al., 2004) for comparison. Virus suspensions obtained from 16 samples by the fast plant extraction procedure supplemented the HNB-RT-LAMP as well. All samples except sample 6 and 7 were collected in the Weihenstephan PPV isolate collection. Samples 6 and 7 were derived from M. Cambra, Spain. The samples 1 to 20 were assigned previously to a PPV subgroup, whereas samples 21, 22 and 23 were not yet tested.

IV-6.4 Increasing the reliability of RT-LAMP by the detection of an internal control

For the quality control of the samples used for the detection of PPV by RT-LAMP,

primer sets were developed to target two different plant genes as an internal control. The RT-PCR primers established by Menzel et al. (2002) and Sánchez-Navarro et al. (2005) hybridize to the *nad5* gene (accession number D37958) and *Rbcl* gene (accession number AF206813), respectively. PrimerExplorer V4 was used to generate primers targeting these sequences particularly at the same locations as the primers for detection by RT-PCR. As LAMP primers recognize more specific sites than PCR primers, the newly designed primers were analysed for their specificity by BLAST.

The primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) with the inner primers being HPLC purified. The reaction conditions were the same as for the general detection of PPV by RT-LAMP.

IV-7. Visualisation by gel electrophoresis

The reaction products derived from the diverse RT-PCR protocols as well as from CRCA and RT-LAMP were visualized by gel electrophoresis (2 % agarose (Biozym Scientific, Hess. Oldendorf, Germany), TBE buffer, ethidium bromide). GeneRuler™ 50bp DNA Ladder (Fermentas, St. Leon-Rot, Germany) was used as size marker in all cases. The gels were run at a voltage of 90 V for 45 minutes. Afterwards the images of the gels were taken by a Gel Hood Imager 2009 (Intas, Göttingen, Germany).

V Results

V-1. Differentiating the isolates of the Weihenstephan PPV isolates collection by RT-PCR

Up to now, the Weihenstephan PPV isolate collection contains 48 different isolates. Thirty five of those derived from several sites in Germany, mainly from Baden-Württemberg and Rhineland-Palatinate. Each three isolates were taken in Czech Republic and in Serbia. The remaining isolates were collected in Switzerland, Italy, Slovakia, Romania, Poland (Fig. V-1-1), Egypt and Canada. The virus is maintained onto infected *Prunus domestica* 'Hauszwetsche' and 'Katinka' trees, which are cultivated in an insect-proof greenhouse.

The isolates were assigned to the PPV strains by PCR using strain specific primers based on the protocols published by Szemes et al. (2001) and Šubr et al. (2004) with minor modifications. For comparison, general detection of PPV was also done by the method of Wetzel et al. (1991b) modified by Bühler (2007).

Using the primers P1 and P2 (Wetzel et al., 1991b) all samples of the Weihenstephan PPV isolate collection were infected by PPV. 46 out of the 48 probes were tested positively for PPV using the method of Szemes et al. (2001), whereas only 33 positives were found in the first round by the general PPV primers M3-5', M4-5' and M2-3'. The remaining 13 positives were just detected in the nested PCR by the differentiating primer pairs (Fig. V-1-2).

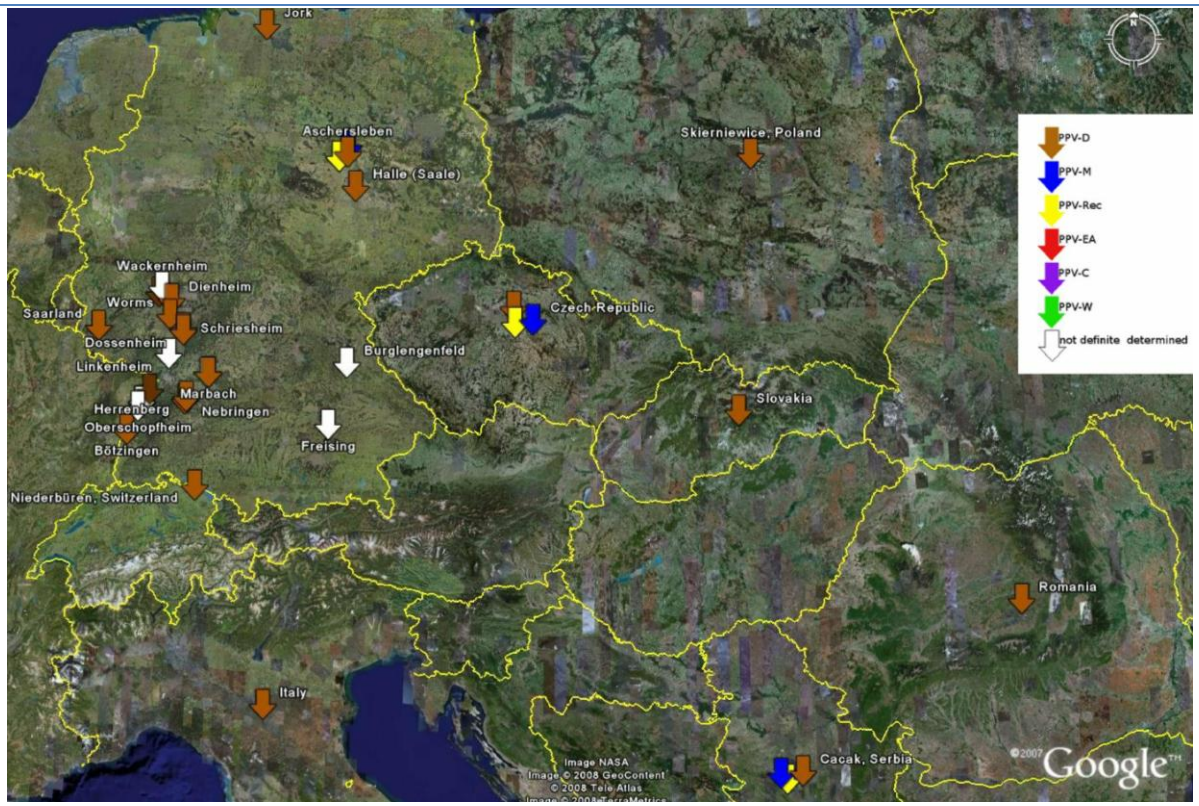


Fig. V-1-1: Map of Central and Eastern Europe
Each arrow indicates the location, where one or more isolates were collected. The colour of an arrow indicates the strain to which the isolates were assigned.

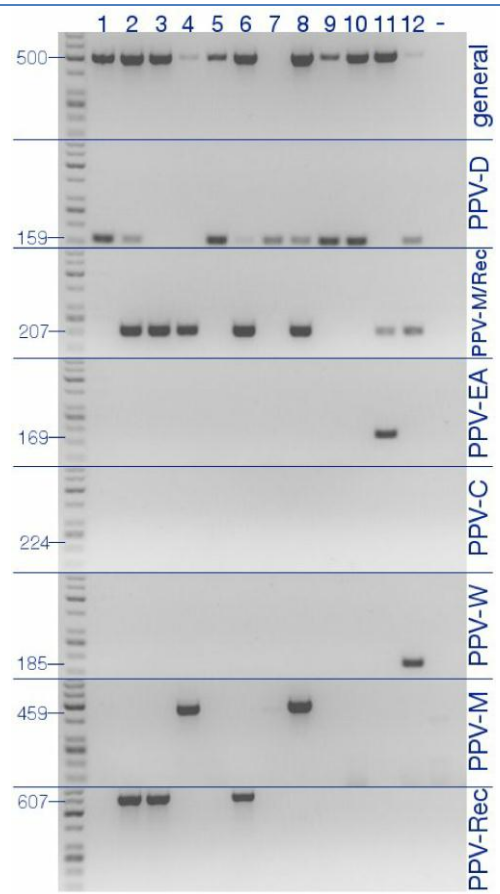


Fig. V-1-2: PCR amplification with strain specific primers
Lanes 1 to 12 are PCR products from isolate 1 to 12 of the Weihenstephan PPV isolate collection (–: negative control). Amplification was done with primers M3-5'/M4-5' and M2-3' (general), M1-5' and M5-3' (PPV-D), M6-5' and M7-3' (PPV-M/Rec), M8-5' and M9-3' (PPV-EA), M10-5' and M11-3' (PPV-C), M14-5' and M15-3' (PPV-W), mM5 and mM3 (PPV-M), mD5 and mM3 (PPV-Rec).

The vast majority of 34 isolates was assigned to strain PPV-D. Three samples were classified with strain PPV-M and three with strain PPV-Rec. PPV-C was not detected in any sample. Isolates belonging to the strain PPV-EA were identified once. The strain PPV-W was found once with newly designed primers (Tab. IV-4-1), but this isolate was also detected by the primers of PPV-D and PPV-M. Four further samples could not be clearly assigned to any strain, either because they produced the amplicones of more than one strain or because the differentiation between PPV-M and PPV-Rec was not possible (Fig. V-1-2 and Suppl. Tab. 1).

V-2. Detection of PPV by cascade rolling circle amplification

The detection of PPV by CRCA compromises the circularization of the CLP by a ligase upon hybridization of the probe to PPV cDNA and the amplification of the circularized probe. As a control, the resulting amplicons are subjected to restriction digestion. The results showing the development of the CRCA based PPV diagnosis are presented according to this work flow. In the beginning of the development of the PPV specific CRCA, reaction conditions were adopted from Thomas et al. (1999).

V-2.1 Ligation of the CLP hybridized to PPV cDNA

V-2.1.1 Variation of the cDNA concentration applied to the ligation reaction

Different total amounts of cDNA were tested to obtain the optimal result for positive samples and minimal background for negative samples. cDNA at concentrations of 200 ng, 80 ng and 40 ng resulted in the expected ladder like amplicon pattern after CRCA with each amplicon being a multimer of the CLP and in a single fragment of 80 bp upon restriction digestion (Fig. V-2-1). Unspecific amplification occurred when either applying lower amounts of cDNA of positive control or applying NTC or cDNA of negative control. It resulted likewise in a ladder like fragment pattern. Each amplicon was a multiple of about a half length of the CLP. These amplification products were not completely digested by

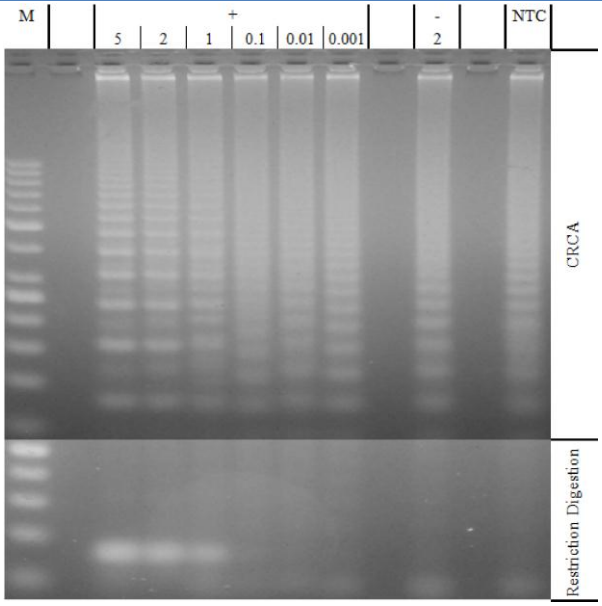


Fig. V-2-1: Influence of varying cDNA volumes on the CRCA upon ligation
The indicated volume of cDNA (in µl) was applied to the ligation reaction containing 0.12 µM CLP PPV 1 and 0.5 U Ampligase®. Two µl of the ligation reaction were transferred to the CRCA mix which included primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment). Restriction digestion was carried out on 4 µl CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

EcoRI resulting in a 40 bp fragment and in CRCA amplicon pattern whose intensity was reduced compared to before restriction digestion due to partial digestion and diluted CRCA products upon applying to the *EcoRI* reaction mix (Fig. V-2-1). The specific accumulation upon adding 200 or 80 ng cDNA of the positive control exceeds that of nonspecific amplification. Using 40 ng of cDNA, the positive sample was not recognized until subsequent restriction digestion. cDNA volumes of 4, 0.4 and 0.04 ng of a positive sample were not detected.

V-2.1.2 Design of the circularizable probe

For the general detection of PPV three CLPs were designed to match the PPV cDNA (CLP PPV 1 - 3, Tab. V-2-1). The spacer segment was the same in each CLP. It contained the recognition site of the endonuclease *EcoRI* followed by the complementary sequence of primer FWD 1a and the homologous sequence of primer REV 2a (Fig. II-3-6). Restriction digestion was used to verify the CRCA products. The terminal regions of the CLP were designed to hybridize to the cDNA complementary to the RNA which encodes for the coat protein. The hybridization site is conserved to all PPV subgroups as observed by

Tab. V-2-1: The sequences of the CLPs for the specific detection of PPV
The colour of the sequence represents the corresponding use: bold and black - 5' PPV specific region; red - restriction site for *EcoRI*; green - hybridisation site of primer FWD; blue - homologous site of primer REV; black - 3' PPV specific region; baltic and black - spacer; underlined - self complementary sites. All CLPs are phosphorylated at the 5'-end. The primers, which can be used for amplification of each CLP, are listed as well.

Name	Sequence (5'-3')	Length	Primer FWD	Primer REV
CLP PPV 1	**ATT GTT GGA TCA TGC GAA ACC CA**G AAT TCA ACG ACT AAT GAC TGG AC C ACC TTG ATG CTA CCT TAG TAT CCA ATA AAG CC	80 nt	1a, 1b, 1c, 3a, 3b, 3c	2a, 2b, 4a, 4b, 4c
CLP PPV 2	**ATT GTT GGA TCA YGC GA**G AAT TCA ACG ACT AAT GAC TGG ACC ACC TTG ATG CTA CCT TAG AGT ATC CAA TAA AGC C	76 nt	1a, 1b, 1c, 3a, 3b, 3c	2a, 2b, 4a, 4b, 4c
CLP PPV 3	**CAA ATT ATG GCA CAT TTC AGT AAC** GAA TTC AAC GAC TAA TGA CTG GAC CAC CTT GAT GCT ACC TTA AAC CCA CTT TTA GA	80 nt	1a, 1b, 1c, 3a, 3b, 3c	2a, 2b, 4a, 4b, 4c
CLP PPV 4	**ATT GTT GGA TCA YGC GA**G AAT TCG ACT CGT CAT GTC TCA GCT CTA GT *A CGC* TGA TCT TAG TGT CAG GAT ACG GGA GTA TCC AAT AAA GCC	90 nt	5	6
CLP PPV 5	**ATT GTT GGA TCA YGC GA**G AAT TCT TAG TTA GGC TCG CTG TCA GGA CC *A CTC* CTA CTG AGC CTA CGA GAA CGA CGA GTA TCC AAT AAA GCC	90 nt	7	8
CLP PPV 6	**ATT GTT GGA TCA YGC GA**G AAT TCA ACG ACT AAT GAC TGG ACC A*AG* ACC ACC TTG ATG CTA CCT TAG GCT TTA TTG GA*C A*GA GTA TCC AAT AAA GCC	96 nt	1a, 1b, 1c, 3a, 3b, 3c	2a, 2b, 4a, 4b, 4c

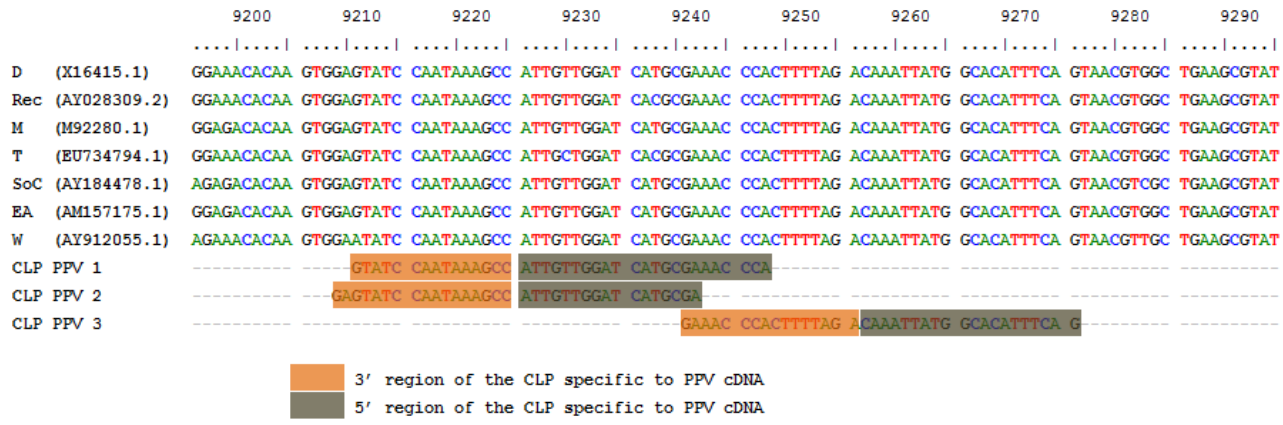


Fig. V-2-2: Localisation of CLPs within the RNA of PPV-D, -Rec, -M, -T, -C, -EA and -W
Within this section the hybridization sites of CLP PPV 1, CLP PPV 2 and CLP PPV 3 are located. The brown shading indicates the 5' target specific sequence of the CLP, the orange shading indicates the 3' arm of the CLP. The CLP PPV 4, CLP PPV 5 and CLP PPV 6 bear the same target specific regions as CLP PPV 2. The terminal sections of the CLPs are complementary to cDNA derived from PPV RNA.

alignment (Fig. V-2-2). Sequence specificity was checked by BLAST analysis. The target specific ends varied in length and the hybridization region on the PPV cDNA.

All CLPs detected PPV correctly since gel electrophoresis of CRCA products of each CLP resulted in numerous fragments with multiple length of the CLP. In negative control and NTC, a high rate of background amplification was observed as indicated by more fragments than obtained by the positive control. The lengths of the additional fragments corresponded to multiples of about the half length of the CLPs. CRCA was followed by restriction digestion for better discrimination of PPV positive and PPV negative samples. Moving the hybridization region resulted in lower signal strength, whereas the variation of the length of the PPV complementary region caused hardly an alternation but did not change the intensity of the background signal either (Fig. V-2-3 and Fig. V-2-4).

The ligation of CLP PPV 2 was examined at different temperatures in the range of 54 °C and 66 °C. A ligation temperature of 56, 58 or

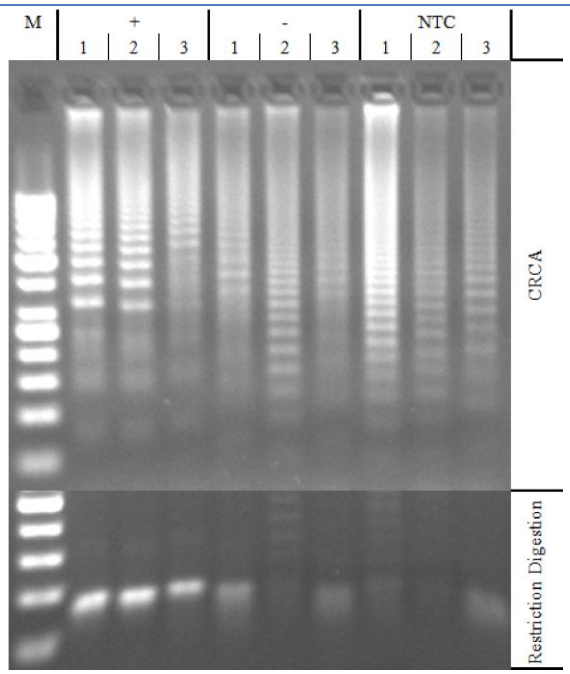


Fig. V-2-3: Functionality of the CLPs for the detection of PPV
Two µl of cDNA were applied to the ligation reaction containing 0.12 µM of the respective CLP and 0.5 U Ampligase®. Two µl of the ligation reaction were transferred to the CRCA mix including primers FWD 1c and REV 2b and 1.6 U *Bst* DNA polymerase (large fragment). Restriction digestion was carried out on 4 µl CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

60 °C resulted in a slightly enhanced amplification rate. This was visible as recently as restric-

tion digestion was done (Fig. V-2-5). The variation of ligation temperature had no influence on the background amplification.

Further variation in the CLP design based on CLP PPV 2 by introducing of two nucleotides between the sequences for primer hybridization did not result in an improvement concerning the background amplification (Tab. V-2-1, Fig. V-2-4). The CLP PPV 4 and CLP PPV 5 were amplified by primers FWD 5 and REV 6 and primers FWD 7 and REV 8, respectively. They were 24 nt in length, which lead to an enhanced amplification rate compared to primers FWD 1a and REV 2a used for amplification of CLP PPV 2. This was also observed for the nonspecific amplification in negative and NTC.

To minimize background amplification, CLP PPV 6 was created, which contained a hairpin compared to CLP PPV 2. The hairpin was located at the 3'-end of the CLP and was formed by a stem with 12 nt in length (Tab. V-2-1). With this length, the hairpin should be stable at the am-

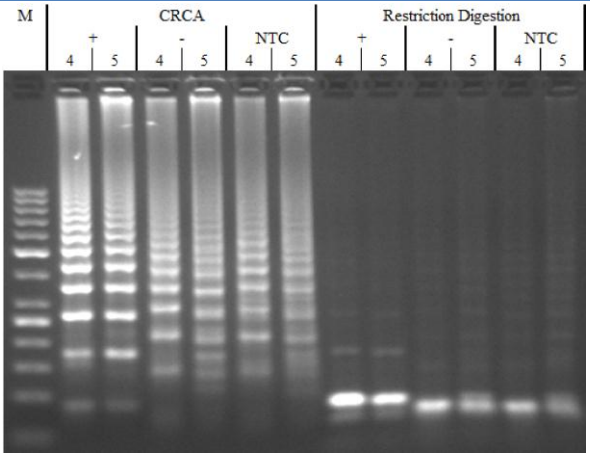


Fig. V-2-4: Testing of further CLPs
CLP PPV 4 or CLP PPV 5 were applied to the ligation at a concentration of 0.12 µM. The CRCA utilized 2 µl of the ligation reaction, either primers FWD 5 and REV 6 (CLP PPV 4) or primers FWD 7 and REV 8 (CLP PPV 5) and 1.6 U *Bst* DNA polymerase (large fragment). An aliquot of 4 µl of the CRCA product was transferred to restriction digestion. + - positive control, - - negative control, NTC - no template control, M - size marker

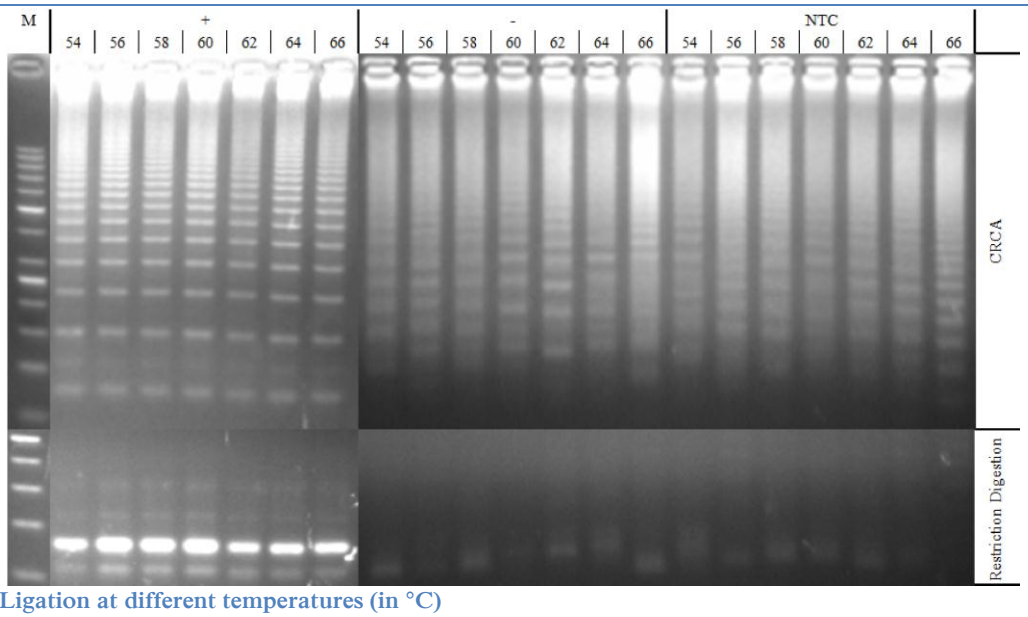


Fig. V-2-5: Ligation at different temperatures (in °C)
A ligation reaction was composed of 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®. After initial denaturetion it was incubated at the indicated temperatures for 15 min. The following CRCA contained 2 µl of the ligation reaction, primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment). Restriction digestion was carried out on 4 µl CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

plification temperature allowing elongation of the 3'-end. However, this hairpin construct did not have an inhibitory effect on the background amplification at a CRCA temperature of 63, 65 or 67 °C. On the contrary, in positive, in negative as well as in NTC no differences were visible regarding the amplicon pattern after CRCA and restriction digestion, respectively (Fig. V-2-6).

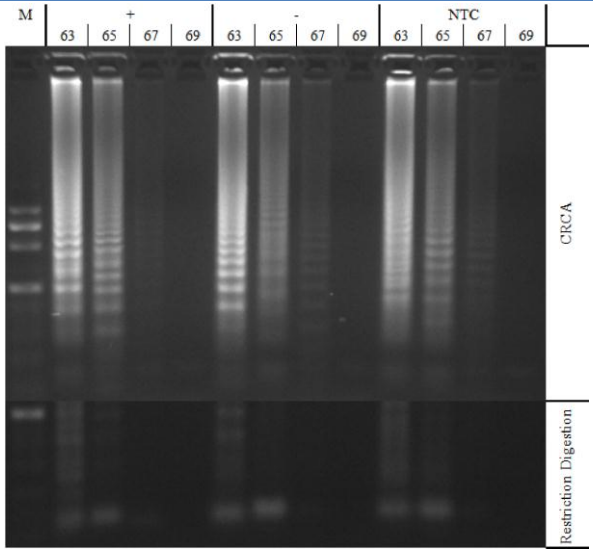


Fig. V-2-6: Increasing temperatures of CRCA to amplify CLP PPV 6 containing a hairpin construct cDNA (2 µl), CLP PPV 6 (0.12 µM) and Ampligase® (0.5 U) were applied to the ligation reaction. A CRCA reaction contained 2 µl of the ligation reaction, primers FWD 1c and REV 2b and 1.6 U *Bst* DNA polymerase (large fragment). It was incubated for 1 h at the temperatures (in °C) indicated. Restriction digestion was carried out on 4 µl CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

V-2.1.3 Determination of the optimal CLP concentration

CLP PPV 2 was used to evaluate the influence of CLP concentration on specific and non specific amplification. This CLP was chosen due to the equal length of target specific regions (Tab. V-2-1) representing the basic design of a CLP. With rising CLP concentration from 0.06 µM up to 0.36 µM the amplification rate of the positive control increased (data not shown). Background amplification was visible in negative control and NTC in each case with 0.06 µM CLP PPV 2 resulting at the lowest signal intensity but still visible.

To avoid the background amplification, lower concentrations of CLP PLPV 2 from 0.12 µM down to 0.012 nM were examined. With decreasing CLP concentration the rate of background amplification was reduced as well (Fig. V-2-7). Amplification was observed neither in the positive control nor in the negative control at the lowest CLP concentration of 0.012 nM. Twelve and 1.2 nM of CLP PPV 2 resulted in a similar rate of amplification of the positive control, which was slightly reduced compared to 120 nM CLP PPV 2. The decrease of the CLP concentration to 0.12 nM caused a minor amplification of the PPV positive sample (Fig. V-2-7). However, background amplification was visible at all concentration except 0.012 nM CLP PPV 2.

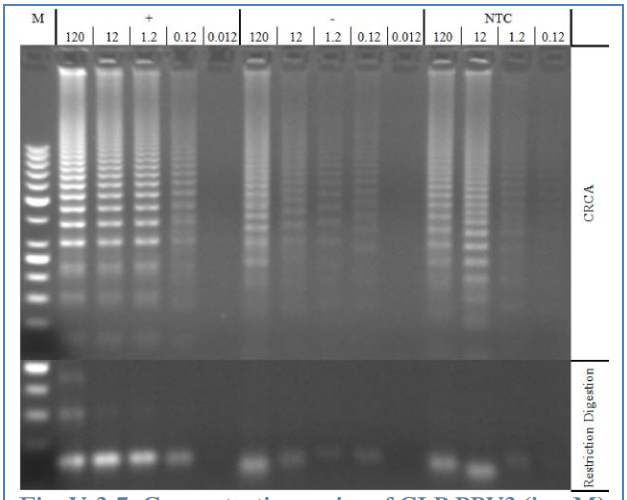


Fig. V-2-7: Concentration series of CLP PPV2 (in nM) Decreasing concentrations of the CLP were circularized by Ampligase® (0.5 U). Two µl of the ligation reaction were transferred to the CRCA mix which included primers FWD 1c and REV 2b and 1.6 U *Bst* DNA polymerase (large fragment). Subsequent restriction digestion was carried out on 4 µl CRCA product. + - positive control, - - negative control, NTC - no tem-plate control, M - size marker

V-2.1.4 Ligases suitable for specific ring closure upon probe hybridization to the target

There are a number of different ligases available. To test which DNA joining enzyme provides the optimal basis for the following amplification four ligases were compared: Ampligase® Thermostable DNA Ligase, *Taq* DNA Ligase, 9°N™ DNA Ligase and T4 DNA Ligase.

Results obtained by *Taq* DNA ligase were similar to those by Ampligase® Thermostable DNA Ligase (Fig. V-2-8): strong amplification of the positive control was observed, but also background amplification in negative control and NTC, so definite discrimination only was possible after restriction digestion. 9°N™ DNA Ligase resulted in a very weak signal. The T4 DNA Ligase produced DNA circles in either case at the same rate indicating an unspecific reaction (Fig. V-2-8). The length of the fragments was multiples of about 60 bp.

V-2.1.5 Influence of thermal cycling of the ligation on the detection limit of CRCA

A ligation reaction was incubated for 5 min at 95 °C and for 15 min at 64 °C. The ligation was repeated up to six times using a thermal cycler. With each additional cycle of ligation the amplification of the positive control was enhanced clearly (Fig. V-2-9), whereas the background signal of NTC was not changed dramatically.

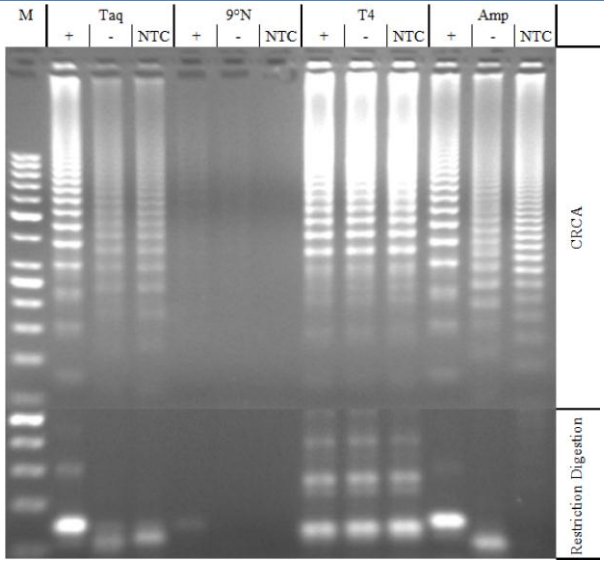


Fig. V-2-8: Comparison of different ligases concerning their applicability in CRCA
CLP PPV 2 (0.12 µM) was ligated by either *Taq* DNA Ligase (Taq, 12 U), 9°N™ DNA Ligase (9°N, 20 U), T4 DNA Ligase (T4, 20 U) and Ampligase® Thermostable DNA Ligase (Amp, 0.5 U). The following CRCA contained 2 µl of the ligation reaction, primers FWD 1c and REV 2b and 1.6 U *Bst* DNA polymerase (large fragment). Finally, 4 µl of CRCA product were digested by EcoRI. + - positive control, - - negative control, NTC - no template control, M - size marker

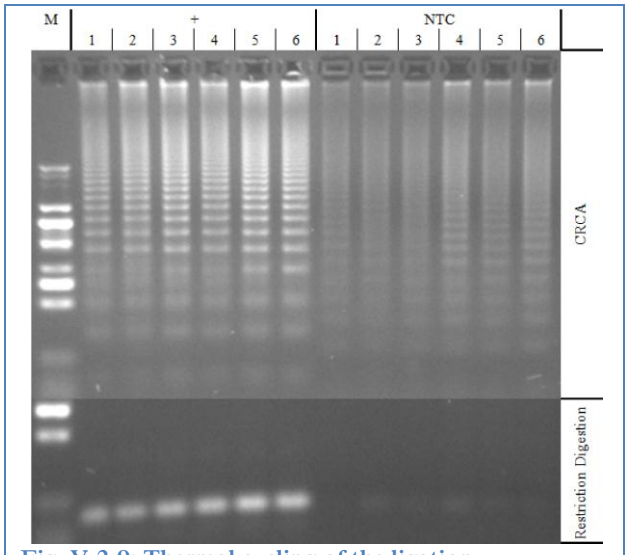


Fig. V-2-9: Thermal cycling of the ligation
The ligation with one reaction containing 0.2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase® was repeated n-fold (n: number of cycles). Two µl of the ligation reaction were transferred to the CRCA mix which included primers FWD 1b pur and REV 9a pur and 1.6 U *Bst* DNA polymerase (large fragment). Fi-nally, 4 µl of CRCA product were applied to restrict-tion digestion. + - positive control, - - negative con-trol, NTC - no template control, M - size marker

V-2.2 Amplification of ligated CLPs

V-2.2.1 Influence of the ligation product concentration on the amplification

Increasing ligation product concentration applied to CRCA up to 40 nM CLP and 26.7 ng cDNA resulted in a considerably enhanced amplification of the positive control. Higher concentration did not enhance the amplification rate further (Fig. V-2-10A). In a negative sample, elevated ligation reaction concentrations led to background amplification. These products were not digested by *EcoRI* (Fig. V-2-10B). Interestingly, in Fig. V-2-10A no amplification was visible in NTC, whereas amplification was observed in Fig. V-2-10B with the same reaction conditions.

Applying the positive control at a concentration of 40 nM CLP and 26.7 ng cDNA, 60 nM CLP and 40 ng cDNA and 80 nM CLP and 53.3 ng cDNA of the ligation reaction to CRCA resulted in the estimated fragment pattern with high signal intensity and in fragments with half length multiples and low signal intensity. The amplicons visible in negative and no template control exhibited similar intensities. Restriction digestion of the amplification products resulted in the expected single fragment for the positive control. The nonspecific amplification was not digested as the fragment pattern was still visible. The lower fragment intensity was caused by the dilution of the amplification product with the digestion mix. Background amplification was observed not only in negative and NTC but also in positive control.

V-2.2.2 Improvement of CRCA by the use of additives

Concentration series with betaine, DMSO and DTT revealed that the addition of up to 0.75 M betaine to the CRCA reaction mix enhanced the amplification slightly. Higher concentrations had an inhibitory effect (Fig. V-2-11A). The additive DMSO gave the best result at a concentration of 1 % with concentrations higher than 3 % showing dramatically decreasing amplification (Fig. V-2-11B). Adding DTT at concen-

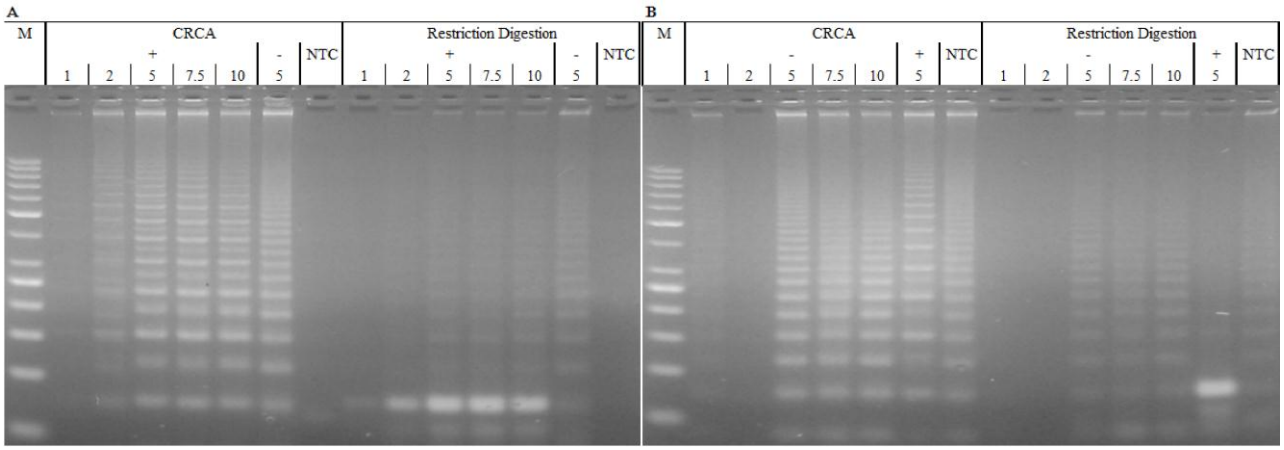


Fig. V-2-10: Influence of varying ligation product volumes applied to the CRCA
CLP PPV 1 was ligated at a concentration of 0.12 µM on 2 µl cDNA by 0.5 U Ampligase®. Increasing volumes of the ligation reaction were transferred to the CRCA mix which included primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment) as indicated. Restriction digestion was carried out on 4 µl CRCA product. + - positive control, A - increasing ligation product volume derived from a PPV positive sample, B - increasing ligation product volume derived from a PPV negative sample, - - negative control, NTC - no template control, M - size marker

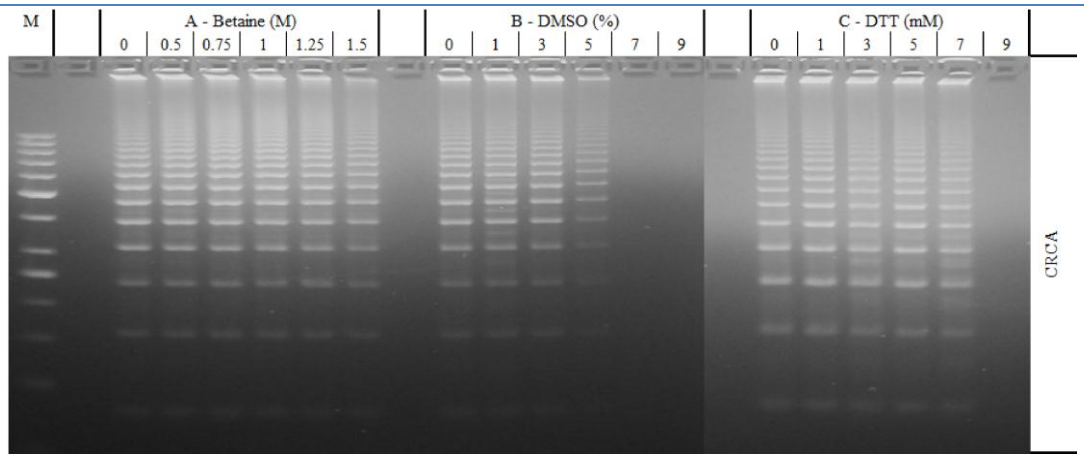


Fig. V-2-11: Optimisation of the CRCA by including additives
Two µl of the ligation reaction (2 µl cDNA, 0.12 µM CLP PPV 1, 0.5 U Ampligase®) were applied to the CRCA reaction (primers FWD 1a and REV 2a, 1.6 U *Bst* DNA polymerase (large fragment)) supplemented by either betaine (A), DMSO (B) or DTT (C) at the denoted concentration. M - size marker

trations up to 7 mM had no visible effect on the expected fragments. However, additional amplicons were observed at concentrations from 3 to 7 mM. No Signal was obtained from CRCA supplemented with 9 mM DTT (Fig. V-2-11C). Further tests using betaine at concentra-tions of 0.6, 0.75 and 0.9 as an additive showed that any addition enhanced the amplification in positive reactions (Fig. V-2-12). Background amplification was reduced by 0.75 M betaine in negative control. In NTC, no amplification was visible except upon addition of 0.6 M betaine (Fig. V-2-12).

Ectoin as well as ET SSB were examined for their use in CRCA. Ectoine and ET SSB were inhibitory to the amplification at the concentrations tested (ectoine: 0.25, 0.5, 0.75 and 1 mM; ET SSB: 0.1, 0.25 and 0.5 µg; data not shown).

V-2.2.3 Influence of CRCA incubation time on the amplification

Incubation time of CRCA was optimized to avoid the false positive signals yet to achieve a high detection limit. After 45 min incubation, the

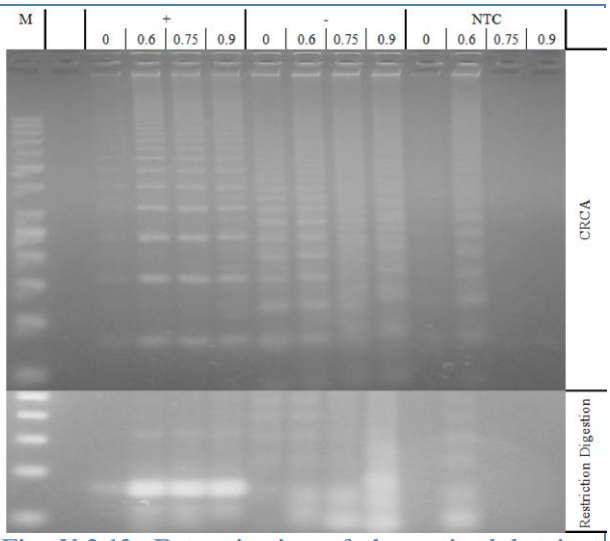


Fig. V-2-12: Determination of the optimal betaine concentration (M) for improved CRCA
After ligation of 0.12 CLP PPV 1 on 2 µl cDNA by 0.5 U Ampligase®, 2 µl were subjected to CRCA (primers FWD 1a and REV 2a, 1.6 U *Bst* DNA polymerase (large fragment)) containing betaine at varying concentrations. An aliquot (4 µl) was transferred to restriction digestion. + - positive control, - - negative control, NTC - no template control, M - size marker

signal of the positive control was visible with a longer reaction time resulting in a higher rate of amplification. However, background amplification in negative control and NTC also appeared after 45 min (Fig. V-2-13).

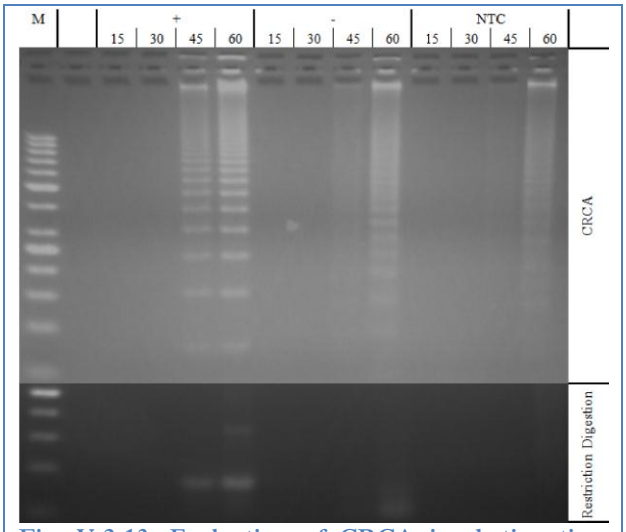


Fig. V-2-13: Evaluation of CRCA incubation time (min)
A 2 µl aliquot of the ligation reaction (2 µl cDNA, 0.12 µM CLP PPV 1, 0.5 U Ampligase®) completed the CRCA reaction (0.75 M betaine, primers FWD 1a and REV 2a, 1.6 U *Bst* DNA polymerase (large fragment). The amplification was incubated for up to 60 min as indicated. For confirmation, a restriction digestion was conducted. + - positive control, - - negative control, NTC - no template control, M - size marker

V-2.2.4 Varying primers and primer design for optimal CRCA performance

Variations and modifications in the design of the primers were analysed especially concerning their influence on background amplification. All designed primers (Tab. V-2-2) were specific just to the CLPs and not to plum or to PPV.

First, the length of the primers was altered from 18 nt for primers FWD 1a and REV 2a to 20 nt for primers FWD 1c and REV 2b. Since the CLP was not adapted, primers FWD 1c and REV 2b exhibited 4 nt complementary to each other at their 5'-end (Tab. V-2-3). The second approach was to utilize a primer FWD which hybridized to the homologous sequence of primer REV 2a and a primer REV which was homologous to the primer FWD 1a binding site. Primer FWD 3a hybridized to the recognition site of primer REV 2a initiating the CRCA and primer REV 4a was homologous to the primer FWD 1a

Tab. V-2-2: Primers, developed for the amplification of ligated CLPs
Bases depicted in red denote the bases substituted by LNA bases in LNA primers. Underlined bases denote the bases forming the stem in the hairpin construct, *italic bases* the loop forming ones.

Primer	Sequence (5'-3')	Length	CLP PPV	2nd primer
FWD 1a	GTC CAG TCA TTA GTC GTT	18 nt	1, 2, 3, 6	REV 2a
REV 2a	CAC CTT GAT GCT ACC TTA	18 nt	1, 2, 3, 6	FWD 1a, 1b
FWD 1b	CCA GTC ATT AGT CGT TGA A	19 nt	1, 2, 3, 6	REV 2a
REV 2a	CAC CTT GAT GCT ACC TTA	18 nt	1, 2, 3, 6	FWD 1a, 1b
FWD 1c	TGG TCC AGT CAT TAG TCG TT	20 nt	1, 2, 3, 6	REV 2b
REV 2b	ACC ACC TTG ATG CTA CCT TA	20 nt	1, 2, 3, 6	FWD 1c
FWD 1c HP	ACG ATG AC *T GAC G* GT CAT CGT TGG TCC AGT CAT TAG TCG TT	41 nt	1, 2, 3, 6	REV 2b
REV 2b HP	ACT GGT GAG *GA T* CTC ACC AGT ACC ACC TTG ATG CTA CCT TA	41 nt	1, 2, 3, 6	FWD 1c
FWD 3a	AGG TAG CAT CAA GGT GGT	18 nt	1, 2, 3, 6	REV 4a
REV 4a	CGA CTA ATG ACT GGA CCA	18 nt	1, 2, 3, 6	FWD 3a
FWD 3b	TAA GGT AGC ATC AAG GTG	18 nt	1, 2, 3, 6	REV 4b
REV 4b	TTC AAC GAC TAA TGA CTG G	19 nt	1, 2, 3, 6	FWD 3b
FWD 3c	TAA GGT AGC ATC AAG GTG GTC	21 nt	1, 2, 3, 6	REV 4c
REV 4c	AAC GAC TAA TGA CTG GAC CAC	21 nt	1, 2, 3, 6	FWD 3c
FWD 5	ACT AGA GCT GAG ACA TGA CGA GTC	24 nt	4	REV 6
REV 6	GCT GAT CTT AGT GTC AGG ATA CGG	24 nt	4	FWD 5
FWD 7	GGT CCT GAC AGC GAG CCT AAC TAA	24 nt	5	REV 8
REV 8	TCC TAC TGA GCC TAC GAG AAC GAC	24 nt	5	FWD 7
REV 9a	ACC TTA GAG TAT CCA ATA AAG CC	23 nt	2	FWD 1c
REV 9b	CCT TAG AGT ATC CAA TAA AGC CAT	24 nt	2	FWD 1c
REV 9c	AGA GTA TCC AAT AAA GCC ATT GT	23 nt	2	FWD 1c

Tab. V-2-3: Sequence of CLP PPV 2 and the primers designed for the amplification of the CLP
The orientation as well as the hybridization site of the primers is indicated. The primers FWD and REV which were commonly used together are stacked. Colouration of CLP PPV 4 is same as in Tab. V-2-1.

CLP PPV 2	5'-**ATTGTTGGATCAYGCGA**GAATTCAACGACTAATGACTGGACCACCTTGATGCTACCTTAGAGTATCCAATAAAGCC-3'
Primer FWD 1a	3'-TTGCTGATTACTGACCTG-5'
Primer REV 2a	5'-CACCTTGATGCTACCTTA-3'
Primer FWD 1b	3'-AAGTTGCTGATTACTGACC-5'
Primer REV 2a	5'-CACCTTGATGCTACCTTA-3'
Primer FWD 1c	3'-TTGCTGATTACTGACCTGGT-5'
Primer REV 2b	5'-ACCACCTTGATGCTACCTTA-3'
Primer FWD 3a	3'-TGGTGGAACTACGATGGA-5'
Primer REV 4a	5'-CGACTAATGACTGGACCA-3'
Primer FWD 3b	3'-GTGGAACTACGATGGAAT-5'
Primer REV 4b	5'-TTCAACGACTAATGACTGG-3'
Primer FWD 3c	3'-CTGGTGGAACTACGATGGAAT-5'
Primer REV 4c	5'-AACGACTAATGACTGGACCAC-3'
Primer FWD 1c	3'-TTGCTGATTACTGACCTGGT-5'
Primer REV 9a	5'-ACCTTAGAGTATCCAATAAAGCC-3'
Primer FWD 1c	3'-TTGCTGATTACTGACCTGGT-5'
Primer REV 9b	AT-3' 5'-CCTTAGAGTATCCAATAAAGCC
Primer FWD 1c	3'-TTGCTGATTACTGACCTGGT-5'
Primer REV 9c	ATTGT-3' 5'-AGAGTATCCAATAAAGCC

binding site. Primers FWD 3c and REV 4c were 3 nt longer each and were complementary to the 3'-end of Primers REV 4c and FWD 3c, respectively (Tab. V-2-3).

Elongation of the primers resulted in enhanced signal intensity of specific amplification as well as of background amplification (Fig. V-2-14). The exchange of the hybridization site of primer FWD and primer REV did not reduce the nonspecific amplification. It has to be noted, that the amplicons derived from primers FWD 3a and REV 4a and primers FWD 3b and REV 4b were larger than the amplicons of primers FWD 1a and REV 2a because more than one round of the circularized CLP had to be released for annealing of the primer REV. Restriction digestion resulted in the same fragment length (Fig. V-2-14). The fragment pattern of background amplification was similar in all cases but the signal intensity of background amplification derived from primers FWD 3a and REV 4a was weaker compared to the one obtained by other primers (Fig. V-2-14).

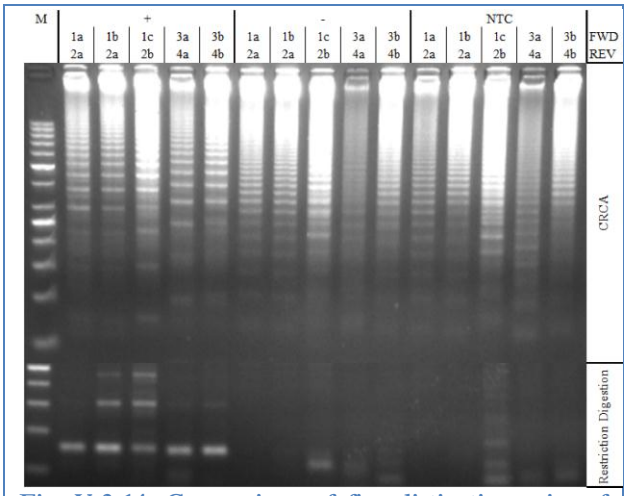


Fig. V-2-14: Comparison of five distinctive pairs of primer for the amplification via CRCA
Ampligase® (0.5 U) circularized 0.12 CLP PPV 2 in the presence of 2 µl cDNA. Two µl of the ligation reaction were transferred to CRCA (0.75 M betaine, 1.6 U *Bst* DNA polymerase (large fragment)). The utilized primers are denoted. + - positive control, - - negative control, NTC - no template control, M - size marker, FWD - forward primer, REV - reverse primer

One approach in optimizing the amplification by different sets of primers was to use primer FWD 1c as initial primer and primer REV 9 as reverse primer. Primer REV 9a was homo-

logous to the 3' target specific arm of the CLP PPV 2 and to the 6 nt of the primer REV 2a binding site next to the 3' PPV specific region (Tab. V-2-3). Primers REV 9b and REV 9c were designed to bind with their 3'-end onto the 5' arm of the CLP at a length of 2 nt or 5 nt, respectively.

The three primer combinations resulted in the correct amplification of the closed CLP in the positive control with primers FWD 1c and REV 9c showing the highest rate of amplification (Fig. V-2-15). Background amplification was observed in varying degrees: primers FWD 1c and REV 9a exhibited the same amplicon pattern typical for background amplification. The amplicons were digested to a fragment of about 60 nt (Fig. V-2-15). Using primers FWD 1c and REV 9a just these sequences and the restriction site were involved. The background amplification in NTC applying primer FWD 1c together with primers REV 9b or REV 9c was nonspecific amplification as well. However, the fragment pattern was similar to that of the positive control as nearly the whole CLP is amplified during background amplification. This was particularly obvious in the case of primer REV 9c.

To reduce background amplification, primers FWD 1c and REV 2b were modified to contain phosphoroamidite (C3), 18-atom hexa-ethyleneglycol (C18), or a hairpin (HP) at the 5'-end. Primers containing a hairpin construct inhibited the amplification in any case (Fig. V-2-16), whereas the primers modified by C3 and C18 did not influence the amplification of the positive control. Though, the background amplification was not influenced either (Fig. V-2-16). HPLC purification of the unmodified primers led to an overall increased amplification rate.

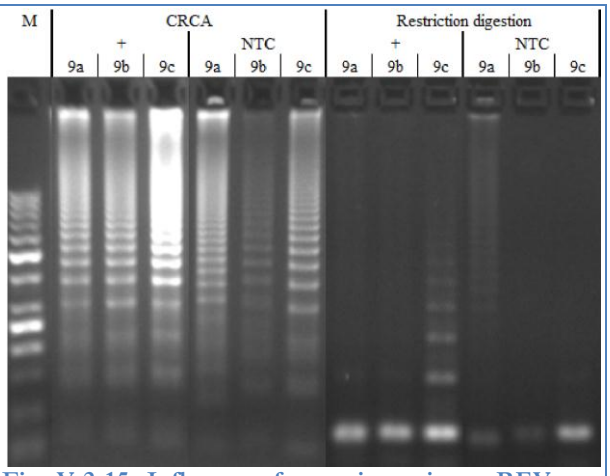


Fig. V-2-15: Influence of spanning primers REV on background amplification
The ligation reaction contained 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®. The amplification reaction contained 0.75 M betaine, primers FWD 1c pur and REV as indicated, 1.6 U *Bst* DNA polymerase (large fragment). The result was proven by restriction digestion. . + - positive control, - - negative control, NTC - no template control, M - size marker

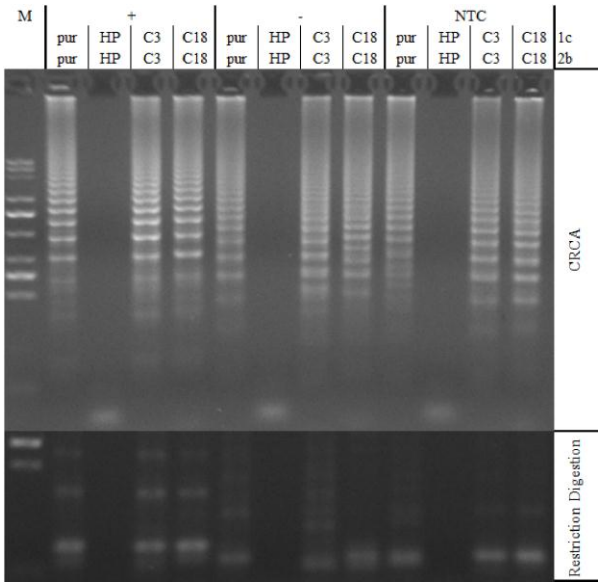


Fig. V-2-16: Evaluation of 5' modifications introduced to the primers
Ligation was conducted with 0.12 µM CLP PPV 2, 2 µl cDNA and 0.5 U Ampligase®. An aliquot of 2 µl was applied to the CRCA reaction containing 0.75 M betaine, 1.6 U *Bst* DNA polymerase (large fragment) and primers FWD 1c or REV 2b either modified by a hairpin construct (HP), by C3 phosphoroamidite or by 18-atom hexa-ethyleneglycol (C18). Restriction digestion was done with 4 µl of the CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

The combination of primer FWD 1c containing a hairpin and primer REV 2b without hairpin did not improve the result of two primers with hairpin (Fig. V-2-17). Primers FWD 1c and REV 2b HP caused a strong decrease in signal intensity in the positive control and no amplification in negative control and NTC (Fig. V-2-17). Indeed, improving the amplification conditions using these primers led to background amplification again (data not shown).

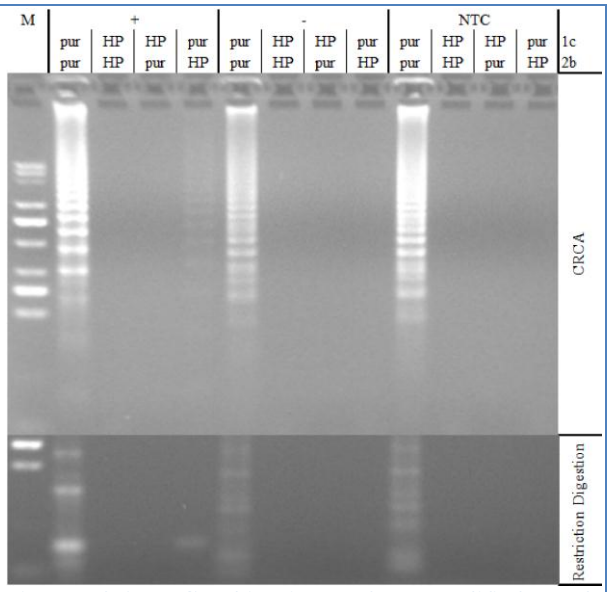


Fig. V-2-17: Combination of unmodified and modified primers containing a hairpin
cDNA (2 µl) was applied to the ligation of 0.12 µM CLP PPV 2 by 0.5 U Ampligase®. CRCA utilized 2 µl ligation reaction, 0.75 M betaine, 1.6 U *Bst* DNA polymerase (large fragment) and primers FWD 1c or REV 2b with or without a hairpin as indicated. EcoRI was used for digestion of 4 µl CRCA product. + - positive control, - - negative control, NTC - no template control, M - size marker

Primers FWD 1a and REV 2a were modified by two locked nucleic acid (LNA) bases replacing DNA bases in the middle of the primers. These primers increased the rate of CRCA of circular and of linear CLPs. It was sufficient to apply one LNA primer and one without this modification. Thereby, primers FWD 1a LNA and REV 2a resulted in a higher rate of amplification than primers FWD 1a and REV 2a LNA (Fig. V-2-18).

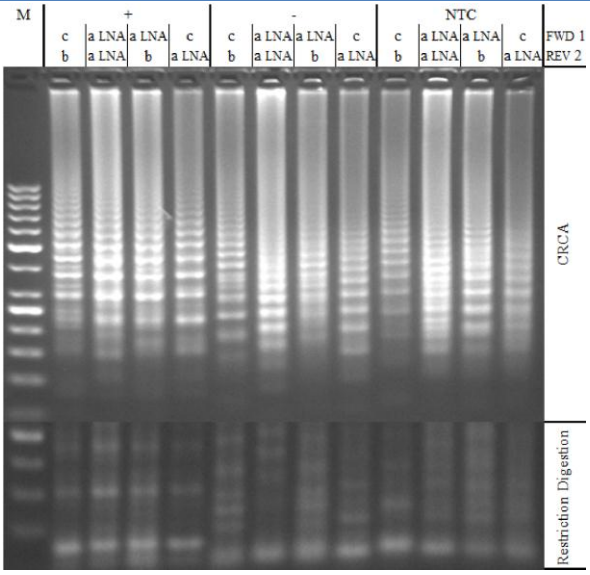


Fig. V-2-18: Influence of LNA bases incorporated in the primers on the CRCA
Ampligase® (0.5 U) catalysed the nick closure of CLP PPV 2 (0.12 µM) in the presence of cDNA (2 µl). CRCA was carried out with *Bst* DNA polymerase (large fragment, 1.6 U), 0.75 M betaine, 2 µl ligation reaction and primers FWD 1a and REV 2a or primers FWD 1a LNA and REV 2a LNA or a combination of the primers. The CRCA product (4 µl) was applied to restriction digestion. + - positive control, - - negative control, NTC - no template control, M - size marker

Using RNA primers instead of DNA primers did not result in unspecific amplification. However, no amplification at all was observed using either two RNA primers or one RNA primer and one DNA primer (data not shown).

Up to now, equimolar concentrations of primers FWD and REV were used. Here, on the one hand primer FWD 1c pur was applied at a concentration of 1 µM and primer REV 9a pur at concentrations of 0.25, 0.5 and 0.75 µM. On the other hand the concentration of primer FWD 9a pur remained unchanged and the primer FWD 1c pur concentration was varied. Reducing the concentration of one of the primers down to 0.5 µM improved the amplification (Fig. V-2-19). Further

decline to 0.25 µM reduced the rate of amplification. Lowering the primer FWD 1c pur concentration rose the amplification more than that of primer REV 9a pur. Thereby, 1 µM of primer FWD 1c pur and either 0.5 µM or 0.75 µM of primer REV 9a pur showed less background amplification than vice versa (Fig. V-2-19).

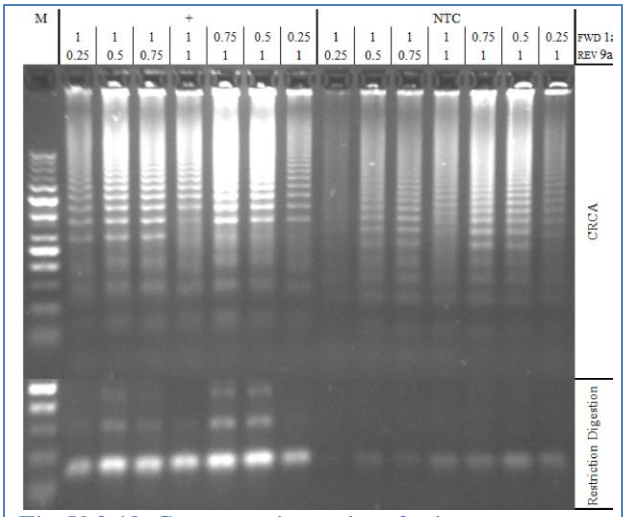


Fig. V-2-19: Concentration series of primers
A ligation reaction contained 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®. Amplification was carried out with 0.75 M betaine, 1.6 U *Bst* DNA polymerase (large fragment) and primers FWD 1c pur and 9a pur with the indicated concentrations. The amplification was proven by restriction digestion. + - positive control, NTC - no template control, M - size marker

V-2.2.5 Comparisons of DNA polymerases regarding strand displacement during CRCA

The range of polymerases that meet the demand of high strand displacement activity required for CRCA is limited. *Bst* DNA Polymerase was tested against DisplaceAce™ DNA Polymerase, IsoTherm™ DNA Polymerase, both active at 65 °C, and Phi29 DNA Polymerase with its temperature optimum at 37 °C (Fig. V-2-20).

The CRCA performed by the *Bst* DNA Polymerase yielded a high amount of DNA. The incubation time in case of IsoTherm™ and DisplaceAce™ DNA Polymerase were twice as long as that of *Bst* DNA Polymerase, but the signal intensity using these thermophilic enzymes was weaker. Phi29 DNA Polymerase did not synthesize any DNA although using exonuclease resistant primers (Fig. V-2-20).

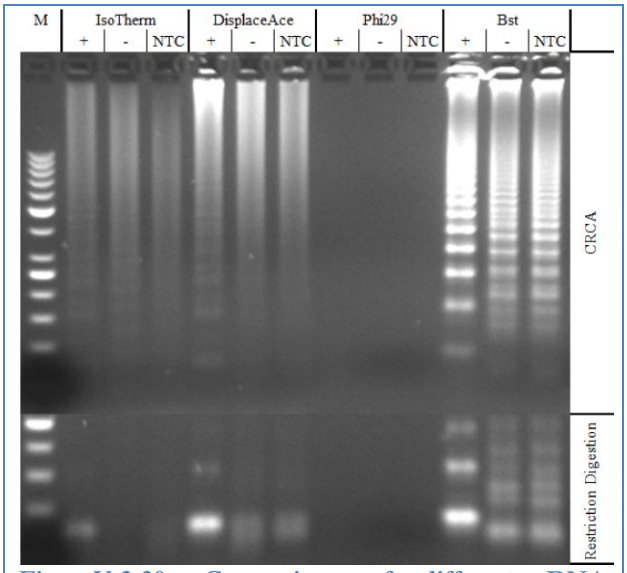


Fig. V-2-20: Comparison of different DNA polymerases applied to CRCA
CLP PPV 2 at a concentration of 0.12 µM was ligated on 2 µl cDNA by 0.5 U Ampligase®. The following CRCA reaction contained 2 µl ligation reaction, 0.75 M betaine, primers FWD 1c and REV 2b and either IsoTherm™ DNA Polymerase (IsoTherm), DisplaceAce™ DNA Polymerase (DisplaceAce), Phi29 DNA Polymerase (Phi29) or *Bst* DNA polymerase (large fragment) (Bst). 4 µl of the CRCA reaction were digested using *EcoRI*. + - positive control, - - negative control, NTC - no template control, M - size marker

Another DNA polymerase capable to displace a DNA strand is Vent (exo-) DNA Polymerase. Therefore, a trial with increasing units of enzyme was conducted. The amplification activity was rather low using standard reaction conditions and 3 U Vent (exo-) DNA Polymerase. No background signal was observed for NTC (Fig. V-2-21). A further increase of enzyme resulted in background amplification (Fig. V-2-21).

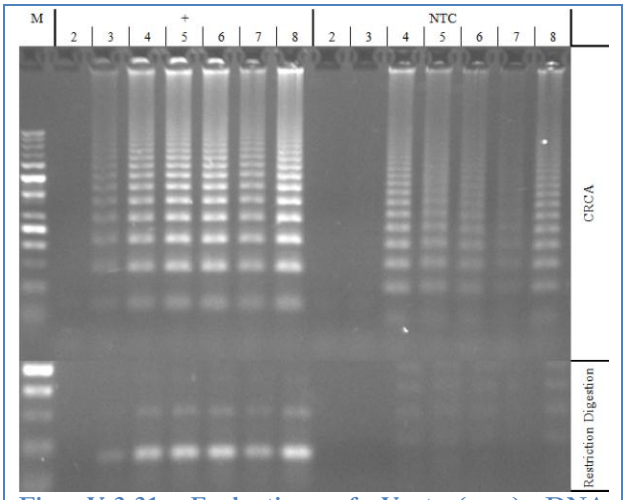


Fig. V-2-21: Evaluation of Vent (exo-) DNA polymerase for its use in CRCA
A ligation reaction contained 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®. The CRCA reaction comprised 2 µl ligation reaction, 0.75 M betaine, primers FWD 1c pur and REV 9a pur and Vent (exo-) DNA polymerase at a concentration as indicated. Amplification was confirmed by restriction digestion. + - positive control, NTC - no template control, M - size marker

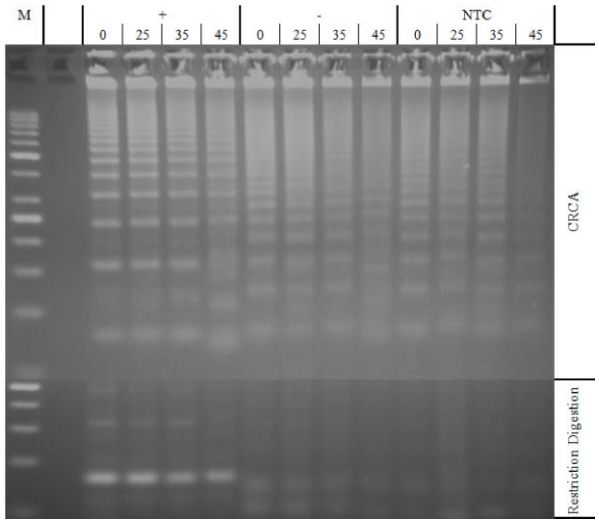


Fig. V-2-22: Reduction of background amplification by exonucleolysis of linear CLPs after ligation
Two µl cDNA were used as target for the ligation of 0.12 µM CLP PPV 2 by 0.5 U Ampligase®. Not circularized CLPs were degraded by Exonuclease I at the concentration (U) denoted. Subsequent CRCA contained 0.75 M betaine, primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment). Restriction digestion was performed on 4 µl amplification product. + - positive control, - - negative control, NTC - no template control, M - size marker

V-2.3 Improving the signal to background ratio by exonucleolysis of linear CLPs

Exonuclease I was tested for reducing the background amplification by digestion of linear CLP prior to CRCA. The enzyme was applied to 3.5 µl of the ligation reaction at concentrations of 25, 35 and 45 U. The amplification of circularized CLPs was not affected using 25 or 35 U Exonuclease I. The yield of CRCA was slightly reduced by 45 U of enzyme. The removal of linear CLPs was not sufficient at either concentration as demonstrated by Fig. V-2-22.

V-2.4 Analysis of the unspecific amplification mechanism

Sequencing of amplification products revealed that sequences derived from the positive control comprised tandem repeats of the CLP sequence as expected (Fig. V-2-23C, Suppl. Fig. 3). The sequence obtained from the negative control exhibited also tandem repeats. However, each repeat consisted of the sequence of primer FWD 1a, the complementary sequence of primer REV 2a and a spacer of about 3 - 5 nt. In most cases this spacer was part of the restriction site, which is located downstream of primer FWD 1a on the CLP. Sometimes the spacer was the nucleotide triplet TAC (Fig. V-2-23A, Suppl. Fig. 1). One sequence was made of a mixture of both variants of tandem repeats (Fig. V-2-23B, Suppl. Fig. 2). Several times, the restriction site of *EcoRI* was included in amplicons derived from background amplification as restriction digestion resulted in a fragment with the length at least 40 bp and a maximum of 70 bp.

To discover the components which were causal for the background amplification, either the

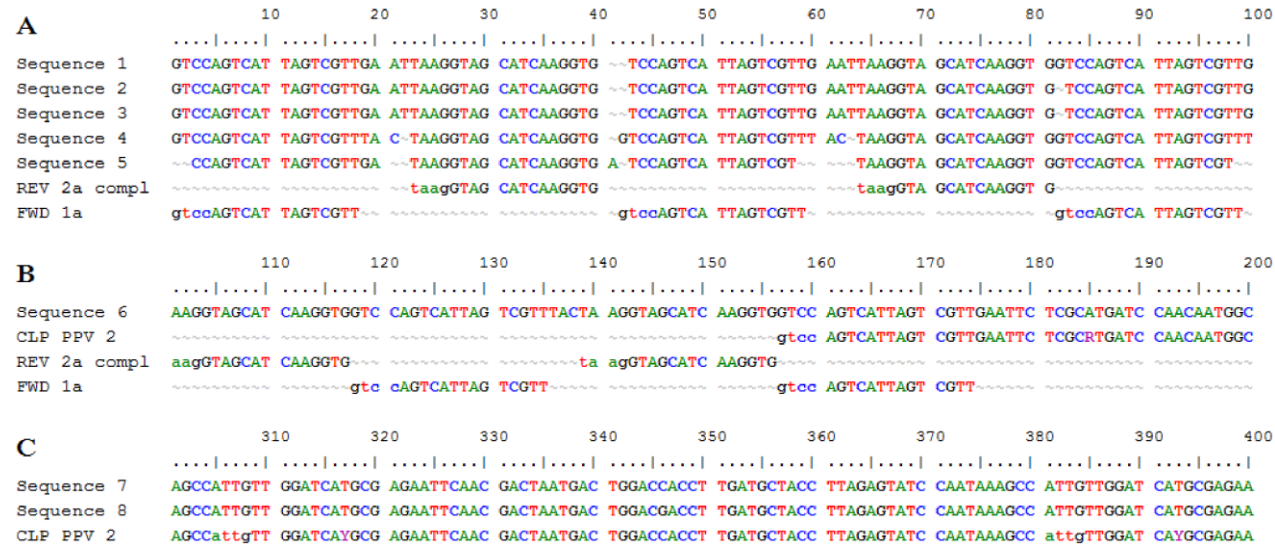


Fig. V-2-23: Sequences of amplicons derived from CRCA
Ampligase® (0.5 U) catalysed the ligation of 0.12 µM CLP PPV 2 on 2 µl cDNA. CRCA contained 2 µl ligation reaction, 0.75 M betaine, primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment). Cloned amplicons were sequenced. A - amplicons derived from unspecific amplification, B - amplicon containing specific as well as unspecific amplification, C - amplicons obtained from specific amplification

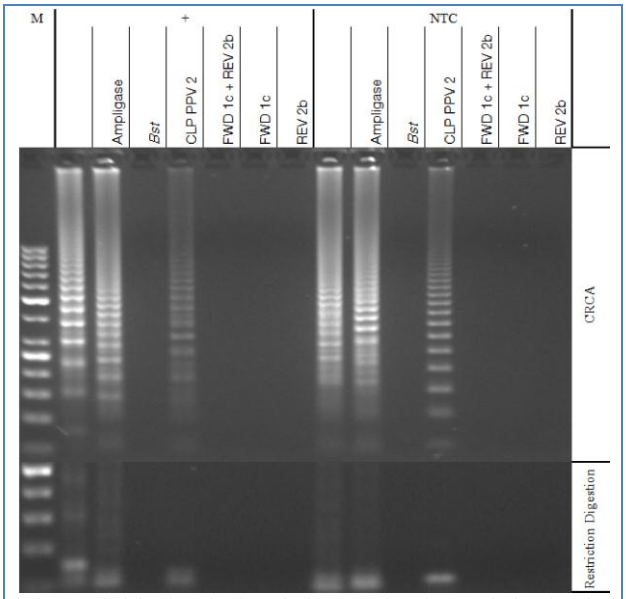


Fig. V-2-24: Analysis of the mechanism of the background amplification
The ligation reaction contained 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®. A volume of 2 µl was transferred to the CRCA (0.75 M betaine, primers FWD 1c and REV 2b and 1.6 U *Bst* DNA polymerase (large fragment). The amplification was proven by restriction digestion. The denoted constituent was omitted from the reaction. + - positive control, NTC - no template control, M - size marker

ligase, the polymerase, the CLP, primer FWD, primer REV or both primers were omitted from the reaction. For correct amplification all five constituent were necessary resulting in the fragment pattern with multimeric length of the CLP after CRCA and a single fragment at 76 bp after restriction digestion (Fig. V-2-24). No ligase resulted in unspecific amplification in any case. Omitting either the polymerase or the primers no amplification was observed. CRCA without template, e.g. the CLP, resulted in unspecific amplification. Compared to the background amplification derived from normal reactions, the fragment pattern was more distinctive and the fragments were digested completely with no visible dilution effect (Fig. V-2-24).

V-3. Loop-mediated isothermal amplification (LAMP)

Since the reason for background amplification during CRCA was not clarified despite

various attempts, another isothermal technique was used for PPV detection. Varga and James, 2006b) established a PPV-RT-LAMP protocol, which was advanced in this work by a homogenous visualisation of the reaction product (Goto et al., 2009) and a simplified procedure to obtain RNA applicable to LAMP (Lee et al., 2009).

Initial trials using the published RT-LAMP reaction conditions showed the suitability of this detection method. Already after an incubition time of 40 min, a faint amplicon pattern was visible in the positive control upon gel electrophoresis. At 60 min a clear signal could be observed. Increasing the reaction time up to 80 and 100 min resulted in a similar amplification rate (Fig. V-3-1). In the negative control, no ladder like fragment pattern was found anytime. However, a single fragment with the length of at least 2,000 bp was visible in any case in the same signal intensity (Fig. V-3-1).

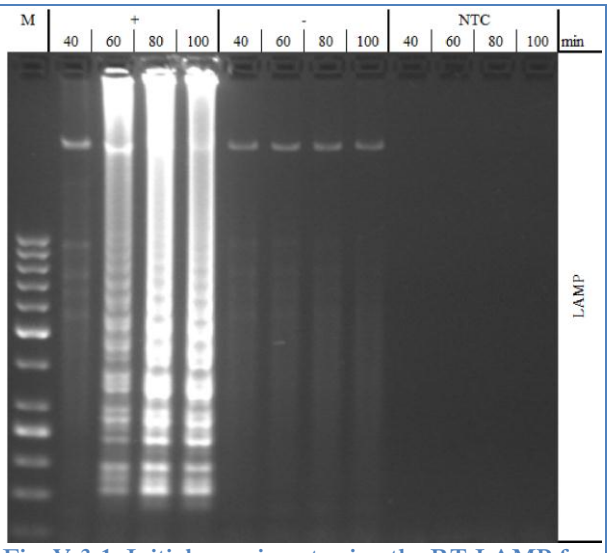


Fig. V-3-1: Initial experiment using the RT-LAMP for the detection of PPV
The RT-LAMP reactions according to Varga and James (2006b) was incubated at 63 °C for 40, 60, 80 or 100 min. + - positive control, - - negative control, NTC - no template control, M - size marker

Sequence analysis of PPV isolates covering all PPV subgroups known so far revealed a few nucleotide exchanges in the range of the RT-LAMP primers. However, none of the exchanges were located at critical positions at the 3'-end of F3, B3, F2 and B2 sequences and at the 5'-end of F1 and B1 sequences to inhibit primer extension (Suppl. Fig. 4).

V-3.1 Visualisation of RT-LAMP amplicons without the need for gel electrophoreses

V-3.1.1 Visualisation of DNA synthesis during RT-LAMP

The determination of the turbidity derived from the accumulation of pyrophosphate during DNA amplification was carried out without any modification of the RT-LAMP reaction conditions. The turbidity was observed at daylight after completion of the reaction (Fig. V-3-2 left). Upon centrifugation of the reaction tubes the pyrophosphate was pelletised and, therefore, was more convenient to recognize than the turbidity (Fig. V-3-2 right).

The increasing turbidity due to pyrophosphate is accompanied by decreasing magnesium concentration which can be visualized by the metal ion indicator calcein. Adding 25 µM calcein and 0.5 mM $MnCl_2$ to RT-LAMP reaction mix resulted in a colour change (Fig. V-3-3) at daylight from light orange in negative samples to light green in positive samples and by illumination with UV light from light green to fluorescent green. Applying cDNA to the modified RT-LAMP reaction mix resulted in the expected colour change in the positive control with the negative control and NTC remaining unchanged (Fig. V-3-3). Using RNA as template, positive control

and NTC showed the same result. However, there was also a colour change in the negative control. Gel electrophoresis did not reveal a positive RT-LAMP amplification in the negative control but a weak single band at about 2,000 bp in case of RNA but not in the case of cDNA (Fig. V-3-3).

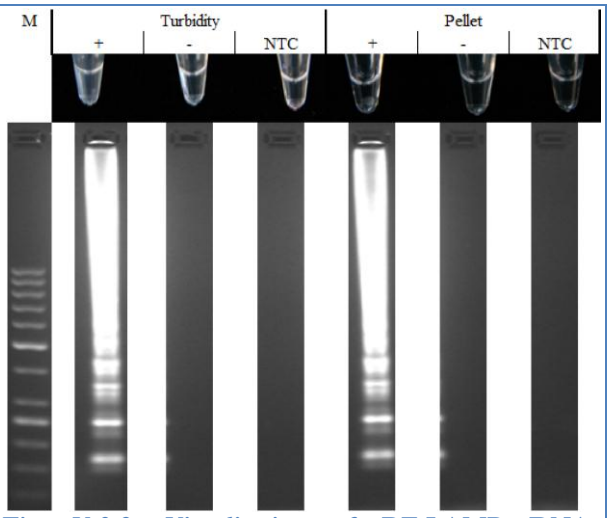


Fig. V-3-2: Visualisation of RT-LAMP DNA synthesis by pyrophosphate turbidity
Upon RT-LAMP, clear reaction tubes were evaluated with respect to the turbidity of the reaction mix (left). The turbidity is caused by the pyrophosphate which is a by-product during DNA amplification and is accumulated during the reaction. Upon centrifugation, a pyrophosphate pellet is formed (right). + - positive control, - - negative control, NTC - no template control, M - size marker

Hydroxy naphthol blue can supplement the RT-LAMP reaction for homogenous visualisation instead of calcein. HNB induces a colour change from purple in negative reactions to blue in positive samples. The colour change is visible at day light. The type of template, whether RNA or cDNA, had no influence on the colour change from purple in negative control and NTC to blue in positive control (Fig. V-3-4). The results of the gel electrophoresis were in perfect agreement to the colour change (Fig. V-3-4). Interestingly, the fragment in the negative control previously described was weaker.

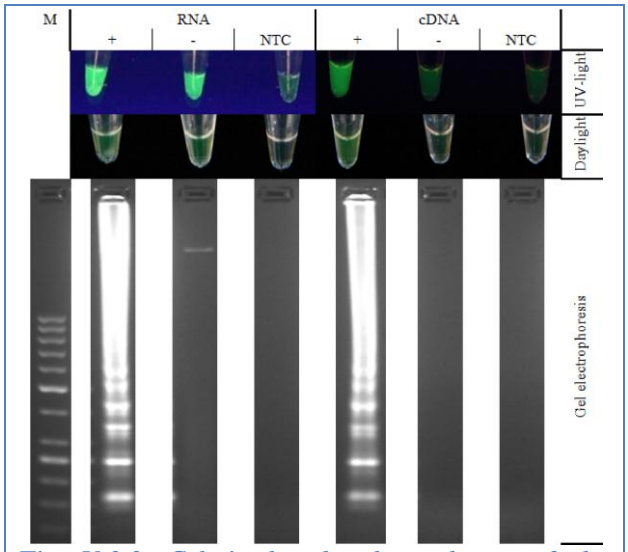


Fig. V-3-3: Calcein based colour change of the reaction mix upon DNA amplification
RT-LAMP is supplemented by 25 µM calcein and 0.5 mM $MnCl_2$. The pyrophosphate binds to the magnesium resulting in a decreasing Mg concentration, which is indicated by calcein by a colour change. This is visible either at daylight or under UV-light. DNA amplification is indicated by light green (daylight) or fluorescent green (UV-light), no amplification by light orange or light green, respectively. + - positive control, - - negative control, NTC - no template control, M - size marker

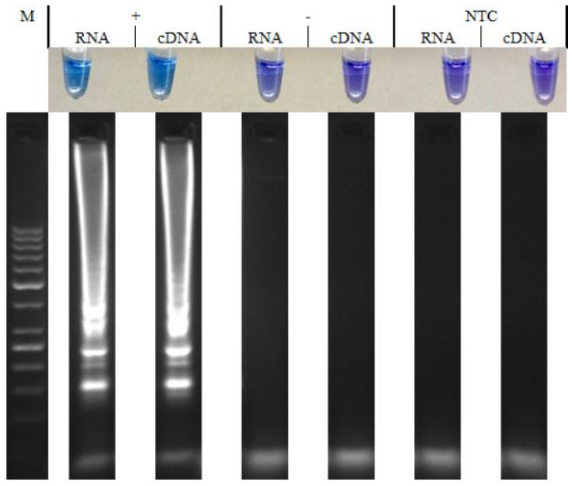


Fig. V-3-4: Homogenous visualisation of DNA synthesis during RT-LAMP by hydroxy naphthol blue HNB (120 µM) is added to the RT-LAMP reaction mix. Its colour change from purple to blue indicates DNA synthesis by RT-LAMP due to declining magnesium concentration as accumulating pyrophosphate, a DNA synthesis by-product, complexes with the Mg. RNA or cDNA was used as template for the reaction. + - positive control, - - negative control, NTC - no template control, M - size marker

The amplification of nucleic acids can be monitored chromatographically by LFDs as well. The LFDs tested exhibited a control line and the testing line. The control line appeared in any case as expected. However, the testing line of the positive control was visible only very faintly despite a high yield of DNA synthesis as proven by gel electrophoresis (Fig. V-3-5). Negative control and NTC showed no signals either on the testing line neither of the LFD nor upon gel electrophoresis.

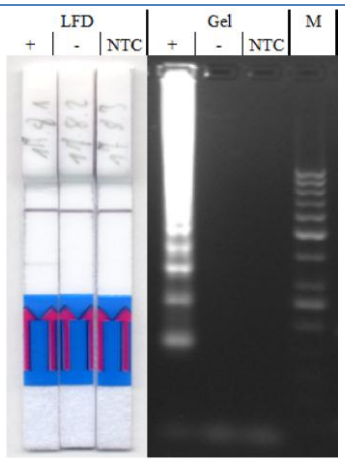


Fig. V-3-5: Replacement of gel electrophoresis by lateral flow devices
RT-LAMP is followed by visualisation of the amplicons by LFDs instead of gel electrophoresis. FITC labelled probes move along the LFD. If they have specifically hybridized to biotinylated DNA molecules, the streptavidin coated testline appears. Otherwise the FITC probes move further on to the control line resulting in a colouration. LFD - lateral flow device, Gel - gel electrophoresis, + - positive control, - - negative control, NTC - no template control, M - size marker

V-3.1.2 Optimisation of the HNB-RT-LAMP detection system

Optimisation was performed to identify RT-LAMP conditions with unambiguous HNB staining results and well performing LAMP conditions.

HNB serves to visualise the actual Mg^{2+} concentration and its decrease by pyrophosphate precipitation during positive LAMP amplification. This is dependent on start concentrations of Mg^{2+} and HNB, but also on dNTPs, which compete with HNB for Mg^{2+} chelatization but which are consumed during the reaction. Moreover, magnesium concentration has an influence on the performance of the polymerase. For HNB-RT-LAMP optimisation, HNB staining blue was added to the RT-LAMP protocol of Varga and James (2006b) in a concentration of 0.06 mM, 0.12 mM, 0.18 mM and 0.24 mM (Fig. V-3-6A). HNB did not affect the RT-LAMP reaction with RNA template in any case. With increasing HNB concentration the colour intensity of the RT-LAMP reaction changed from light blue to dark blue in the case of the positive sample (RNA derived from an artificially PPV inoculated tree) and from light purple to dark purple in the case of the negative (RNA derived from a healthy tree) and NTC. This result was confirmed by two replications.

The influence of the dNTP concentration on DNA amplification and subsequent on the colour change due to HNB was tested with 1.4 mM, 1.2 mM, 1.0 mM and 0.8 mM of each dNTP in triplicate (Fig. V-3-6B). The rate of amplification was negatively affected by using a lower concentration of dNTPs. The colour intensity was higher at elevated dNTP concentrations. The lowest concentration of only 0.8 mM dNTPs produced ambiguous results.

The effect of varying magnesium concentrations (7 mM, 8 mM, 9 mM and 10 mM) on the reaction was analysed in triple repetition (Fig. V-3-6C). The use of 8 mM of magnesium resulted in a slightly enhanced amplification. The colour change from purple to blue was more visible using lower magnesium concentrations.

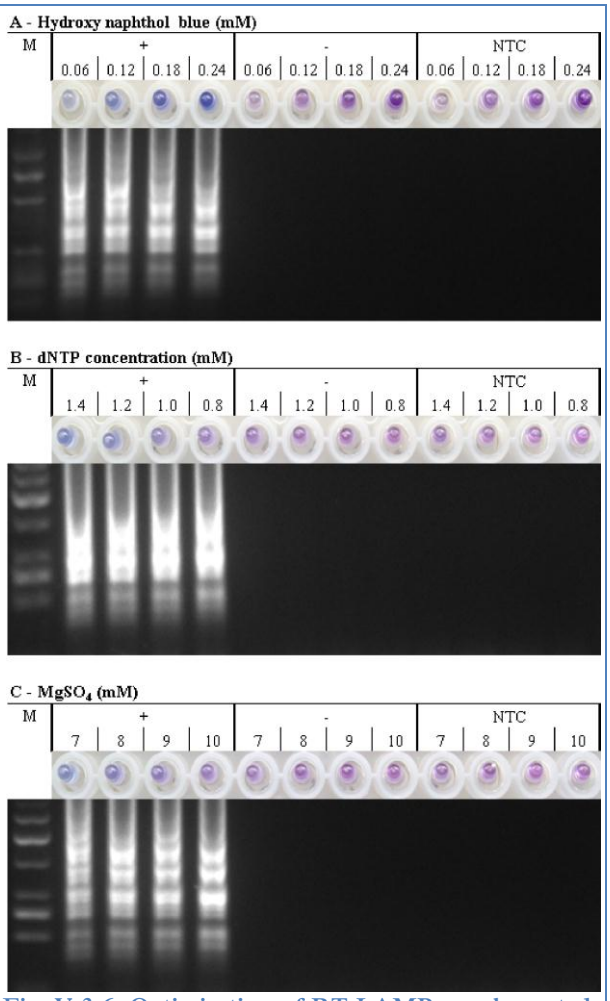


Fig. V-3-6: Optimisation of RT-LAMP supplemented by HNB
A – RT-LAMP protocol according to Varga and James (2006b) amended with 0.06 mM, 0.12 mM, 0.18 mM or 0.24 mM hydroxy naphthol blue (HNB); B – RT-LAMP with 0.12 mM HNB and 1.4 mM, 1.2 mM, 1.0 mM or 0.8 mM of each dNTP; C – RT-LAMP with 0.12 mM HNB, 1.0 mM dNTPs and 7 mM, 8 mM, 9 mM or 10 mM magnesium ($MgSO_4$). The blue colour of the reaction mix indicates a decrease in Mg^{2+} concentration upon RT-LAMP reaction. The result of the colour change was verified by gel electrophoresis. + - positive control, - - negative control, NTC - no template control, M - size marker, incubation time: 1 h

Combining the results from the three approaches the optimised concentrations with 0.12 µM HNB, 1 mM dNTPs and 8 mM $MgSO_4$ applied to the RT-LAMP resulted in a definite colour change from purple to blue upon DNA amplification based on PPV RNA (Fig. V-3-6).

Finally, a time course of HNB-RT-LAMP using the optimized reaction conditions was conducted in triplicate to evaluate the optimal incubation time to detect PPV in samples exhibiting low virus titre. At the same time a colour change of the reaction mix upon unspecific amplification should be avoided to prevent the detection of false positive signals. Using 10^{-5} diluted RNA as template, a colour change was observed after 1 h of incubation (Fig. V-3-7), but it was still difficult to distinguish the positive control from the negative one. After 2 h the difference of the colouration was unambiguous. In contrast, unspecific amplification in negative and NTC was found by colour change and by gel electrophoresis only after incubation of the HNB-RT-LAMP reaction for 5 h or longer (Fig. V-3-7). However, the determination of colour change of a sample must be assessed by comparison to a negative control or NTC performed at equal conditions.

V-3.1.3 Detection limit of the HNB-RT-LAMP test

HNB-RT-LAMP and RT-PCR were compared with regard to their respective detection limits. The plasmid pICPPV-NK-GFP was used as template at concentrations from 100 pg/µl down to 10 ag/µl. Using the HNB-RT-LAMP, 1 fg/µl plasmid was detected in each case. Once, the lower concentration of 100 ag/µl was detected. The detection limit of the RT-PCR was similar with one reaction detecting even 10 ag/µl. The colour change of the HNB-RT-LAMP reaction from purple to blue for positive samples was according to the presence of expected fragment pattern obtained by gel electrophoresis (Fig. V-3-8).

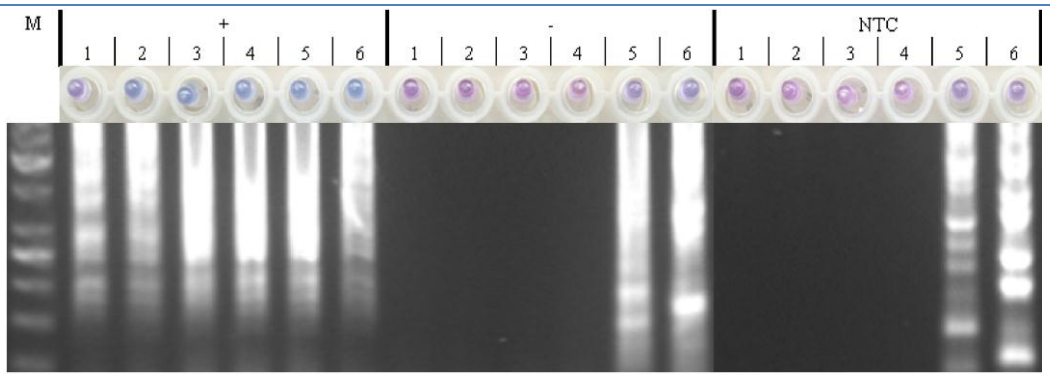


Fig. V-3-7: Time course of HNB-RT-LAMP
HNB-RT-LAMP was incubated for the time indicated (in h) to determine the optimal duration of the LAMP based amplification. Optimal incubation time is characterized by a reaction with HNB based colour change in even slight positive samples but simultaneously with no unspecific amplification, which would lead to a colour change as well. + - positive control, - - negative control, NTC - no template control, M - size marker

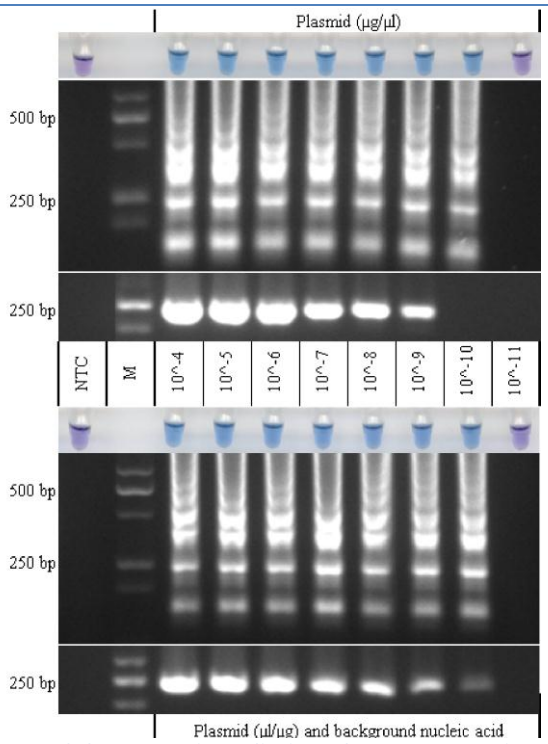


Fig. V-3-8: Detection limit of HNB-RT-LAMP compared to RT-PCR
HNB-RT-LAMP and RT-PCR were carried out with a serially diluted plasmid containing the genome of PPV as template. The colour change of the HNB-RT-LAMP reaction mix from purple to blue upon amplification indicated a positive result. A purple reaction mix indicated a negative result. This was proven by gel electrophoresis demonstrating the ladder like fragment pattern of LAMP amplicons. The amplification using RT-PCR resulted in a single amplicon. The analysis was done in absence (above) and presence (below) of background nucleic acid. NTC - no template control, M - size marker

In the presence of background cDNA the overall detection limit of the RT-PCR decreased to 100 ag/µl plasmid and in two out of three trials to 10 ag/µl plasmid. Applying plasmid and background RNA to the HNB-RT-LAMP protocol the minimal detection limit differed in each of the replications with 10 fg/µl, 100 ag/µl and 10 ag/µl plasmid. Again, the colour of the reaction mix and the gel analysis were in perfect agreement (Fig. V-3-8).

V-3.2 Virus suspensions obtained by a fast plant extraction procedure as template for HNB-RT-LAMP

V-3.2.1 Comparison of HNB-RT-LAMP and RT-PCR using the virus suspension as template

Apart from the direct visualisation of RT-LAMP reactions by HNB, a further aim was to test the simplification of the RNA extraction procedure. For comparison, a virus suspension obtained by a fast plant extraction procedure and RNA derived from both this plant extract and from the conventional RNA extraction were applied to HNB-RT-LAMP, to one-step multiplex

RT-PCR and to cDNA synthesis, respectively. The cDNA was used as template for RT-PCR. Twenty four genotypes with each one artificially PPV infected tree showing clear symptoms and one PPV free tree without any symptoms per genotype were tested.

In all positive samples (artificially infected plants showing symptoms) PPV was detected by HNB-RT-LAMP and RT-PCR regardless of the type of sample preparation. After applying the virus suspension obtained by the fast plant extraction procedure to the one-step multiplex RT-PCR no false positives were observed. The one-step multiplex RT-PCR failed to discover PPV seven times out of 24 (false negatives). Employing the fast plant extraction procedure, all detection methods identified the negative ones correctly. Applying extracted RNA to HNB-RT-LAMP and RT-PCR resulted in false positive signals in seven samples (Fig. V-3-9 and Tab. V-3-1) as these samples were derived from healthy trees. PPV was detected in all cases by one-step

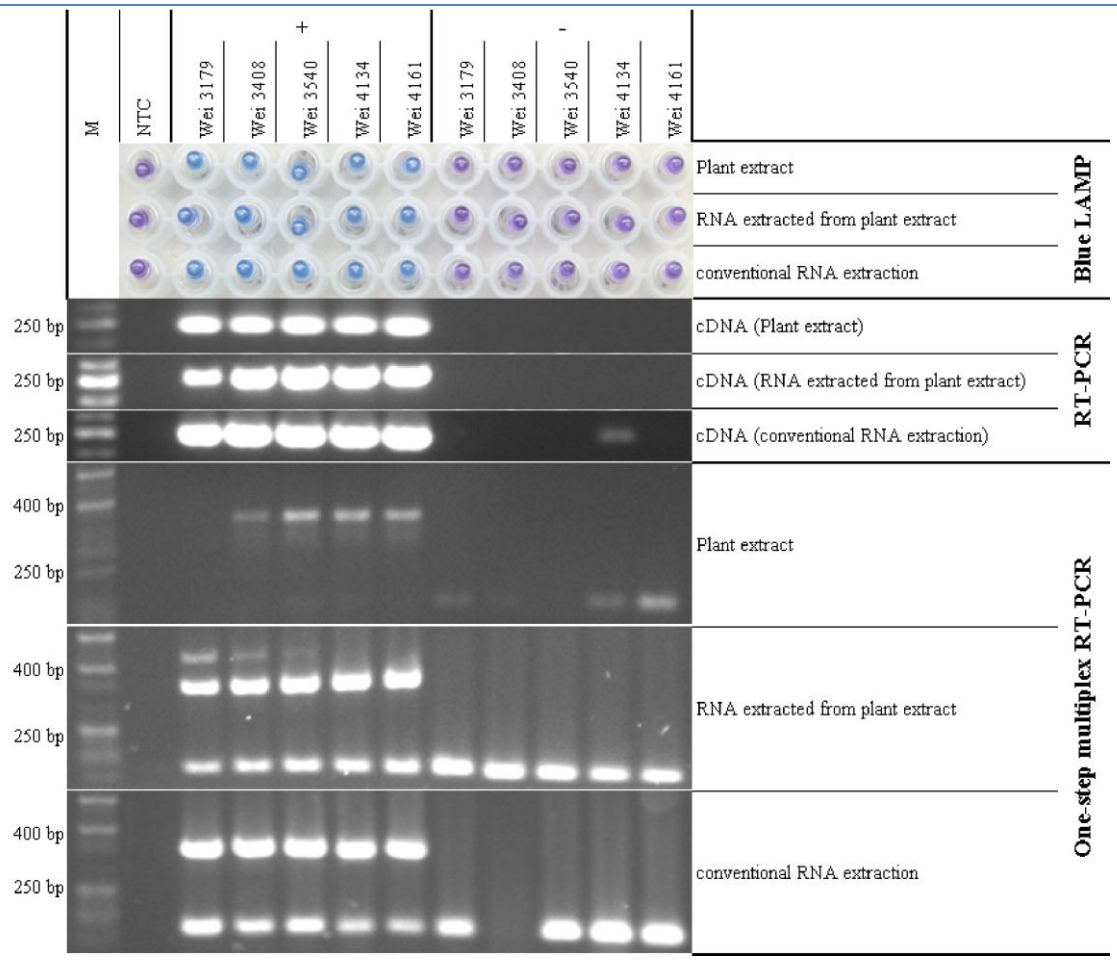


Fig. V-3-9: Influence of the template type on results with different nucleic acid amplification protocols
Virus suspension obtained by the fast plant extraction procedure, RNA extracted from plant extract and conventionally extracted RNA were applied to HNB-RT-LAMP, one-step multiplex RT-PCR and cDNA synthesis. The cDNA was applied to HNB-RT-LAMP and RT-PCR. Using HNB-RT-LAMP the colour of the reaction mix reports the result: purple - negative, blue - positive. In the case of RT-PCR and one-step multiplex RT-PCR the reaction products were analysed by the use of gel electrophoresis. Using RT-PCR the size of the PPV specific amplicon was 243 bp. The one-step multiplex RT-PCR was able to detect three viruses (PPV: 345 bp, PDV: 220 bp, PNRSV: 425 bp) and an internal control (*nad5*: 181 bp). NTC - no template control, M - size marker

Tab. V-3-1: Comparison of PPV detection protocols using template from different extraction techniques HNB-RT-LAMP, RT-PCR and one-step multiplex RT-PCR (Multiplex) were supplemented with virus suspension, RNA extracted from plant extract, conventionally extracted RNA or cDNA derived from the different extraction methods. Summarised results of primary data on method evaluation given in Suppl. Tab. 2.

	Sensitivity			Spezificity		
	Plant extract	RNA extracted from plant extract	conventional RNA extraction	Plant extract	RNA extracted from plant extract	conventional RNA extraction
Blue LAMP	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	22/24 (0,92)	23/24 (0,96)
RT-PCR	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	22/24 (0,92)
Multiplex	17/24 (0,71)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)	24/24 (1,00)

multiplex RT-PCR using extracted RNA as template.

The results of the extraction trial were used to calculate the sensitivity and specificity of each detection method tested based on the definition of Altman and Bland (1994). Using this approach, sensitivity was optimal (100 %) for all tested combinations of RNA extraction method and nucleic acid amplification technique but only 71 % for the fast plant extraction procedure combined with the one-step multiplex RT-PCR. Complete specificity was achieved by applying the virus suspension to HNB-RT-LAMP and RT-PCR and by using the one-step multiplex RT-PCR. Specificity was slightly reduced in the case of conventionally extracted RNA used in HNB-RT-LAMP and RT-PCR (Tab. V-3-1).

The combination of virus suspension and one-step multiplex RT-PCR failed to amplify the internal control *nad5* 21 times out of 48. Neither PPV nor *nad5* were detected in six PPV positive samples (sample preparation or reaction failed). *nad5* was not found in eleven PPV negative samples (sample preparation, internal control or reaction failed). Four times PPV was recognized while *nad5* was not detected (internal control failed). Besides PPV, the one-step multiplex RT-PCR discovered the infection with PDV and PNRSV in some cases. The internal control was detected in all cases and 46 out of 48 cases using RNA extracted from the virus suspension or conventionally extracted, respectively, as template for one-step multiplex RT-PCR. The two samples with no detectable internal control were PPV negative (Tab. V-3-1).

V-3.2.2 Influence of the virus suspension on HNB-RT-LAMP performance

Samples composed of different ratios of plant material obtained from a healthy tree and from a PPV infected tree were applied to the fast plant extraction method. The virus dilution and the RNA derived thereof were diluted and used as template used in HNB-RT-LAMP and RT-PCR, respectively.

The sample derived from the healthy tree resulted in no signal upon HNB-RT-LAMP and RT-PCR (Fig. V-3-10). There was also no difference in the obtained signals by HNB-RT-LAMP and RT-PCR after applying the dilutions of the sample composed solely of infected plant material and the sample composed of one half of the infected leaves and the other half of healthy leaves in any repetition. All dilutions of RNA were detected by HNB-RT-LAMP when using the 1:10

(PPV positive:PPV negative) sample. HNB-RT-LAMP using the virus suspension and RT-PCR in two out of three replications failed to detect the 10^{-6} dilution of this sample. The 1:50 (PPV positive: PPV negative) sample was detected at dilutions down to 10^{-4} by the virus suspension in HNB-RT-LAMP in all replications. At a dilution of 10^{-6}, RT-PCR resulted in no amplification. RNA was still detectable by HNB-RT-LAMP at a dilution of 10^{-5} in two trails and at a dilution of 10^{-6} in one trial.

V-3.2.3 Exemplary orchard PPV screening using HNB-RT-LAMP

Reliability of the HNB-RT-LAMP protocol was tested with orchard samples. For this, 102 samples were collected in an orchard with natural infection rate. Four leaves per sample were stored in a filter extraction bag. The fast plant extraction procedure was set up as described above and the virus suspension was applied directly to HNB-RT-LAMP and one-step multiplex RT-PCR for comparison.

HNB-RT-LAMP and one-step multiplex RT-PCR were used to analyse the plant extracts of 102 samples from an orchard with natural infection rate. HNB-RT-LAMP showed positive signals in 13 and no signals in 89 samples. Using one-step multiplex RT-PCR, eight samples were PPV positive and 48 samples were truly negative as *nad5* was amplified in both cases. In the remaining 46 samples amplification failed, neither of PPV RNA nor of the *nad5* was detectable. The eight samples in which PPV was detected by one-step multiplex RT-PCR were also proved PPV positive by HNB-RT-LAMP.

V-3.3 Differentiating the PPV subgroups by HNB-RT-LAMP

Up to now, seven subgroups of PPV are characterized: D, Rec, M, T, C, EA and W. To assign an isolate to one of the subgroups, strain specific primer sets were developed using PrimerExplorer V4 and checked for their specifity by BLAST analysis (Tab. V-3-2, Suppl. Fig. 5, Suppl. Fig. 6, Suppl. Fig. 7, Suppl. Fig. 8).

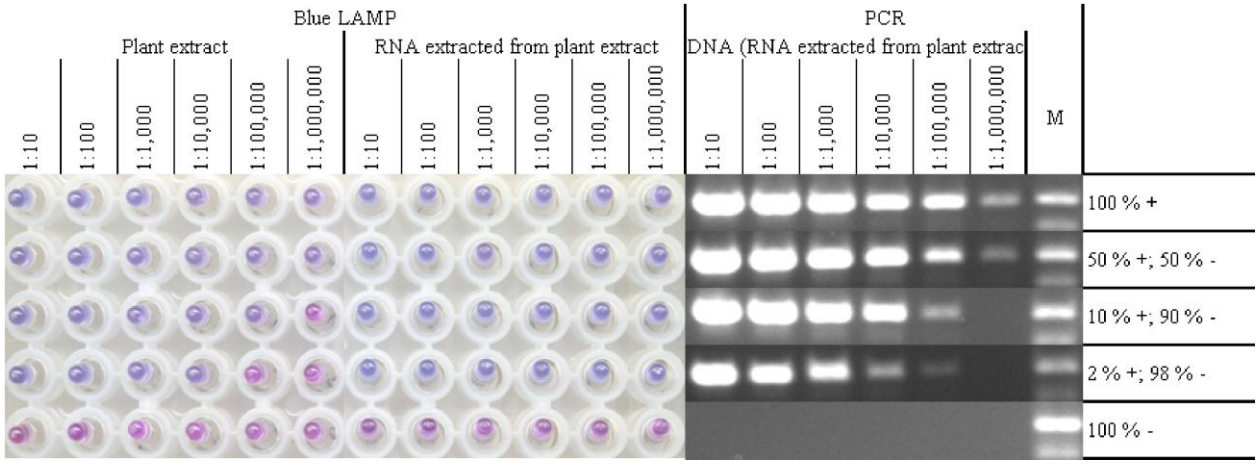


Fig. V-3-10: Influence of the virus suspension compared to RNA on HNB-RT-LAMP and RT-PCR. Samples comprising varying ratios of plant material derived from an artificially PPV inoculated tree to plant material of a healthy tree were subjected to the fast plant extraction procedure. The homogenates were either applied to conventional RNA extraction or serially diluted to HNB-RT-LAMP. The RNA was also diluted and used for HNB-RT-LAMP and cDNA synthesis which was followed by PCR. The colour of the HNB-RT-LAMP reaction mix indicated the presence (blue) or absence (purple) of PPV. + - plant material derived from a PPV infected tree, - - plant material derived from a healthy tree, M - size marker

The seven primer sets were tested for their ability to discriminate between the PPV subgroups. The HNB-RT-LAMP reaction conditions were the same as for the general detection of PPV with minor modifications. Betaine was replaced by 3 mM trehalose and incubation time was shorten to a maximum of 40 min since preliminary tests exhibited unspecific amplification upon longer incubation. Reaction temperature was increased to 64 °C. RNA of 23 samples and 4 controls as well as virus suspension obtained by the fast extraction procedure of 16 samples and 4 controls were applied to the reactions. A NTC was included as well. The samples used for evaluation of strain specific HNB-RT-LAMP reactions were also tested by HNB-RT-LAMP and

Tab. V-3-2: Sequences of the primers used for strain differentiation by HNB-RT-LAMP
Green coloured bases indicate, that this base is different in the other strain. Red coloured bases indicate different bases in five other strains. Nucleotides which were assigned for specificity in PrimerExplorer V4 for primer generation are depicted in small letters.

Primer	Sequence (5'-3)	Length (nt)
F3 PPV-D	AGC AAC AAC TCA ACC agc a	19
B3 PPV-D	CCT TTC CCT TCA CCT TTG Gc	20
FIP PPV-D	Gcg cgT TTG AGt TGC TAG GTG AAT TTC aca ggt gtc AGg acc	42
BIP PPV-D	ACa aac AGA GAC AGG GAC GTC GCA TTG cct tca aaC GTG GC	41
FL PPV-D	TGC ATC CTC ATT ACC ATA TGT TCC	24
BL PPV-D	GCA GGA TCA ATT GGA ACT TTt aCA G	25
F3 PPV-Rec	AGG ACG ATG AAG AAG TYG	18
B3 PPV-Rec	TCC ATA AAC TCC AAA AGA YTG	21
FIP PPV-Rec	CTG GTT GAg ttg TTG CCA CAA TGC AGG aaa acc cac TG	38
BIP PPV-Rec	CTG CAa ttc aaa cca caa CAC CGa gaa acT GGT CTT atc GCA	42
FL PPV-Rec	CTG CCG GTG CAG TTA C	16
F3 PPV-M	GAA TTT gca ctg cCC ATG	18
B3 PPV-M	GAA AGC ATC TGc TCA CCA	18
FIP PPV-M	CCA CCC ATC TGG TAA AGC TCT GCG TTG GAA TGA CAA AAT TCA GA	44
BIP PPV-M	cGG TTC TCA GTT CGA TAG CTC TCA CTC TTC CAT gaa ggc c	40
FL PPV-M	AGT AAC TTG TCC CAA CCA C	19
BL PPV-M	ACG CAG TTC TCA ACA TCC	18
F3 PPV-T	AcG TCA ARC TCT Taa ttA GAA GC	23
B3 PPV-T	GCc gaa agC ATT GAT GTG	18
FIP PPV-T	CCA GGT GAY AAA ACa CTC ATA AGC TAT cta tAA ACC ACA Agt gAT GGA G	49
BIP PPV-T	GCA TTA TTC AAT AGC GGT TCg cTA TtG CTG CCA AAC TAT GAG Ag	44
FL PPV-T	TGG TTC CTC CTT GAR CAC TT	20
F3 PPV-C	gcg aaa acc gag AAC AAA G	19
B3 PPV-C	GAT Ctc cAT AAC CCC AAG C	19
FIP PPV-C	CCA ACT Tcg ggA TGT ACA Tcc CAG GAG CTa TGG TTC ATG TCt	42
BIP PPV-C	TTG AGT GGG Ata ggT CAc ATG GTC aAC CAT cGA TGC ACA A	40
BL PPV-C	CAA TTC ACA GAT TGG AAG CA	20
F3 PPV-EA	CGA TGA AGA Rga agt aGA TG	20
B3 PPV-EA	gga gGT gta gta gtT Gtt gtg	21
FIP PPV-EA	AGt TTG cTG AGT TGT TGt ggt AGG gAG GCC ttt aGT CAC	39
BIP PPV-EA	aac ctt aca agC TAC Gca ggC TGA Ggc act gtc cta gtg	39
FL PPV-EA	AAC AAT TGG CTG CTG TGT AG	20
BL PPV-EA	CAC TCC AGC GAC GAC TG	17
F3 PPV-W	CAT AGT gaT GCA CCA GGC A	19
B3 PPV-W	GcT Tca cgc tcG GTT GAG	18
FIP PPV-W	cgg tgG ATG gag gtg ttg ttg cgA CGA gga AGA TGA CGA tcc	42
BIP PPV-W	acc ACa ggt aaC AGC CAc atc gTG TTG CTG Gtg taA Aga cGG	42
FL PPV-W	TAG AAG AGC AGG CCT GCC	18
BL PPV-W	CCT GCA GAG CAC AAC GTC	18

RT-PCR (Wetzel et al., 1991b, Bühler, 2007) for general detection of PPV as well as by RT-PCR for strain typing (Szemes et al., 2001, Šubr et al., 2004). Strain W was detected by the primers described in V-1. For strain T no PCR primers have been developed yet.

In the sample C- 1 and NTC, PPV was not detected by either method used (Tab. V-3-3, Suppl. Fig. 9, Suppl. Fig. 10). In C- 2, which should have been negative as well, PPV was detected by HNB-RT-LAMP (RNA) and RT-PCR (Wetzel et al., 1991b, Bühler, 2007). However, strain specific reactions gave no signals. PPV was recognized in sample 21 by HNB-RT-LAMP (virus suspension) and RT-PCR. In contrast, HNB-RT-LAMP (RNA) resulted in positive signals for samples 22 and 23 (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10). HNB-RT-

Tab. V-3-3: Differentiation of PPV samples by strain specific primer sets applied to HNB-RT-LAMP
The seven primer sets developed for the specific detection of one of the PPV subgroups were examined in HNB-RT-LAMP using a modified HNB-RT-LAMP for the general detection of PPV (incubation at 64 °C for 40 min, 3 mM trehalose) and compared to strain typing by RT-PCR. A - colour change to blue by HNB-RT-LAMP, B - gel electrophoresis of HNB-RT-LAMP amplicons, C - RT-PCR according to Wetzel et al. (1991b), D - RT-PCR according to Szemes et al. (2001), E - RT-PCR according to Šubr et al. (2004), * - virus suspension used as template; C+ - positive control, C- - negative control, NTC - no template control, ? - isolate not assigned to a PPV subgroup previously; ++ - positive signal, + - weak positive signal, - - no signal, U - unspecific signal

Sample	Nominal Strain	PPV in general						PPV-D						PPV-Rec					PPV-M					
		LAMP				PCR		LAMP				PCR		LAMP				PCR	LAMP				PCR	
		A	B	A*	B*	C	D	A	B	A*	B*	D	E	A	B	A*	B*	E	A	B	A*	B*	D	E
1	Rec	++	++			++	++	-	U			++	++	+	++			++	+	++			++	-
2	D	++	++			++	++	+	++			++	++	-	U			-	-	-			-	-
3	M	++	++			++	++	-	-			++	++	+	U			-	+	++			++	++
4	EA	++	++			++	++	-	U			-	+	-	U			-	-	-			-	-
5	W	++	++			++	-	-	-			-	-	-	U			-	-	-			-	-
6	C	++	++			++	++	-	-			-	-	+	U			-	-	-			-	-
7	T	++	++			++	++	-	-			-	-	-	-			-	+	++			++	-
8	D	++	++	+	++	++	++	++	++	+	++	++	++	-	-	-	U	-	-	-	+	++	-	-
9	D	++	++	+	++	++	++	++	++	+	++	++	++	-	U	-	U	-	-	-	-	-	-	-
10	D	++	++	+	++	++	++	-	-	+	++	++	++	-	U	-	U	-	-	-	-	-	-	-
11	Rec	++	++	+	++	++	++	-	-	-	-	-	-	++	++	+	++	++	-	-	-	-	++	-
12	Rec	++	++	+	++	++	++	-	-	-	-	-	-	-	-	+	++	++	-	-	-	-	++	-
13	M	++	++	+	++	++	++	++	++	-	-	++	++	+	U	-	U	-	+	++	+	++	-	-
14	M	++	++	+	++	++	++	-	-	-	-	-	-	-	U	-	U	-	++	++	+	++	++	++
15	C	++	++	+	++	-	-	-	-	-	-	-	-	-	U	-	U	-	-	-	-	-	-	-
16	EA	++	++	+	++	++	++	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-
17	W	++	++	+	++	++	+	-	-	-	-	-	-	+	U	-	U	-	-	-	-	-	-	-
C+ 1	?	++	++	+	++	+	++	-	-	-	-	-	-	+	+	+	++	+	-	-	-	-	++	-
C- 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	U	-	U	-	-	+	-	-	-	-
18	W	++	++	-	-	++	-	-	-	-	-	-	-	-	U	-	U	-	-	-	-	-	-	-
19	EA	++	++	+	++	++	++	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-
20	Rec	+	++	+	++	++	++	-	U	-	-	-	-	++	++	+	U	+	-	-	-	-	++	-
21	?	-	-	+	++	++	-	-	+	+	++	++	++	-	-	-	-	-	-	-	-	-	-	-
22	?	++	++	-	-	++	-	-	+	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
23	?	+	++	-	-	-	-	+	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
C+ 2	?	++	++	+	++	++	++	++	++	+	++	++	++	-	-	-	-	-	-	-	-	-	-	-
C- 2	-	+	++	-	-	+	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-	-	-
NTC	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-

Tab. V-3-3 continued

Sample	Nominal Strain	PPV-T LAMP A	PPV-T LAMP B	PPV-T LAMP A*	PPV-T LAMP B*	PPV-C LAMP A	PPV-C LAMP B	PPV-C LAMP A*	PPV-C LAMP B*	PPV-C PCR D	PPV-EA LAMP A	PPV-EA LAMP B	PPV-EA LAMP A*	PPV-EA LAMP B*	PPV-EA PCR D	PPV-W LAMP A	PPV-W LAMP B	PPV-W LAMP A*	PPV-W LAMP B*	PPV-W PCR D
1	Rec	-	-			-	U			-	-	-			-	-	U			-
2	D	-	-			-	-			-	-	-			-	-	U			-
3	M	-	-			-	-			-	-	-			-	-	-			-
4	EA	-	-			-	-			-	+	++			++	-	U			-
5	W	-	-			-	-			-	-	-			-	+	++			++
6	C	-	-			++	++			++	-	-			-	-	-			-
7	T	+	++			-	U			-	-	-			+	-	-			-
8	D	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-
9	D	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	U	-
10	D	-	-	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-
11	Rec	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-
12	Rec	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	U	-	U	-
13	M	-	U	-	-	-	-	-	-	-	-	-	-	U	-	-	U	-	-	-
14	M	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	U	-	-	-
15	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	EA	-	-	-	U	-	-	-	U	-	++	++	+	++	++	-	-	-	-	-
17	W	-	-	-	U	-	-	-	U	-	-	-	-	-	-	+	++	-	++	++
C+ 1	?	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-
C- 1	-	-	-	-	-	-	-	-	U	-	-	U	-	-	-	-	-	-	-	-
18	W	-	-	-	-	-	U	-	-	-	-	-	-	-	-	++	++	-	-	++
19	EA	-	-	-	-	-	-	-	-	-	++	++	+	++	++	-	U	-	-	-
20	Rec	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	U	-	-	-
21	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C+ 2	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C- 2	-	-	-	-	-	-	-	-	U	-	-	-	-	-	-	-	-	-	-	-
NTC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

LAMP (virus suspension), RT-PCR (Wetzel et al., 1991b, Bühler, 2007) and/or RT-PCR (Szemes et al., 2001) did not detect PPV in samples 5, 15 and 18. The remaining samples including C+ 1 and C+ 2 were PPV positive regardless which nucleic acid amplification protocol was used (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

Samples 2, 9 and C+ 2 were assigned to PPV-D by all methods tested but not to one of the other strains. Samples 21 - 23 were assigned to PPV-D as well. Though, not every of these samples were recognized by all techniques. Based on these techniques, sample 8 was classified to PPV-D but, based on HNB-RT-LAMP (virus suspension), also to PPV-M. PPV-D and PPV-M specific HNB-RT-LAMPs (RNA and virus suspension) both detected sample 13. Despite the nominal strain PPV-M of this sample, it was identified as PPV-D by the two strain typing RT-PCRs. These protocols also recognized samples 1 and 3 as PPV-D. Though, the cDNA of those samples used for RT-PCR were supposed to be contaminated as the RNA applied to HNB-RT-LAMP gave no PPV-D signal (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

The PPV-M specific primers of Szemes et al. (2001) detected not only PPV-M but also

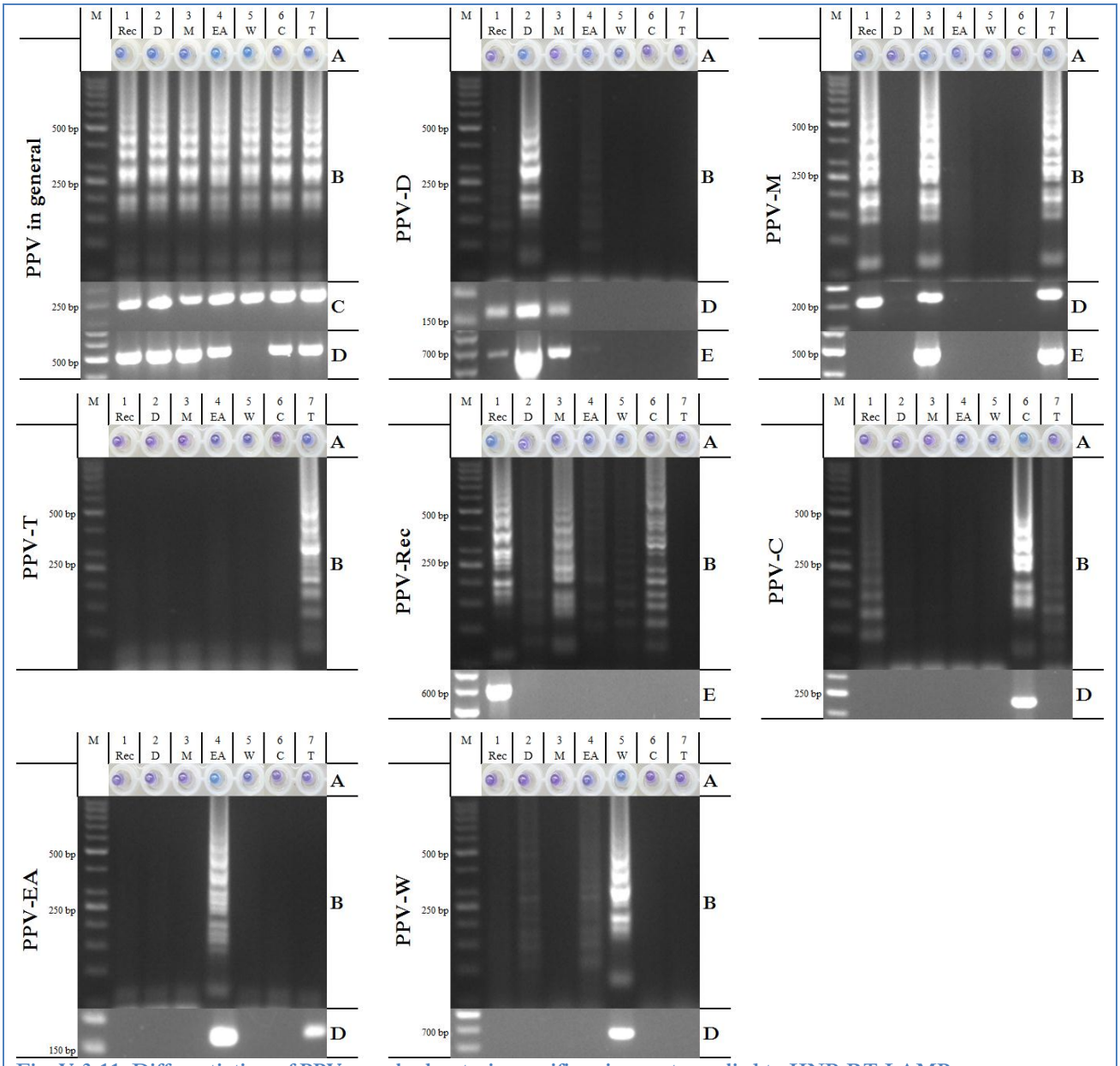


Fig. V-3-11: Differentiation of PPV samples by strain specific primer sets applied to HNB-RT-LAMP
The seven primer sets developed for the specific detection of one of the PPV subgroups were examined in HNB-RT-LAMP using a modified HNB-RT-LAMP protocol for the general detection of PPV (incubation at 64 °C for 40 min, 3 mM trehalose) and compared to strain typing by RT-PCR. A - colour change to blue by HNB-RT-LAMP, B - gel electrophoresis of HNB-RT-LAMP amplicons, C - RT-PCR according to Wetzel et al. (1991b), D - RT-PCR according to Szemes et al. (2001), E - RT-PCR according to Šubr et al. (2004); M - size marker

PPV-Rec and PPV-T. Only samples 3 and 14 were assigned to PPV-M by all detection methods. PPV-M positive signals were obtained for samples 1 and 7 by HNB-RT-LAMP (RNA) (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

The PPV-T specific primer set used in HNB-RT-LAMP resulted in an amplification signal just in the case of sample 7 (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

PPV-Rec recognition was in agreement by all detection methods for samples 1, 11 and C+ 1. It was not detected in sample 12 (RNA) and 20 (virus suspension) by HNB-RT-LAMP. Specific HNB-RT-LAMP amplification was found in NTC. In most of the remaining samples, unspecific amplification derived from HNB-RT-LAMP was observed (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

PPV-C was recognized by all techniques in sample 6, but not by any method in sample 15 despite general detection by HNB-RT-LAMP. Unspecific amplification was found in many samples (virus suspension) applied to HNB-RT-LAMP (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

Samples 4, 16 and 19 were assigned to PPV-EA by HNB-RT-LAMP (RNA and virus suspension) as well as by nested RT-PCR. Specific amplification in other samples appeared twice (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

PPV-W specific detection was observed in samples 5 and 17 in all cases, in sample 18 solely by HNB-RT-LAMP (RNA) and nested RT-PCR but not by HNB-RT-LAMP (virus suspension. Beside, HNB-RT-LAMP (RNA) resulted in unspecific amplification several times (Tab. V-3-3, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

The colour change from purple to blue of the HNB-RT-LAMP reaction mix due to DNA synthesis was obvious in all cases of specific amplification, but difficult to observe in samples exhibiting unspecific amplification. However, in most cases the intensity of unspecific amplification was rather weak compared to specific amplification (Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

Applying RNA to HNB-RT-LAMP led to a colour change from purple to dark blue in several samples (4, 5, 9, 10, 11, 13, 15, 18, 19, C+ 1 and C- 2). This was not observed for single reactions but for any reaction (Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10).

V-3.4 Evaluation of an internal control to support of the reliability of HNB-RT-LAMP

The primer sets developed to target either the *nad5* or the *Rbcl* gene of plants (Tab. V-3-4) were proved by BLAST analysis to be specific for the designated target. The primer F3 for detecting *nad5* was designed to link two exons and, therefore, allow the detection of the spliced

Tab. V-3-4: Primers for the detection of internal control genes by HNB-RT-LAMP
The primer sets were developed to target either the *Rbcl* gene or the *nad5* gene. Detection of one of these genes by HNB-RT-LAMP is indicative for the quality of the template used as these genes are constitutively expressed. Bold nucleotides are equal in the primers of Menzel et al. (2002) and Sánchez-Navarro et al. (2005)

Primer	Sequence (5'-3)	Length (nt)
F3 *Rbcl*	TGG GGG TTC CTA TCG TAA T	19
B3 *Rbcl*	ACG TAA CGC TTT AGC TAG T	19
FIP *Rbcl*	CCA TTA TCT CGG CAA TAA TGA GCC CAT GAT TAC TTA ACA GGG GGA T	46
BIP *Rbcl*	**CAC CGT GCA ATG CAT GCA G**AC ACG AAA GTG CAT ACC ATG	39
F3 *nad5*	**GAT GCT TCT TGG GGC TTC TTG TT**	23
B3 *nad5*	TCC CAG GAA TAA TTG AAG AGA	21
FIP *nad5*	ATA TGG AAT AAA GAT GGA CCA AGC TCC CGA CCG TAG TGA TGT T	43
BIP *nad5*	GAT CCG CAT AGC CCT CGA TTT TAT **CTC CAG TCA CCA ACA T**	40

mRNA of the gene rather than genomic DNA containing the introns as well. The target regions of the primers for *Rbcl* gene detection are located within the coding sequence of the *Rbcl* gene.

HNB-RT-LAMP targeting the *nad5* gene resulted in amplification in all samples tested. Indeed, a ladder like amplicon pattern was observed in all cases, but there were differences regarding signal intensity of single fragments or the number of fragments despite all samples were derived from *Prunus domestica* trees (data not shown).

The *Rbcl* gene was detected by HNB-RT-LAMP in all samples either PPV positive or PPV negative but not in NTC (Fig. V-3-12). However, unspecific amplification was found in the no template control as this amplicon pattern was quite different to the specific ampli-fication. With this weaker DNA amplification a colour change did not occur in contrast to the amplification of the *Rbcl* gene in the plant samples (Fig. V-3-12). Using virus suspension or RNA as template in HNB-RT-LAMP had no influence on the amplification of the *Rbcl* gene.

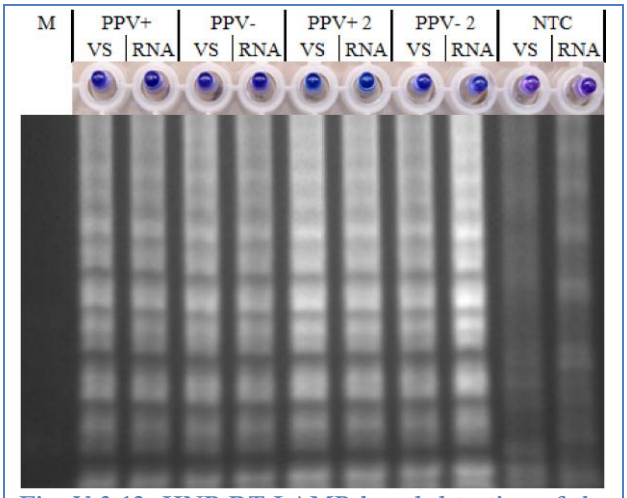


Fig. V-3-12: HNB-RT-LAMP based detection of the *Rbcl* gene for internal control
Either virus suspension (VS) or RNA derived from PPV positive or PPV negative samples were applied to HNB-RT-LAMP containing primers specific for the *Rbcl* gene. The reaction conditions were the same as for the general detection of PPV by HNB-RT-LAMP. Blue reaction mix - DNA amplification indicated by HNB, purple reaction mix - no DNA amplification indicated by HNB, NTC - no template control, M - size marker

VI Discussion

VI-1. Evaluation of cascade rolling circle amplification as a test system for PPV

A new detection system was developed for the detection of PPV. It combined the specific ligation of a CLP hybridized with its terminal segments to the target and the signal amplification via CRCA. The aim for the development of a new detection method was to reduce the need for costly technical equipment and toxic reagents, to decrease the detection limit, to reduce the hands on time for one sample.

VI-1.1 Ligation of the CLP hybridized to PPV cDNA

VI-1.1.1 CRCA with cDNA

For specific detection of PPV via CRCA, the PPV RNA had to be reversely transcribed to cDNA prior to the specific ligation of linear CLPs to the target. cDNA synthesis was initiated by primer cDNA 1 which annealed on PPV RNA about 100 bp upstream to the intended ligation site for CLP PPVs 1 and 2 to facilitate the slip off of the circularized CLP from the cDNA. This is essential for the amplification by CRCA as targets with no nearby end or circular targets like plasmids inhibit or prevent amplification of the ligated CLP via CRCA (Banér et al., 1998).

The cDNA concentration applied to the ligation reaction was adjusted to 80 ng in all subsequent experiments to ensure the detection even of samples with low PPV titre. Background amplification did not depend on the amount of cDNA and was observed in all samples which were either negative (negative control or NTC) or positive, but virus concentration was near or below the detection limit. The length of amplicons derived from nonspecific amplification was multiples of about 40 bp (Fig. V-2-1). This is consistent with about the half of the CLP used indicating the involvement of linear CLPs. The intensity of background amplification was similar in all cases irrespective of the cDNA concentration.

VI-1.1.2 Characteristics of the circularizable probes

The CLPs designed for the specific detection of PPV consisted of two terminal regions complementary to the target sequence linked by a restriction site and the region used for the amplification by CRCA (Fig. II-3-6). The 5' PPV specific sequence of CLP PPV 1 was 23 nt in length, whereas the 3' sequence was 15 nt in length according to the recommendations of Thomas et al. (1999), Faruqi et al. (2001) and Szemes et al. (2005). In contrast, CLP PPV 2 had target specific sequence equal in length (17 nt) similar to the basic CLP design of Nilsson et al. (1994). CLP PPV 3 was similar to CLP PPV 1 with a length of 24 nt and 14 nt for the 5' and 3' terminal region. CLP PPV 4 - 6 were equal to CLP PPV 2.

The 5' and 3'-end of CLP PPV 2 were equal in length, however, the melting temperature (T_m) of the 3' arm was about 6 °C below the T_m of the 5'-end. CLP PPV 1 exhibited a higher difference in T_m of the 5' specific region compared to the 3'-end, but the results obtained by CLP PPV 1 and CLP PPV 2 were similar (Fig. V-2-3). Since sequences of target (PPV) and non target (plant, other phytopathogens) differed by more than one nucleotide the T_m of the 3' complementary region for the discrimination of target and non target sequences is not as essential as it is for

using CLPs and CRCA for PPV strain typing similar to SNP typing (Faruqi et al., 2001). CLP PPV 3 was similar to CLP PPV 1 regarding the length of the terminal segments, but resulted in weaker signal intensity (Fig. V-2-3), because the 3' region had a lower T_m compared to that of CLP PPV 1.

There seemed to be an optimal temperature at about 58 °C for ligation. Considerable higher or lower temperatures during ligation resulted in a lower amount of DNA after CRCA (Fig. V-2-5). However, ligation temperature was adjusted to 64 °C for higher sequence specificity in subsequent trials and was maintained for following CLPs with terminal regions equal to the target complementary segments of CLP PPV 2. The background amplification was not changed by different ligation temperatures. This supports the hypothesis of linear CLPs being involved in the nonspecific reaction.

Alsmadi et al. (2003) observed CRCA based background amplification as well. To minimize the background signal, they incorporated a hairpin construct at the 3'-end of the CLP. The hairpin was stable at the ligation temperature. Upon recognition of the target sequence the hairpin was unfolded allowing ligation. Otherwise, the hairpin was elongated during RCA resulting in a double stranded CLP, which was not able to act as template for unspecific amplification. The design of CLP PPV 6 was based on this approach. However, background amplification occurred even in the positive control (Fig. V-2-6). This indicated that the hairpin was stable at the temperature used in ligation, since no amplification occurred which was derived from ligation of CLP PPV 6 to the target. By shortening the hairpin and enlarging the loop which is target specific ligation could proceed. However, if the hairpin has been stable at the ligation temperature (64 °C) it should also have been stable at the CRCA temperature. Nevertheless, non specific amplification occurred in any case at low temperatures (63 and 65 °C) with decreasing intensity at increasing temperatures (Fig. V-2-6). This seems to be due to too high temperatures preventing optimal primer annealing.

For general detection of PPV various designs of the CLP can be used. Equal length terminal regions (CLP PPV 2) as well as differences up to ten nucleotides in length between 5'-end and 3'-end (CLP PPV 1) showed comparable detection of PPV. Ligation was possible at a broad range of temperature. The major problem with CRCA approach was the high rate of background amplification which could not be solved by a hairpin construct under the conditions tested as it has been shown by Alsmadi et al. (2003). Since this non specific reaction was obtained with different CLP differing in target complementary sequences (Fig. V-2-2) and using different primers (Fig. V-2-4) the mechanism of this background amplification seemed not be dependent on specific sequences.

The background amplification could be minimized by a reduced concentration of CLP PPV 2. However, this was accompanied by a decreased amplification of ligated CLPs (Fig. V-2-7). As a consequence, the concentration of CLP applied to the ligation reaction was kept unchanged at 0.12 µM, since samples exhibiting a very low virus titre may be incorrectly declared as negative, when using a low CLP concentration. This is again an evidence for the hypothesis mentioned above regarding the linear CLP being a template for the background amplification as a declining CLP concentration is accompanied by reduced background amplification.

VI-1.1.3 Specific circularization of CLPs upon hybridization to the target

Of the ligases tested, Ampligase® Thermostable DNA Ligase and *Taq* DNA ligase showed similar suitability regarding specific ligation of the CLPs. The background amplification of Ampligase® based CRCA seemed to be higher than of *Taq* DNA ligase (Fig. V-2-8).

Amplification was hardly observed upon ligation by 9°N™ DNA Ligase. A longer incubation of this enzyme driven reaction or the addition of more enzyme can improve the amplification. Background amplification was not visible as well (Fig. V-2-8). This observation was contrary to that with other ligases. It did not support the idea of unligated CLPs being responsible for the background amplification as no unspecific amplification was found.

The usage of T4 DNA ligase resulted in CRCA like amplification in all controls to the same extend (Fig. V-2-8). Therefore, this ligase seems to be able to join ssDNA without a template. Similar results were reported by Kuhn and Frank-Kamenetskii (2005) who assumed a low yield side activity of the enzyme. However, solely high intensity of amplification was observed in this study. This may cause problems in sensitive detection methods based on ligation because background amplification and false positive signals will occur. *E. coli* DNA ligase, *Taq* DNA ligase and Ampligase® do not exhibit such an activity (Kuhn and Frank-Kamenetskii, 2005) as shown in this study as well. However, the amplicon length could not solely be explained by the ligation of the CLP without template. The obtained amplicons differed in size from the multiples of CLP derived from correct ligation by Ampligase® Thermostable DNA Ligase or *Taq* DNA ligase which was proofed by *EcoRI* digestion.

The background amplification could not be traced backed to the action of a single ligase as all ligases tested yielded in varying degree of amplification, either specific or nonspecific. There seemed to be several mechanisms of background amplification as, when using Ampligase® Thermostable DNA Ligase, in negative and NTC the amplicon pattern differed with the fragments of the negative control being digested to a single 50 bp fragment while the fragments of the NTC were not digested (Fig. V-2-8).

The detection limit of CRCA can be increased by the thermal cycling of the ligation (Fig. V-2-9), but this is contrary to the effort to minimize the need for technical equipment.

VI-1.2 CRCA of the circularized CLPs

VI-1.2.1 Influence of the ligation product volume on the amplification

Another possibility to adjust the detection limit of CRCA and to reduce background amplification is to varying volume of the ligation reaction added to the amplification reaction. More than 40 nM CLP and 26.7 ng cDNA did not result in a higher amplification rate as it was observed for 8 nM CLP and 5.3 ng cDNA, 16 nM CLP and 10.7 ng cDNA and 40 nM CLP and 26.7 ng cDNA. This was also case for the background amplification as well. Background amplification was also found in the positive control, but specific amplification dominated (Fig. V-2-10). Accurate discrimination of positive and negative samples was just possible after restriction digestion. However, this made the protocol more laborious and more time consuming.

Non specific amplification was characterized by fragments with multiples of about a half a length of the CLP used, whereas specific amplification resulted in fragments, which were as long as multiples of the CLP. Restriction digestion of amplicons derived from specific amplification resulted in a single fragment as long as the CLP. DNA derived from background amplification was not digested or was digested to a fragment, which was about half as long as the CLP. Assuming the linear CLP was involved in the nonspecific reaction, the mechanism, which led to the background amplification, seemed variable as in this experiment amplicons derived from background amplification were not digested but in other experiments they were (Fig. V-2-8). This indicated, that in some cases the restriction site is not amplified, but in other it is.

A ligation product of 16 nM CLP and 10.7 ng cDNA was chosen for the following experiments to compromise with detection limit and background amplification.

VI-1.2.2 Improvement of CRCA by the use of additives

A number of additives are known to enhance the amplification rate, to reduce background amplification, to enable amplification or sequencing of GC-rich sequences and/or to minimize the effect of inhibitors on the reaction. For new application the best working additive as well as the optimal concentration has to be determined experimentally. In the case of PPV detection by CRCA, betaine, DMSO, DTT, ectoine and ET SSB were tested. ET SSB was not promoting the correct amplification (data not shown). It was inhibitory to the amplification even at a dosage of 0.1 µg in contrast to other studies (Inoue et al., 2006, Mikawa et al., 2009) which showed optimized amplification upon addition of 3 µg *Tth*SSB-F255P protein. Zhang et al. (2001a) and Zhang et al. (2001b) observed a positive effect of DMSO on the CRCA which was proofed in this study as well. However, the concentration used (6 %) was already inhibitory here. Similar to the promotion of specific amplification in PCR by betaine, DMSO and DTT (Viguera et al., 2001, Kang et al., 2005) these additives can also improve the efficiency of CRCA (Fig. V-2-11). However, background amplification was not completely inhibited.

In following experiments betaine was used at a concentration of 0.75 M, because its addition resulted both in enhanced amplification rate and in reduced background amplification.

The *Taq* MutS protein is able to detect mispriming events. It binds to the mismatched nucleotide and blocks the amplification via a polymerase (Mitani et al., 2007). If the background amplification could be traced back to mispriming, *Taq* MutS would be a promising tool to avoid this background amplification.

VI-1.2.3 Influence of incubation time on CRCA

Specific and nonspecific amplification occurred almost simultaneously after 45 min (Fig. V-2-13). In contrast, Pickering et al. (2002) detected background amplification 70 min later than specific amplification. They also observed the first positive signal as early as after 24 min.

The signal intensity of the background amplification was weaker than of the amplification of circular CLPs. This indicated that amplification occurred in the presence of circular as well

as linear CLP, whereby the amplification of closed CLPs is preferred.

VI-1.2.4 Varying primers and primer design for optimal CRCA

Primer FWD is the primer initiating the rolling circle amplification. Primer REV is necessary for the cascade like amplification (Fig. II-3-6). The longer primers FWD 1c and REV 2b enhanced the amplification rate, either specificly or unspecificly, at the same temperature compared to primers FWD 1a and REV 2a (Tab. V-2-2, Tab. V-2-3, Fig. V-2-14) due to a higher T_m. Primers FWD 3a, b and c were created to be complementary to the homologous site of primer REV in the basic CLP design (Fig. II-3-6). Primers REV 4a, b and c were de-veloped to be homologous to the complementary site of primer FWD. Interchanging the recognition sites of the primers was tested to avoid the background amplification. However, unspecific amplification occurred upon CRCA using these primers as well, even though primers FWD 3a and REV 4a resulted in a reduced rate of background amplification (Tab. V-2-2, Tab. V-2-3, Fig. V-2-14).

Primers REV 9a, b and c were designed to be homologous the 3'-end of the CLP and to span to 5'-end of the CLP (Tab. V-2-3) and, thereby, to reduce background amplification. This approach failed. In contrast, the unspecific amplicon pattern derived from primer REV 9c was hardly distinguishable from the specific amplification upon CRCA and restriction digestion as well (Fig. V-2-15). Restriction digestion of background amplification based on primers FWD 1c and REV 2b resulted in a fragment of about 40 bp which is equal to the length of the two primers and the restriction site (Fig. V-2-14). The fragment derived from restriction digestion of primers FWD 1c and REV 9c indicates that all parts of the CLP except the 5' arm but including the bases of primer REV 9c spanning to the 5'-end of the CLP anticipated in the nonspecific reaction. It was not tested to use reverse primers which were homologous to the 5'-end of the CLP and which contain few bases at the 3'-end that span to the 3'-end of the CLP.

Knott et al. (2002) described the suppression of background amplification by exchanging the DNA primers for RNA primers since *Bst* DNA polymerase is capable to start DNA synthesis at RNA primers. A similar observation was made for Phi29 DNA polymerase (Takahashi et al., 2009). This could not be confirmed as no amplification was initiated by RNA primers at all (data not shown).

Modifying the 5'-end of hexamers either with C3 phosphoroamidite or with 18-atom hexaethyleneglycol and an additional base completely suppresses the amplification without template in WGA using Phi29 DNA polymerase (Brukner et al., 2005). Introducing these modifications in primers FWD 1c and REV 2b had no effect on the CRCA reaction either specific or unspecific compared to unmodified primers (Fig. V-2-16). However, in absence of template DNA, Brukner et al., 2005) observed DNA synthesis based on self-priming of the primers used which is avoided by the use of modified primers. In the case of background amplification derived from CRCA, there was a template in any case assuming not ligated CLPs were involved in background amplification. Self-priming was not causal for unspecific amplification because especally primer FWD could anneal to the CLPs which were not ligated.

Hairpins can be incorporated not only in the CLP but also in the primers. In contrast to the

hairpin in the CLP, the hairpins in the primers are located in the 5'-end. Hairpin structures are stable at elevated temperatures depending on loop size (Varani, 1995) as well as depending on the length and the GC content of the stem (Tyagi and Kramer, 1996). More than 10 nt of the single stranded 3'-end complementary to the template are sufficient for effective annealing and elongation in PCR. The section of the stem, which is located within the primer, and parts of the loop are also complementary to the target. In subsequent cycles the hairpin is opened by replication or hydrolysis depending on the polymerase used (Nazarenko et al., 1997). This is similar to the Ampliflour technology (Pickering et al., 2002). The incorporation of a hairpin construct at the 5'-end diminishes the background amplification when using ThermoSequenase II. Using *Bst* DNA polymerase, an Ampliflour construct works better (Knott et al., 2002). The primers FWD 1c HP and REV 2b HP with the hairpins linked to the 5'-end completely inhibited amplification. A signal was observed solely in the case of primers FWD 1c and REV 2b HP for specific as well as for unspecific reaction (Fig. V-2-17). However, the design of the hairpin can be varied in many ways, e.g. incorporating of the hairpin in the target specific sequence of the primer or modifying the hairpin of primer FWD 1c HP.

Oligonucleotides, in which desoxyribonucleotides and ribonucleotides, respectively, are substituted by LNAs, exhibit an increased melting temperature and an increased specificity to their complementary strand (Singh et al., 1998). The position of the LNA base within the primer is essential for the result compared to conventional primers. A 3' LNA base worsens the result. One or two LNA bases in the middle of the oligonucleotide improve the amplification via PCR. In contrast, a higher specificity regarding SNP is achieved by a LNA base located at the 3'-end of the primer (Latorra et al., 2003b). Incorporation of two LNA in the primers increased the amplification rate but did not reduce background amplification (Fig. V-2-18). Since just a few bases were changed to LNA bases, there is the possibility that the variation of the number, the position of the LNA bases within the primers as well as of the length of LNA containing primers may result in the reduction of background amplification (Latorra et al., 2003a).

The modifications introduced so far influenced the specific as well as the unspecific accumulation of amplicons via CRCA either in a supporting or inhibitory manner. However, none of them reduced or even diminished the background amplification while maintaining or improving the amplification of ligated CLPs. The possibilities for modifying oligonucleotides to avoid the formation of artefacts were not exhausted. The incorporation of other nucleotide analogues such as 2'-O-Me RNA bases, 5-nitroindole and abasic sites can prevent the amplification of unspecific fragments. The analogues should be located at least at the sixth position from the 3'-end of the primer and can affect one or several bases (Ball et al., 1998, Stump et al., 1999, Faruqi et al., 2001, Knott et al., 2002, Lage et al., 2003).

As specific amplification as well as background amplification were improved or impaired to the same extent by all primers and modified primers used, it was not possible to trace back the background amplification to the action of the two primers. The amplification of the CLPs is affected by the T_m and the concentration of the primers. An uneven concentration of primers FWD and REV resulted in an increased amplification rate (Fig. V-2-19). Thereby, a higher concentration of

primer FWD 1c than primer REV 9a at a ratio of 1:0.5 resulted in reduced background amplification. In contrast, a ratio of 1:0.25 resulted in a decreased amplification (Fig. V-2-19).

VI-1.2.5 Evaluation of DNA polymerases for their use in CRCA

Bst DNA polymerase was used for the amplification of ligated CLPs upon hybridization on PPV cDNA. Other polymerases (Phi29 DNA polymerase, IsoTherm™ DNA polymerase, DisplaceAce™ DNA polymerase and Vent (exo-) DNA polymerase) were examined in order to find a polymerase which is more applicable in CRCA regarding reduced or even prevented background amplification.

Phi29 DNA Polymerase was not suitable for the use in CRCA for the detection of PPV as no amplification was observed using this enzyme (Fig. V-2-20). Compared to *Bst* DNA polymerase, Phi29 DNA Polymerase shows low efficiency and sensitivity in CRCA (Lizardi et al., 1998, Zhang et al., 2001a, Zhang et al., 2001b). However, Phi29 DNA polymerase is a very good enzyme for RCA because of its high processivity (Banér et al., 1998, Lizardi et al., 1998). In addition, Sequenase and Vent (exo-) DNA polymerase can amplify a DNA circle which is topological linked to the target (Kuhn et al., 2002) as well as a CLP linked to a long ssDNA (Lizardi et al., 1998) similar to *Bst* DNA polymerase (Lizardi et al., 1998, Thomas et al., 1999, Zhang et al., 2001b). In contrast, Phi29 DNA polymerase has to shove off the CLP at a nearby end (Banér et al., 1998). Therefore, the starting point of the cDNA introduced by primer cDNA 1 during cDNA synthesis is probably too far away for Phi29 DNA polymerase but not for *Bst* DNA polymerase.

The weak signal intensity of IsoTherm™, a modified variant of *Bst* DNA polymerase and DisplaceAce™ DNA Polymerase (Fig. V-2-20) could be improved by longer incubation or applying more enzyme. Another DNA polymerase capable to displace a DNA strand and, therefor, applicable to CRCA is Vent (exo-) DNA Polymerase (Lizardi et al., 1998, Kuhn et al., 2002). However, this characteristic is rather weak compared to *Bst* DNA polymerase as more units of enzyme have to be applied to achieve a similar result (Fig. V-2-21) as observed by Wang et al., 2005). Addition of T4 gene 32 protein to Vent (exo-) DNA Polymerase or *Bst* DNA polymerase catalyzed CRCA may support the reaction according to Lizardi et al. (1998) and Wang et al. (2005).

Further polymerases capable to displace a downstream strand are Klenow fragment of *E coli* DNA polymerase I, *Aac* DNA polymerase (*Alicyclobacillus acidocaldarius*) (Mitani et al., 2007) and Sequenase (Kuhn et al., 2002). These enzymes were not tested for their suitability in CRCA for the detection of PPV. According to Zhang et al. (1998), *Taq* DNA polymerase is able to displace a downstream strand despite its 5'-3' exonuclease activity. However, this was not found for CRCA in this work (data not shown).

VI-1.3 Exonucleolysis of linear CLPs prior to CRCA

Exonucleolysis had a minor effect on background amplification at the concentration of exonucleases and incubation time used here (Fig. V-2-22) despites numerous reports advising this procedure (Nilsson et al., 1994, Zhang et al., 1998, Banér et al., 2003, Hardenbol et al., 2003, Dahl et al., 2004, Millard et al., 2006). The concentration of at least 25 U was high compared to

10 U (Tong et al., 2007, Kaocharoen et al., 2008, Steain et al., 2009, Wang et al., 2010) or 0.5 U (Szemes et al., 2005). Another difference was that the last four working groups used a combination of exonuclease I and exonuclease III which uses dsDNA as substrate. Szemes et al. (2005) incubated the exonucleolysis for 2 h instead of 30 min. Since genomic DNA (Szemes et al., 2005) and PCR products (Kaocharoen et al., 2008) were used it seemed unlikely that the failure of exonucleolysis here could be attributed to excessive amount of cDNA. Najafzadeh et al. (2011) reported a single, weak amplicon when exonucleolysis was omitted. This is in contrast to the observation in this work as a ladder like pattern was visible upon gel electrophoresis.

Another approach to remove unreacted CLPs to avoid background amplification is a so-called solid phase RCA (Wang et al., 2005). mRNA hybridize to oligo(dT) which are coated to magnetic beads. Upon recognition of the mRNA by the CLP, the magnetic beads are used for removing non hybridized CLP prior to the ligation and amplification (Wang et al., 2005). A hairpin construct at the 3'-end of the CLP (Alsmadi et al., 2003) has already been shown to not work with the design used in this study.

VI-1.4 Examination of background amplification

Holbrook et al. (2005) discovered amplification in the no template control during WGA similar to reactions containing template DNA. The unspecific amplification was assigned to the random hexamer primers acting as template. As a consequence, definite verification was only possible after downstream applications similar to restriction digestion used in the present work. Linear target isothermal multimerization and amplification (LIMA) utilizes *Bst* DNA polymerase, two primers and a linear template at isothermal conditions and results in ladder like fragment pattern (Hafner et al., 2001). As in the obtained sequences derived from nonspecific CRCA, the tandem repeats of LIMA vary in length from reaction to reaction. CLP which were not ligated are linear templates as well. Therefore, the observed unspecific amplification could correspond to LIMA.

Sequencing of nonspecific amplicons revealed, that the DNA molecules contained tandem repeats of the sequence of primer FWD, up to 5 nucleotides downstream the primer FWD binding site on the CLP and the complementary sequence of Pamp REV (Fig. V-2-23, Suppl. Fig. 1) which is similar to the results of Knott et al. (2002). The background amplification relies on the polymerase and both forward and revers primers and their interaction with unligated CLPs (Fig. V-2-24) which is consistent with the observations of Knott et al. (2002). The ligase seems not to be involved in the generation of nonspecific amplicons since omitting this enzyme resulted in background amplification in any case (Fig. V-2-24). No amplification was found when the primer FWD 1c initiating the CRCA was left out. No signal was observed as well when omitting primer REV 2b. However, the single-strand was produced by this reaction as the cascade like amplification initiated by primer REV 2b depends on RCA. The single strand released by RCA was not stained by ethidium bromide as this dye stains ssDNA to minor degree compared to dsDNA. However, the reactions without CLP resulted in unspecific amplification as well (Fig. V-2-24). The reason for this observation can be either carry over contamination of CLPs or a second mechanism of unspecific amplification without the need for a template.

To avoid the use of two primers the RCA can be initiated by the target itself (Banér et al., 1998). The amplification of target primed RCA rate can be enhanced by adding a reverse primer which is complementary to the released single strand (Cheng et al., 2008). Phi29 DNA polymerase exhibits 3'-5' RNase activity. Therefore, target primed RCA can be conducted also on a RNA target, as the CLP hybridizes to the RNA strand which is digested in the 3'-5' direction until the DNA/RNA hybrid is reached (Lagunavicius et al., 2009, Li et al., 2009a, Takahashi et al., 2010).

Several working groups (Liang et al., 2004, Zyrina et al., 2007, Tan et al., 2008, Murakami et al., 2009) observed background amplification without template DNA and primer using a DNA polymerase and a (nicking) endonuclease in a single reaction. This was tested for several DNA polymerases including *Bst* DNA polymerase and numerous endonucleases and nicking endonucleases. Ogata and Miura (1997) found template/primer independent amplification when using a DNA polymerase derived from *Thermococcus litoralis* upon incubation for at least 1 h. Unspecific amplification without template DNA and primer using *Bst* DNA polymerase was observed after 13 h of incubation (Zyrina et al., 2007). The background amplification obtained by the CRCA of PPV specific linear CLPs involved a template and the primers as shown by sequencing (Fig. V-2-23, Suppl. Fig. 1). However, Fig. V-2-24 shows amplification in absence of the CLP. These amplicons were not sequenced. Therefore, contaminating CLP could have led to the amplification.

Polymerases either DNA polymerases (Zaphiropoulos, 1998, Viguera et al., 2001) as well as reverse transcriptases (Houseley and Tollervey, 2010) are able to switch to another template or to slip to another region of the template and to proceed with elongation of the former synthesized strand resulting in a recombination event. In subsequent cycles of PCR various amplicons arise containing tandem repeats of a part of the template (Zaphiropoulos, 1998). It is hypothesised that during a single elongation step the polymerase switches to an upstream position of the same template creating a loop structure which is stabilized by the newly synthesized complementary strand. This loop is amplified in a RCA manner resulting in the observed multimers (Fig. VI-1-1, Zaphiropoulos, 1998). The amplicons derived from background amplification during CRCA varied in length and in the sequence of the spacer which linked the primer sequences, but there was virtually no variation in the repeats within an amplicon (Fig. V-2-23; Suppl. Fig. 1). This fit to the hypothesis of Zaphiropoulos (1998). However, *Bst* DNA polymerase has strong strand displacement activity compared to the *Taq* DNA polymerase used in the work of Zaphiropoulos (1998). Polymerases with this characteristic do not tend to slip off or to slip along the template (Viguera et al., 2001). A second argument against this mechanism being causal for background amplification is the missing of homologous sequences within a CLP which are necessary for template switching as well (Viguera et al., 2001).

If the polymerase would slip to a region within the hybridization site of primer REV 2a no amplification would occur since primer REV 2a could not bind with its 3'-end. In contrast, if the polymerase slipped upstream the hybridization site of this primer, amplicons with multiple lengths of the linked primers up to amplicons with

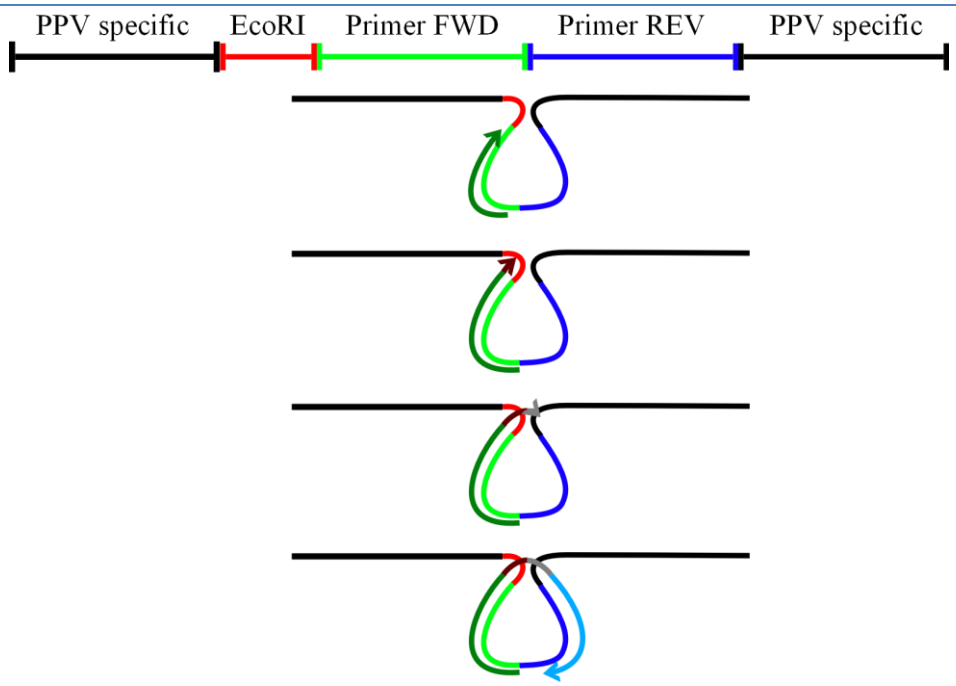


Fig. VI-1-1: Template switch of the polymerase
During elongation of primer FWD, the polymerse switches upstream to the CLP by what a loop structure arises which is amplified in CRCA similar to ligated CLP (according to Zaphiropoulos (1998) and Viguera et al. (2001))

multiple lengths of linked primers and the 3'-end of the PLP could arise. However, amplicons as short as possible were generated containing the two priming sites and up to six additionale bases predominantly derived from the *EcoR*I restriction site. Assuming the background amplification during CRCA is based on a mechanism similar to that hypothesized by Zaphiropoulos (1998), several recombination events may potentially occure though the one comprising the smallest number of nucleotides would be amplified. As background amplification is hardly observed in the case of ligated CLPs specific amplification dominates over the unspecific amplification. Though, this assumption was not proved.

Chimeras formed during multiple displacement amplification (MDA) consist mainly of inverted sequences. The polymerase displaces downstream 5'-ends of presynthesized DNA. The 3'-end can also be displaced by branch migration. As a consequence, the free 3'-end is able to hybridize to a single stranded 5' template resulting in the chimeric DNA strand. The complementarity can be reduced to a few nucleotides. This mechanism occurs preferentially in the same amplicon molecule (Lasken and Stockwell, 2007). The background amplification observed in CRCA cannot be explained solely by this mechanism. The linear CLP exhibits just one priming site. So there is no strand displacement, because the polymerase runs off the CLP.

VI-1.5 Analytical discrimination between CRCA and unspecific amplification

The avoidance of background amplification is crucial for visualisation of accumulated amplicons. One the one hand, there would be no need for restriction digestion. On the other hand, visualisation via fluorescent dyes like SYBR® Green can be applied directly to the CRCA reac-

tion as it is done in real time PCR. However, these dyes intercalate into dsDNA irrespective of the sequence. Negative samples would be typed as positive ones due the background amplification. Without restriction digestion, the CRCA protocol for detecting PPV is still laborious.

VI-1.6 Ligation of the CLP to PPV RNA

RNA has to be extracted from the samples, which is then reversely transcribed to cDNA. The cDNA is used as target for the ligation of the CLP and the CLP is finally amplified via CRCA. There are two possibilities to omit the reverse transcription. The first one is to ligate the CLP directly on PPV RNA either by chemical ligation (Abe et al., 2008) or enzymatically. T4 DNA ligase is able to join DNA in a DNA/RNA hybrid to a very low extent (Kleppe et al., 1970). It is even possible to detect SNP in RNAs via specific ligation of a CLP on the RNA target using T4 DNA ligase (Nilsson et al., 2000), but the RNA templated ligation proceeded more slowly than ligation on DNA, even in the presence of $MnCl_2$ (Nilsson et al., 2001, Li et al., 2008, Tang et al., 2008). Ampligase® Thermostable DNA Ligase and T4 RNA ligase are also able to join nicks in a DNA strand hybridized to a RNA strand (Christian et al., 2001). The second possibility is to modify the design of the CLP for the detection of RNA targets to form a three way junction (3WJ) (Leontis et al., 1991). The target specific sequences are located between the terminal regions. Upon hybridization to the target RNA a 3WJ is formed with two arms containing the target RNA/CLP DNA hybrid. The third arm is made of the terminal segments of the CLP hybridized to an internal complementary sequence. CRCA is possible after ligation of the nick. Similar to this approach is the invention of suicide cassettes (Lohmann et al., 2007) and turtle probes (Stougaard et al., 2007). A 3WJ is used by Murakami et al. (2012) for RNA detection as well. A 3WJ primer and a 3WJ template are designed to be complementary at a length of 7 nt but they bind to each other just in the case of annealing of both oligonucleotides to the RNA target. Afterwards, the 3WJ primer is elongated by a DNA polymerase along the 3WJ template. A nicking enzyme introduces a nick at which the polymerase can start again releasing a signal primer which initiates a RCA via a preformed circular probe.

VI-1.7 Conclusion

The main task arising during the optimisation of the CRCA technique for the detection of PPV was to reduce the background amplification which occurred in the absence of circularized CLPs. This made the differentiation of positive and negative samples more complicated since the electrophoresis fragment pattern was similar in both cases. To overcome this problem restriction digestion had to be conducted making the CRCA protocol more laborious. Besides the evaluation of ligases, polymerases and additives the CLPs and primers were varied and modified. None of these attempts resulted in a complete suppression of background amplification. Restriction digestion of CRCA amplicons allowed for the definite discrimination of PPV positive and PPV negative samples. However, this is an additional working step resulting in a more laborious and time consuming procedure to detect PPV which is contrary to the aim of obtaining a high-throughput monitoring system.

Due to the gained results and knowledge of CRCA it seems quite difficult to establish an isothermal RNA based detection system for PPV

based on the ligation of a CLP followed by its amplification by CRCA which also includes a simplified sample preparation, i.e. RNA extraction, and simplified visualisation of amplicons. Therefore, another isothermal nucleic acid amplification, LAMP, already published for the detection of PPV (Varga and James, 2006b), was examined for its suitability for simplification of sample preparation and visualisation.

VI-2. Loop-mediated isothermal amplification for PPV detection

As shown above, it was not possible to avoid the background amplification despite various approaches using CRCA. As a consequence, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) protocol for the detection of PPV (Varga and James, 2006b) was tested. Initial testing of control samples by RT-LAMP revealed the suitability of this protocol (Fig. V-3-1).

In the following, the RT-LAMP protocol for the detection of PPV was combined with a simplified sample preparation procedure (Lee et al., 2009) and homogenous visualisation (Goto et al., 2009) to obtain a fast, sensitive, reliable and inexpensive PPV detection system avoiding the need for sophisticated laboratory equipment and the use of toxic chemicals.

VI-2.1 Homogenous visualisation of the LAMP product

As seen in Fig. V-3-1 the amplicons of RT-LAMP can be visualized by gel electrophoresis and ethidium bromide staining like the amplicons of PCR and CRCA. However, this procedure is time consuming and requires costly technical equipment (chambers for gel electrophoresis, power supply, and gel documentation) and a toxic reagent (ethidium bromide).

VI-2.1.1 Comparison of methods to visualise RT-LAMP driven DNA synthesis

One aim of this work was to combine the isothermal nucleic acid amplification based detection of PPV with a homogenous visualisation.

Fluorescent dyes such as SYBR® Green I were not tested for direct visualisation of RT-LAMP as they have to be added in high concentration after termination of the reaction for naked eye evaluation (Iwamoto et al., 2003, Sun et al., 2006, Saleh, 2009, Zhao et al., 2010). This increases the cost of the detection system as well as the risk of cross contaminations and is contrary to the aim to make the new detection protocol as simple as possible for the use of the method in the orchard. However, such intercalating dyes can be used for real-time monitoring of the RT-LAMP reaction in laboratories which already use qPCR for analysis (Tomlinson et al., 2007, Cai et al., 2011) for quantification of the virus titre. Alternatively, machines for qLAMP are available as well. This is advantageous as its software is adapted to LAMP which is not the case for real-time thermal cyclers.

The LFD technology requires post amplification handling of the RT-LAMP products similar to fluorescent dyes. The additional costs are high, too, as a biotinylated primer and a FITC labelled probe as well as the LFDs are needed. Furthermore, the visualisation of amplicons by LFDs did not work well in this study (Fig. V-3-5) compared to the reports of other working groups (Jaroenram et al., 2008, Kiatpathomchai et al., 2008, Ding et al., 2010, Tomlinson et al., 2010d).

The other approaches for homogenous visualisation, turbitiy, calcein and hydroxy naphthol blue, rely on an intrinsic characteristic of LAMP. Very high amounts of DNA are synthesised during LAMP compared to PCR (Mori et al., 2001). This is accompanied by the accumulation of pryophasphat which precipitates with magnesium present in the reaction buffer. The turbidity is caused by the magnesium-pyrophosphate-complexes which can be observed with the naked eye without additional equipment or reagents (Fig. V-3-2). Nonetheless, low PPV titre in a sample may lead to questionable turbidity or no turbidity at all.

The calcein (Yoda et al., 2007, Tomita et al., 2008) or HNB (Goto et al., 2009) driven colour change caused by the declining magnesium concentration in the reaction is more obvious and is visible at daylight. However, using RNA as template for the calcein supplemented RT-LAMP resulted in a colour change from light green to fluorescent green not only in the positive control but also in the negative control (Fig. V-3-3). This was not observed for cDNA. Gel electrophoresis revealed a single, slight and lengthy amplicon in the negative RNA control (Fig. V-3-1, Fig. V-3-3). Since in NTC no signal occurred but this fragment was also visible in the positive control (Fig. V-3-1) it raised probably from unspecific DNA synthesis. Though, this single fragment did not interfere with the typical LAMP amplicon pattern upon gel electrophoresis as the background amplification did in CRCA. Such an unspecific amplification leading to a colour change during calcein-RT-LAMP was not observed in other studies (Yoda et al., 2007, Le et al., 2010, Nemoto et al., 2010). Indeed, this fragment was found in the negative RNA control applied to HNB-RT-LAMP as well, but there was no colour change from purple to blue as in the positive control (Fig. V-3-4). This indicated a higher sensitivity of calcein to decreasing Mg concentration compared to HNB. However, the amplicon obtained by RT-LAMP seemed to be unspecific, whereby the calcein colour change resulted in a false positive signal. On the other hand, the RT-LAMP for the detection of PPV should be improved to avoid the unspecific amplification permanently as there are examples without this kind of amplification in negative controls. If the sensitivity of calcein to declining Mg contreations is as high as assumed, calcein can be applied to PCR as well. This approach should be tested.

As RT-LAMP supplemented with HNB showed the best results under the conditions used for comparison of different visualisation systems, HNB was choosen for the use in RT-LAMP based detection of PPV. For this reaction, the name Blue LAMP is proposed (Hadersdorfer et al., 2011).

VI-2.1.2 Optimisation of the Blue LAMP detection system

Reaction conditions for Blue LAMP were varied to optimise the HNB based colour change without impairing the RT-LAMP reaction.

For HNB a concentration of 0.12 mM was finally chosen, since the differentiation between the purple colour of the negative control and the blue colour of the positive control was easier to observe. A concentration of 0.06 mM resulted in a light colour, whereas higher concentrations tended to dye the reaction mix even without amplification bluish (Fig. V-3-6A).

Varga and James (2006b) used 1.4 mM of dNTPs. For a better discrimination between PPV positive and PPV negative samples using Blue LAMP, the dNTP concentration was reduced to 1 mM (Fig. V-3-6B). Some extend of false negative staining was observed with the lowest dNTP concentration of 0.8 mM. In contrast, with higher concentrations some effect of masking of Mg^{2+} may contribute to the colour reaction (Roux, 2009).

The magnesium concentration was kept unchanged at 8 mM, since there was no improvement visible using other concentrations (Fig. V-3-6C).

The reaction time was elongated to 2 h, as samples exhibiting a low virus titre cause an insufficient colour change after 1 h, which is hard to observe. If the incubation time exceeds four hours, unspecific amplification will occur in any case (Fig. V-3-7), which is in agreement to the results of Varga and James (2006b). For LAMP, incubation for 2 h is long. Visualisation of DNA amplification by LAMP is possible within 30 min after starting the reaction (Li et al., 2011b, Luo et al., 2011). For straintyping, the reaction time of 40 min resulted infrequently in unspecific amplification especially in the case of PPV-Rec. In contrast, incubation time of 1.5 h is reported as well (Wang et al., 2011).

The HNB supplemented RT-LAMP resulted in a colour change from purple to blue. It was more obvious in this study than in reports of other working groups (Harper et al., 2010, Bearinger et al., 2011, Luo et al., 2011, Nie et al., 2011) demonstrating the well optimized reaction conditions of Blue LAMP.

Additional simplification can be obtained by preparing the reaction mix in advance and freezing it at -20 °C for later use even with the reverse transcriptase included (data not shown). Storage of a LAMP reaction mix targeting DNA is possible for at least 90 days (Angamuthu et al., 2011). According to Thekisoe et al. (2009) it also seems possible to store the LAMP reaction mix at ambient temperatures up to 37 °C for two weeks without loss of activity.

VI-2.1.3 Detection limit of the Blue LAMP test

Blue LAMP and RT-PCR were compared regarding the detection limit of the methods in absence and in presence of background RNA and cDNA respectively. A plasmid bearing the PPV genome was used as template.

The detection limit of Blue LAMP and RT-PCR was similar both in absence and presence of background RNA or cDNA showing a detection limit of 100 ag plasmid (Fig. V-3-8). This is in accordance with other reports of LAMP methods (Jaroenram et al., 2008, Kubo et al., 2010, Ma et al., 2010). In contrast, the results of Fukuta et al. (2003b), Liu et al. (2008), Boubourakas et al. (2009), Kikuchi et al. (2009), Liu et al. (2009a) and Goto et al. (2010) revealed a lower detection limit of LAMP compared to PCR. Varga and James (2006b) found a higher consistency of real-time RT-PCR near the detection limit than of LAMP. This was also observed here for RT-PCR and Blue LAMP.

Varga and James (2006b) determined the PPV detection limit of LAMP by applying serial dilutions of RNA to the reaction with a minimal detection limit at a dilution of 10^{-4} of standard total RNA amounts. The experiment on the influ-

ence of the virus suspension on Blue LAMP revealed a detectable dilution of 10^{-6} for the virus suspension as well as for RNA. Indeed, direct comparison based on PPV-RNA amount was not possible since in both cases the virus titres of the samples used were not known.

Isolates grouped to PPV-D, -M, -C, -EA and -W were detected by RT-LAMP as already shown by Varga and James (2006b) as well as by Blue LAMP (this work, also demonstrated for PPV-Rec and PPV-T, Fig. V-3-11, Suppl. Fig. 9, Suppl. Fig. 10). Hence, this technique is suitable for the detection of PPV in general since all strains known so far are recognized.

VI-2.2 Evaluation of a fast plant extraction procedure with virus suspensions as template

Capote et al. (2009) and Lee et al. (2009) have demonstrated that a crude plant extract is sufficient to serve as template in real-time RT-PCR and LAMP respectively. The crude plant extract according to Capote et al. (2009) is set up in the presence of a phosphate buffered saline (PBS) buffer, the one used by Lee et al. (2009) in the presence of deionised water. Both procedures were tested in combination with Blue LAMP. The amplification via Blue LAMP was possible regardless which plant extraction procedure was used as proven by gel electrophoresis. As expected, the visualisation of the Blue LAMP product failed in the presence of the PBS buffer, because colour change occurred already after adding the plant extract to the reaction mix due to phosphate included in the buffer (data not shown).

The results of Blue LAMP based on the fast plant extraction procedure displayed no false positive signals (Fig. V-3-9, Tab. V-3-1, Suppl. Tab. 2). Using cDNA derived from such plant extracts gave invalid results neither by Blue LAMP nor by RT-PCR. In contrast, conventional RNA extraction sometimes resulted in false positives indicating additional contamination risks. This problem was also observed by Varga and James (2006b). The risk of any contamination is reduced to a minimum by adding a virus suspension to the Blue LAMP reaction mix because the number of working steps is minimized. Nevertheless, one has to take care of sampling and sample preparation. The use of disposable gloves for taking samples from plants is needed.

The one-step multiplex RT-PCR with virus suspension as template failed to amplify the internal control *nad5* in 45 % of the samples which were tested during the survey of an orchard as well as during the comparison of extraction methods. This indicates that the virus suspension does not serve as an optimal template for the one-step multiplex RT-PCR as for Blue LAMP. Since there was no purification step included in the fast plant extraction procedure, the virus suspension may contain substances which are inhibitory to the *Taq* DNA polymerase used in the one-step multiplex RT-PCR, but not to the *Bst* DNA polymerase (Kaneko et al., 2007). As at least two different methods (RT-PCR, visual control for symptoms) were used for the detection of PPV, the classification of samples as PPV positive or as PPV negative can be assumed to be correct (EPPO, 2004).

Sensitivity and specificity, according to the definition of Altman and Bland (1994), mounted up to 100 %, if the simplified Blue LAMP protocol was used (Tab. V-3-1, Suppl. Tab. 2). Compared to the results of Capote et al., 2009) or compared to the other methods tested, the Blue LAMP combined with the fast plant extraction

procedure exhibited very high sensitivity and specificity, even when considering the number of tests performed in the present work being lower than in the mentioned study. It has to be expected that by enlarging the number of samples both sensitivity and specificity will be less than 100 %.

VI-2.3 Establishing a Blue LAMP protocol for differentiating the PPV subgroups

Sequencing is the most reliable way to differentiate between the PPV subgroups as re-combination events or nucleotide exchanges are observed. Assigning an isolate to a PPV strain by RT-PCR is possible as well. However, several protocols have to be combined to type PPV-D, PPV-Rec, PPV-EA, PPV-C and PPV-W correctly (Szemes et al., 2001, Šubr et al., 2004), V-1, Fig. V-3-11). PPV-M and -T cannot be discriminated either (Fig. V-3-11).

Therefore, new RT-LAMP primer sets were developed specific for each of the strains (Tab. V-3-3). The specificity of all primers was proved by BLAST analysis. The primer sets D, Rec, T, EA, C and W lead to the detection of the respective strain by HNB-RT-LAMP either using virus suspension (Suppl. Fig. 10) or RNA (Suppl. Fig. 9) as template. The primer set for PPV-M also recognized PPV-Rec and PPV-T isolates in some cases (Fig. V-3-11). Though, gel electro-phoresis has to be carried out since unspecific amplification occured several times especially in the case of PPV-Rec using RNA and virus suspension and PPV-C using virus suspension (Suppl. Fig. 9, Suppl. Fig. 10).

Compared to the Blue LAMP for the general PPV detection, the reaction conditions for strain typing Blue LAMP were modified: Betaine was interchanged by 3 mM trehalose by what the incubation time was reduced to 40 min. A further reduction in time will lead to prevent unspecific amplification especially in the case of the PPV-Rec specific LAMP primer set.

Trehalose was more beneficial on the reaction than betaine which is in contrast to the result of Harper et al. (2010) who did not observe an improvement of the amplification due to trehalose. According to the manufacturers, the optimal temperatures for *Bst* DNA polymerase and for SuperScript® III Reverse Transcriptase are 65 °C and 55 °C. However, SuperScript® III Reverse Transcriptase is used at temperatures above 60 °C in Blue LAMP. Trehalose is more supportive on the reaction than betaine in the case of Blue LAMP based PPV detection and strain typing but both additives decrease the T_m of DNA to a similar extent (Spiess et al., 2004, Hart et al., 2010). Trehalose is known to stabilize enzymes and even to increase the activity of enzymes at elevated temperatures at which these enzymes are normally denatured (Carninci et al., 1998). Therefore, SuperScript® III Reverse Transcriptase could be stabilized by this additive at the temperature used for Blue LAMP. The activity of both enzymes could be increased by trehalose as well.

The reaction temperature was increased to 64 °C. A further increase in temperature will result in more stringent reaction conditions to allow amplification just in case of perfect hybridization of the primers. This is shown for differentiating Hepatitis B and C viruses exhibitting a low level of nucleotide polymorphism (Cai et al., 2011) which is of particular importance for distinguishing PPV-M from PPV-Rec and –T. All strain specific Blue LAMP reactions contained the same composition of reagents with equimolar concentration of the primers to simplify reaction

setup similar to (Li et al., 2011b) and to enable a lab on a chip organization of the detection (Lutz et al., 2010, Fang et al., 2011).

A colour change from purple to dark blue was observed for several samples without DNA synthesis (Suppl. Fig. 9). This phenomen appeared only in the case of RNA used as template and was not restricted to single reactions. Applying RNA of these samples derived from a single RNA extraction to any Blue LAMP reaction for strain typing resulted in a colour change in every case. This was observed neither for RNA applied to Blue LAMP for the general detection of PPV nor for virus suspension applied to the Blue LAMP reaction independently of the target (Suppl. Fig. 10). As this colour change does not rely on the temperature or the incubation time the only difference to Blue LAMP is the use of trehalose in strain differentiating Blue LAMPs. The RNA seems not to be involved as not all samples show this reaction. During RNA extraction ethanol is used for RNA clean-up which should be completely removed before resuspending the RNA. However, residuals of ethanol may interfere with the non-reducing disaccharide trehalose but not with the zwitterionic amino acid analogue betaine resulting in Mg complexation and a HNB colour change to blue.

At the moment, seven separate reactions have to be conducted for strain differentiation by Blue LAMP. By adjusting the reaction conditions to avoid unspecific amplification, a multiplex reaction can be developed which is similar or equal to the microchamber mμLAMP system (Fang et al., 2011). Samples are applied to microchambers surrounding a chamber which contains the reaction mix. Via microchannels the reaction mix moves to the chambers containing the samples by capillary forces. For multiplexing, different primer sets can be spotted in the sample chambers. Visualisation is possible by turbidity (Lutz et al., 2010, Fang et al., 2011).

VI-2.4 Evaluation of Blue LAMP based detection of an internal control

Including a control based on a housekeeping gene such as *nad5* in the one-step multiplex RT-PCR (Menzel et al., 2002, Jarošová and Kundu, 2010) or *Rbcl* (Sánchez-Navarro et al., 2005) assures the results obtained if just one of these test systems is used to type negative signals accurately by verifying the integrity of the template used. In contrast, the HNB based colour change occurs in any case of DNA amplification independent from the sequences used for amplification. Therefore, it is not possible to differentiate between detection of PPV and the detection of an internal control in a multiplex Blue LAMP reaction. For Blue LAMP a two reaction system has to be established which is proposed by Tomlinson et al. (2010d) as well. This working group tested a multiplex LAMP reaction coupled to LFD visualisation but the obtained results were not in accordance with single target reactions.

The primers developed by Tomlinson et al. (2010d) for the detection of the cytochrome oxidase gene were not tested as they were used to verify the integrity of plant DNA. The evaluation of the two newly designed primer sets recognizing either *nad5* or *Rbcl* and used in Blue LAMP revealed that the primers detecting *nad5* were not as suitable for its use as the primers for *Rbcl* since the rate of amplification was rather low and detection was not as reliable as the detection of *Rbcl*. Indeed, unspecific amplification was observed during *Rbcl*-Blue LAMP in the no template control but it can be circumvented by adjusting the incubation time to 1 h. No loop

primers were included compared to the detection of PPV or to the PPV subgroup differentiation because the genes used for internal control are expressed constitutively at a high level. Thereby, it is not necessary to increase sensitivity by additional primers.

VI-2.5 Future prospect

The number of protocols for the detection of (phyto-)pathogens based on the LAMP technology is rapidly growing. Besides low technical equipment input, sensitivity, specificity and speed, the homogenous visualisation of amplified DNA either based on turbidity, calceine or HNB and the low susceptibility against potential enzyme inhibitors eliminating the need for laborious nucleic acid extraction account for this. Sole, the development of new primers is elaborate. However, software for this purpose is available.

As shown for strain differentiation of PPV and for the detection of an internal control new primer sets can be applied to the Blue LAMP protocol to obtain reliable results without changing the composition of the reaction mix. Improvement of the LAMP reaction can be achieved by replacing betaine for trehalose. Just incubation temperature and incubation time were modified allowing a rapid establishing of new protocols.

VI-2.6 Conclusion

Simplification of the RT-LAMP protocol of Varga and James (2006b) is achieved by using a virus suspension derived from homogenised plant material in the presence of deionised water as template (Lee et al., 2009). The addition of hydroxy naphthol blue to the LAMP reaction mix allows for the direct visualisation of DNA amplification (Goto et al., 2009). Consequently, Blue LAMP based detection of PPV reduces the time between sampling and result to about two and a half hour. Its detection limit is similar to RT-PCR protocols. The risk of contaminations is minimized because the number of working steps is decreased and the tubes do not have to be opened upon amplification avoiding a source of contamination. This low cost method is not only suitable for a small number of samples but also for large scale surveys. Due to the simplified sample preparation and the isothermal reactions conditions of Blue LAMP coupled with the homogenous visualisation of amplified DNA, there is no need for expensive technical devices such as a thermal cycler or a gel electrophoresis device and toxic reagents like ethidium bromide or chloroform. Therefore, Blue LAMP is a promising tool not only for the detection of PPV but also for the detection of other phytopathogens (bacteria, viruses, viroids and phytoplasmas). It is applicable not only for research institutes and plant protection agencies but also for laboratories with only basic equipment and nurseries.

VII Summary

The Sharka disease is the most important virosis in stone fruit production since caused losses due to loss of earnings and eradications are very high. There are no possibilities for treatment of the disease and its causal agent, the *Plum pox virus* (PPV), respectively. The single way to avoid an infection is based on the hypersensitive resistance but, at the moment, just one cultivar is available bearing this resistance. Accordingly, the prevention of the virus spread is only possible by using virus free plant material and by monitoring the orchards for Sharka regularly. For this, methods are required to reliably detect the virus and which can be used for large numbers of samples.

For the detection of PPV, several protocols are available based either on biological, immunological or molecular biological techniques. Actually, the European Plant Protection Organization recommends at least two techniques for reliably proving the presence of the virus in a tree. The preferred methods should be grafting on indicator plants, DASI-ELISA or RT-PCR. Apart from grafting on indicator plants, which is still the most reliable method for the detection of PPV, molecular biological procedures based on RT-PCR show very low detection limits and can be finished within a few hours but they have to be performed in expensive thermal cyclers and require laborious RNA purification. In contrast, DASI-ELISA is widely used because of its higher reliability and simpler workflow compared to RT-PCR. However, these methods are time-consuming, laborious, costly, insensitive and/or require sophisticated technical equipment and, therefore, must be applied by research or diagnostic laboratories.

Several isothermal nucleic acid amplification techniques were developed in recent years to avoid thermal cycling necessary for PCR. Despite this common characteristic, the mechanism to achieve isothermal reaction conditions is quite different. Because of its simple set-up, cascade rolling circle amplification (CRCA) was adopted for the detection of PPV. High specificity was achieved by enzymatic ligation of the circularizable probe (CLP) to PPV cDNA. The CRCA of the circularized CLP allowed for high sensitivity. However, diverse attempts to inhibit background amplification, which is characteristic for CRCA as well, failed. For correct discrimination of positive and negative samples, a final exonucleolysis has to be carried out. As ligation of the CLP and amplification of closed CLPs have to be performed separately, the number of working steps is additionaly increased compared to RT-PCR. Therefore, CRCA is not suitable for the detection of PPV especially for high-throughput monitoring.

Another isothermal technique is loop-mediated isothermal amplification (LAMP). It allows for the direct detection of PPV RNA similar to one-step RT-PCR (RT-LAMP) avoiding the separated cDNA-synthesis prior to the amplification which is necessary for CRCA and partially for RT-PCR. Beside the isothermal reaction conditions a major step towards high-throughput monitoring of PPV infection is the homogenous visualisation of DNA synthesis. The enormous rate of specific amplification is shown by a colour change from purple to blue by supplementing the reaction with hydroxynaphthol blue creating the Blue LAMP. In contrast to RT-qPCR, no apparatus is needed for the observation of the reaction mix colour as the colour change is visible with the naked eye. As a consequence, the time consuming gel electrophoresis is omitted.

For high-throughput monitoring the conventional RNA extraction prior to the amplification is still too laborious and, therefore, is replaced by a fast plant extraction procedure to obtain a virus suspension which is suitable to serve as template for Blue LAMP but not for RT-PCR.

The combination of Blue LAMP and the fast plant extraction procedure allows now for the detection of PPV within 2.5 hours using basic technical equipment. Due to the small number of working steps and the homogenous visualisation compared to RT-PCR, the risk of cross contaminations is reduced. The colour change is clearly visible even for untrained persons. Because of these benfits, the newly developed Blue LAMP for Sharka detection can not only be used by research institutes and plant protection agencies but also by cooperatives, orchardists and nurseries. They can monitor their orchards independently and can test plant material at the point of delivery from or to the facility. Thus, infected trees can be removed faster from the orchard and the spread of the virus by trade goods is prevented. All the more, as the Blue LAMP protocol can be used for single samples as well as for large numbers of samples due to its speed and simple work flow, it is an excellent tool for high-throughput monitoring for *Plum pox virus* infection in stone fruit production.

The detection of an internal control as presented in this study increases the reliability of the Blue LAMP based diagnosis as the integrity of the virus suspension used is proven as well.

The PPV subgroup differentiation by Blue LAMP introduced in this work enables the detection and distinction of all PPV strains known so far by one protocol. Other methods for this purpose do not cover all strains or are laborious and expensive. The Blue LAMP can easily be expanded by further strains upon discovery.

The accuracy, the specificity, the sensitivity and the simplicity of this method regarding high-throughput monitoring of *Plum pox virus* infection in stone fruit production in general but also for subgroup differentiation was demonstrated by diverse Blue LAMP protocols developed on the basis of specific primer sets.

VIII Zusammenfassung

Die Scharka-Krankheit ist die bedeutendste Virose im Steinobstanbau, da die verursachten Schäden durch Ertragsausfälle und Rodungen sehr hoch sind, gleichzeitig aber keine Möglichkeiten vorhanden sind, die Krankheit bzw. das ursächliche *Plum pox virus* (PPV) zu bekämpfen. Die einzige Möglichkeit eine Infektion zu verhindern liegt in der Hypersensibilitätsresistenz, die bislang allerdings nur in einer Sorte verfügbar ist. Dementsprechend kann nur die Verbreitung des Virus verhindert werden, indem virusfreies Pflanzenmaterial verwendet und Anlagen regelmäßig auf Scharka kontrolliert wird. Dies erfordert Methoden, die zuverlässig das Virus nachweisen können und mit denen auch eine größere Probenzahl bewältigt werden kann.

Für den Nachweis des PPV gibt es zahlreiche Protokolle, die entweder auf biologischen, immunologischen oder molekular-biologischen Techniken beruhen. Die European Plant Protection Organization empfiehlt mindestens zwei Verfahren für die verlässliche Testung auf den Virus. Entweder Veredelung auf Indikatorpflanzen, DASI-ELISA oder RT-PCR sollten verwendet werden. Nach der Verwendung von Indikatorpflanzen sind molekularbiologische Verfahren wie RT-PCR die sensitivsten Verfahren zum Nachweis von PPV und können innerhalb einiger Stunden durchgeführt werden, aber setzen einen teueren Thermocycler und eine aufwendige RNA Aufreinigung voraus. Im Gegensatz wird verbreitet DASI-ELISA wegen seiner vergleichsweisen hohen Verlässlichkeit und einfachen Arbeitsschritten verwendet. Allerdings sind diese Methoden zeit-, kosten- und arbeitsintensiv, insensitiv und/oder setzen eine kostspielige Laborausstattung voraus und werden deshalb von Forschungseinrichtungen oder Diagnoselaboren durchgeführt.

Zahlreiche isothermale Nukleinsäureamplifikationstechniken wurden in den letzten Jahren entwickelt, um die in der PCR notwendige thermische Denaturierung zu vermeiden. Trotz dieser Gemeinsamkeit ist die Herangehensweise sehr unterschiedlich. Auf Grund des einfachen Set-ups wurde die Cascade rolling circle amplification (CRCA) für den Nachweis von PPV angepasst. Hohe Spezifität wurde durch die enzymatische Ligierung einer zum Ringschluss fähigen Sonde (circurlarizable probe (CLP)) auf PPV cDNA erreicht. Die Amplifikation der CLP mittels CRCA erlaubte hohe Sensitivität. Allerdings schlugen alle Versuche, die Hintergrundamplifikation, welche CRCA typisch, zu vermeiden, fehl. Für die eindeutige Unterscheidung von positiven und negativen Proben musste eine Exonukleolysis durchgeführt werden. Da Ligation und Amplifikation der CLP hintereinander durchgeführt werden müssen, war die Zahl der Arbeitsschritte im Vergleich zur RT-PCR zusätzlich erhöht. Deshalb ist die CRCA nicht geeignet für den PPV-Nachweis gerade auch in Hinsicht auf Hochdurchsatz-Monitoring.

Eine weitere isothermale Methode ist die Loop-mediated isothermal amplification (LAMP). Diese ermöglicht den direkten Nachweis von PPV RNA vergleichbar zur one-step RT-PCR (RT-LAMP), wodurch die separate cDNA-Synthese vor der Amplifikation vermieden wird, die für CRCA und teilweise für RT-PCR notwendig ist. Die enorme Amplifikationsrate erlaubt die Visualisierung der DNA Synthese entweder durch die Pyrophosphattrübung oder durch einen durch Hydroxynaphtholblau bedingten Farbumschlag von violett

nach blau (Blue LAMP). Dadurch wird die Gelelektrophorese umgangen. Eine weitere Vereinfachung des Nachweissystems liegt in einem durch eine einfache Probenaufbereitung gewonnenen Pflanzenextrakt, der die RNA Aufreinigung ersetzt. Dieser kann in der Blue LAMP als Template verwendet werden, nicht jedoch in der RT-PCR.

Die Kombination aus Blue LAMP und vereinfachte Probenaufbereitung ermöglicht den Nachweis von PPV innerhalb von 2,5 Stunden mit geringem technischem Aufwand und ist auf Grund weniger Arbeitschritte und der internen Visualisierung kaum anfällig für Verschleppungen. Der Farbumschlag ist auch für ungeübte Augen klar ersichtlich. Die Nachweisgrenze ist ähnlich niedrig wie die der RT-PCR. Durch diese Eigenschaften ist die hier vorgestellte Blue LAMP für den Scharkanachweis nicht nur für Forschungseinrichtungen und Landwirtschaftsämtern geeignet, sondern insbesondere auch für Genossenschaften, Obstbauern und Baumschulen, die ihre Anlagen selbstständig kontrollieren können und Pflanzenmaterial bereits bei Lieferung bzw. vor Auslieferung auf Virusbefall testen können. Dies erlaubt zum einen eine wesentlich schnellere Reaktion auf das Auftreten von PPV in der Obstanlage, zum anderen wird die Verbreitung des Virus durch Handelsware unterbunden. Dies um so mehr, da sich das Blue LAMP Verfahren nicht nur für vereinzelte Proben eignet, sondern auf Grund von Schnelligkeit und geringem Arbeitsaufwand für die Testung zahlreicher Proben in kurzer Zeit eignet und somit für das Hochdurchsatz-Monitoring auf *Plum pox virus* Infektionen im Steinobstanbau.

Der paralle Nachweis einer internen Kontrolle erhöht zudem die Aussagekraft bzw. die Verlässlichkeit der Blue LAMP basierten Diagnose, da die Integrität des verwendeten Pflanzenextrakts bestätigt wird.

Die in dieser Arbeit vorgestellte PPV-Subtypenunterscheidung mittels der Blue LAMP ermöglicht die Erfassung und Unterscheidung aller bisher bekannter PPV-Stämme. Andere Methoden für diesen Zweck decken entweder nicht alle Stämme ab oder sind arbeitsintensiv und teuer. Die Blue LAMP kann nach Entdeckung weiterer Stämme einfach um diese ergänzt werden.

Die Spezifität, die Sensitivität und die Einfachheit dieser Methode hinsichtlich des Hochdurchsatz-Monitorings von *Plum pox virus* Infektionen im Steinobstanbau wurde sowohl für den allgemeinen PPV Nachweis als auch für die Stammunterscheidung anhand diverser auf neu entwickelten spezifischen Primersets beruhenden Blue LAMP Protokolle gezeigt.

References

Abe, H., Kondo, Y., Jinmei, H., Abe, N., Furukawa, K., Uchiyama, A., Tsuneda, S., Aikawa, K., Matsumoto, I., and Ito, Y. (2008). Rapid DNA chemical ligation for amplification of RNA and DNA signal. *Bioconjugate Chemistry*, 19(1):327–333.

Adams, A., Guise, C., and Crossley, S. (1999). *Plum pox virus* detection in dormant plum trees by PCR and ELISA. *Plant Pathology*, 48(2):240–244.

Ahmed, M. U., Saito, M., Hossain, M. M., Rao, S. R., Furui, S., Hino, A., Takamura, Y., Takagi, M., and Tamiya, E. (2009). Electrochemical genosensor for the rapid detection of GMO using loop-mediated isothermal amplification. *Analyst*, 134(5):966–972.

Al-Soud, W. A. and Rådström, P. (2000). Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *Journal of Clinical Microbiology*, 38(12):4463–4470.

Aliotta, J. M., Pelletier, J. J., Ware, J. L., Moran, L. S., Benner, J. S., and Kong, H. (1996). Thermostable *Bst* DNA polymerase I lacks a 3'–>5' proofreading exonuclease activity. *Genetic Analysis: Biomolecular Engineering*, 12(5-6):185–195.

Alsmadi, O. A., Bornarth, C. J., Song, W., Wisniewski, M., Du, J., Brockman, J. P., Faruqi, A. F., Hosono, S., Sun, Z., Du, Y., Wu, X., Egholm, M., Abarzúa, P., Lasken, R. S., and Driscoll, M. D. (2003). High accuracy genotyping directly from genomic DNA using a rolling circle amplification based assay. *BMC Genomics*, 4(1):21.

Altman, D. G. and Bland, J. M. (1994). Diagnostic tests. 1: Sensitivity and specificity. *British Medical Journal*, 308(6943):1552.

An, L., Tang, W., Ranalli, T. A., Kim, H.-J., Wytiaz, J., and Kong, H. (2005). Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. *Journal of Biological Chemistry*, 280(32):28952–28958.

Angamuthu, R., Baskaran, S., Gopal, D. R., Devarajan, J., and Kathaperumal, K. (2012). Rapid detection of Marek's disease viral genome in chicken feathers by loop-mediated isothermal amplification (LAMP). *Journal of Clinical Microbiology*, 50(3):961-965.

Atanasoff, D. (1935). Mosaik of stone fruits. *Pythopathologische Zeitschrift*, 8:259–284.

Ball, S., Reeve, M. A., Robinson, P. S., Hill, F., Brown, D. M., and Loakes, D. (1998). The use of tailed octamer primers for cycle sequencing. *Nucleic Acids Research*, 26(22):5225–5227.

Banér, J., Isaksson, A., Waldenström, E., Jarvius, J., Landegren, U., and Nilsson, M. (2003). Parallel gene analysis with allele-specific padlock probes and tag microarrays. *Nucleic Acids Research*, 31(17):e103.

Banér, J., Nilsson, M., Mendel-Hartvig, M., and Landegren, U. (1998). Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Research*, 26(22):5073–5078.

Baskaran, N., Kandpal, R. P., Bhargava, A. K., Glynn, M. W., Bale, A., and Weissman, S. M. (1996). Uniform amplification of a mixture of deoxyribonucleic acids with varying GC content. *Genome Research*, 6(7):633–638.

Bearinger, J. P., Dugan, L. C., Baker, B. R., Hall, S. B., Ebert, K., Mioulet, V., Madi, M., and King, D. P. (2011). Development and initial results of a low cost, disposable, point-of-care testing device for pathogen detection. *IEEE Transactions on Biomedical Engineering*, 58(3):805–808.

Bühler, C. (2007). Einfluss verschiedener Scharkavirusisolate auf die Ausprägung der Hypersensibilitätsresistenz der Europäischen Pflaume (*Prunus domestica* L.) gegen das Virus. Master's thesis, Technische Universität München.

Blanco, L., Bernad, A., Lázaro, J. M., Martín, G., Garmendia, C., and Salas, M. (1989). Highly efficient DNA synthesis by the phage Phi29 DNA polymerase. Symmetrical mode of DNA replication. *Journal of Biological Chemistry*, 264(15):8935–8940.

Boscia, D., Zeramdini, H., Cambra, M., Potere, O., Gorris, M., Myrta, A., and und V. Savino, B. D. T. (1997). Production and characterization of a monoclonal antibody specific to the M serotype of *Plum pox potyvirus*. *European Journal of Plant Pathology*, 103(5):477–480.

Boubourakas, I. N., Fukuta, S., and Kyriakopoulou, P. E. (2009). Sensitive and rapid detection of *Peach latent mosaic viroid* by the reverse transcription loop-mediated isothermal amplification. *Journal of Virological Methods*, 160(1-2):63–68.

Brukner, I., Paquin, B., Belouchi, M., Labuda, D., and Krajinovic, M. (2005). Self-priming arrest by modified random oligonucleotides facilitates the quality control of whole genome amplification. *Analytical Biochemistry*, 339(2):345–347.

Cai, Z., Lou, G., Cai, T., Yang, J., and Wu, N. (2011). Development of a novel genotype-specific loop-mediated isothermal amplification technique for Hepatitis B virus genotypes B and C genotyping and quantification. *Journal of Clinical Virology*, 52(4):288–294.

Cambra, M., Asensio, M., Gorris, M. T., Perrez, E., Camarasa, E., A., G. J., Moya, J. J., Lopez-Abella, D., Vela, C., and Sanz, A. (1994). Detection of *Plum pox potyvirus* using monoclonal antibodies to structural and non-structural proteins. *EPPO Bulletin*, 24(3):569–577.

Cambra, M., Boscia, D., Myrta, A., Palkovics, L., Navrátil, M., Barba, M., Gorris, M. T., and Capote, N. (2006a). Detection and characterization of *Plum pox virus*: serological methods. *EPPO Bulletin*, 36(2):254–261.

Cambra, M., Capote, Myrta, A., and Llácer, G. (2006b). *Plum pox virus* and the estimated costs associated with Sharka disease. *EPPO Bulletin*, 36(2):202–204.

Canceill, D., Viguera, E., and Ehrlich, S. D. (1999). Replication slippage of different DNA polymerases is inversely related to their strand displacement efficiency. *Journal of Biological Chemistry*, 274(39):27481–27490.

Capote, N., Bertolini, E., Olmos, A., Vidal, E., Martínez, M. C., and Cambra, M. (2009). Direct sample preparation methods for the detection of *Plum pox virus* by real-time RT-PCR. *International Microbiology*, 12(1):1–6.

Carninci, P., Nishiyama, Y., Westover, A., Itoh, M., Nagaoka, S., Sasaki, N., Okazaki, Y., Muramatsu, M., and Hayashizaki, Y. (1998). Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proceedings of the National Academy of Sciences U S A*, 95(2):520–524.

Cervera, M., Riechmann, J., Martin, M., and Garcia, J. (1993). 3'-terminal sequence of the *Plum pox virus* PS and O6 isolates: evidence for RNA recombination within the potyvirus group. *Journal of General Virology*, 74(3):329–334.

Chaudhary, A. A., Hemant, Mohsin, M., and Ahmad, A. (2011). Application of loop-mediated isothermal amplification (LAMP)-based technology for authentication of *Catharanthus roseus* (L.) G. Don. *Protoplasma*. 249(2):417-422.

Cheng, Y., Li, Z., Du, B., and Zhang, X. (2008). Homogeneous and label-free bioluminescence detection of single nucleotide polymorphism with rolling circle amplification. *Analyst*, 133(6):750–752.

Christian, A. T., Pattee, M. S., Attix, C. M., Reed, B. E., Sorensen, K. J., and Tucker, J. D. (2001). Detection of DNA point mutations and mRNA expression levels by rolling circle amplification in individual cells. *Proceedings of the National Academy of Sciences U S A*, 98(25):14238–14243.

Clark, M., Adams, A., Thresh, J., and Casper, R. (1976). The detection of *Plum pox virus* and other viruses in woody plants by enzyme-linked immunosorbent assay (ELISA). *Acta Horticulturae*, 67:51–57.

Clark, M. F. and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, 34(3):475–483.

Compton, J. (1991). Nucleic acid sequence-based amplification. *Nature*, 350:91–92.

Crescenzi, A., d'Aquino, L., Comes, S., Nuzzaci, M., Piazzolla, P., Boscia, D., and Hadidi, A. (1997a). Characterization of the sweet cherry isolate of *Plum pox potyvirus*. *Plant Disease*, 81(7):711–714.

Crescenzi, A., d'Aquino, L., Nuzzaci, M., Ostuni, A., Bavoso, A., Comes, S., Stradis, A. D., and Piazzolla, P. (1997b). Production of strain specific antibodies against a synthetic polypeptide corresponding to the N-terminal region of the *Plum pox potyvirus* coat protein. *Journal of Virological Methods*, 69(1-2):181–189.

Dahl, F., Baner, J., Gullberg, M., Mendel-Hartvig, M., Landegren, U., and Nilsson, M. (2004). Circle-to-circle amplification for precise and sensitive DNA analysis. *Proceedings of the National Academy of Sciences U S A*, 101(13):4548–4553.

Dallot, S., Glasa, M., Jevremovic, D., Kamenova, I., Paunovic, S., and Labonne, G. (2011). Mediterranean and central-eastern European countries host viruses of two different clades of *Plum pox virus* strain M. *Archives of Virology*, 156(3):539–542.

Damsteegt, V. D., Waterworth, H. E., Mink, G. I., Howell, W. E., and Levy, L. (1997). *Prunus tomentosa* as a diagnostic host for detection of *Plum pox virus* and other *Prunus* viruses. *Plant Disease*, 81(4):329–332.

Daubendiek, S. L. and Kool, E. T. (1997). Generation of catalytic RNAs by rolling transcription of synthetic DNA nanocircles. *Nature Biotechnology*, 15(3):273–277.

Daubendiek, S. L., Ryan, K., and Kool, E. (1995). Rolling-circle RNA synthesis: Circular oligonucleotides as efficient substrates for T7 RNA polymerase. *Journal of the American Chemical Society*, 117(29):7818–7819.

Dean, F. B., Hosono, S., Fang, L., Wu, X., Faruqi, A. F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S. F., Egholm, M., and Lasken, R. S. (2002). Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences U S A*, 99(8):5261–5266.

Dean, F. B., Nelson, J. R., Giesler, T. L., and Lasken, R. S. (2001). Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Research*, 11(6):1095–1099.

Deborré, G., Jelkman, W., and Maiss, E. (1995). Biological and molecular biological investigations of several *Plum pox virus* (PPV) isolates. *Acta Horticulturae*, 386:253–262.

del Solar, G., Moscoso, M., and Espinosa, M. (1993). Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. *Molecular Microbiology*, 8(5):789–796.

Detter, J. C., Jett, J. M., Lucas, S. M., Dalin, E., Arellano, A. R., Wang, M., Nelson, J. R., Chapman, J., Lou, Y., Rokhsar, D., Hawkins, T. L., and Richardson, P. M. (2002). Isothermal strand-displacement amplification applications for high-throughput genomics. *Genomics*, 80(6):691–698.

Ding, W. C., Chen, J., Shi, Y. H., Lu, X. J., and Li, M. Y. (2010). Rapid and sensitive detection of infectious spleen and kidney necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Archives of Virology*, 155(3):385-389.

Ding, X., Snyder, A. K., Shaw, R., Farmerie, W. G., and Song, W.-Y. (2003). Direct retransformation of yeast with plasmid DNA isolated from single yeast colonies using rolling circle amplification. *Biotechniques*, 35(4):774–6, 778–9.

Dukes, J. P., King, D. P., and Alexandersen, S. (2006). Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Archives of Virology*, 151(6):1093–1106.

En, F.-X., Wei, X., Jian, L., and Qin, C. (2008). Loop-mediated isothermal amplification establishment for detection of pseudorabies virus. *Journal of Virological Methods*, 151(1):35–39.

EPPO (2004). Diagnostic protocols for regulated pests: *Plum pox potyvirus*. *EPPO Bulletin*, 34:247–256.

EPPO (2006). Current status of *Plum pox virus* and Scharka disease worldwide. *EPPO Bulletin*, 36:205–218.

Esteban, J. A., Soengas, M. S., Salas, M., and Blanco, L. (1994). 3'–>5' exonuclease active site of Phi 29 DNA polymerase. evidence favoring a metal ion-assisted reaction mechanism. *Journal of Biological Chemistry*, 269(50):31946–31954.

Esteban, O., García, J. A., Gorris, M. T., Domínguez, E., and Cambra, M. (2003). Generation and characterisation of functional recombinant antibody fragments against RNA replicase NIb from *Plum pox virus*. *Biochemical and Biophysical Research Communications*, 301(1):167–175.

Fall, J., Chakraborty, G., Kono, T., Maeda, M., Itami, T., and Sakai, M. (2008). Establishment of loop-mediated isothermal amplification method (LAMP) for the detection of *Vibrio nigripulchritudo* in shrimp. *FEMS Microbiology Letters*, 288(2):171–177.

Fang, X., Chen, H., Yu, S., Jiang, X., and Kong, J. (2011). Predicting viruses accurately by a multiplex microfluidic loop-mediated isothermal amplification chip. *Analytical Chemistry*, 83(3):690–695.

Fanigliulo, A., Comes, S., Maiss, E., Piazzolla, P., and Crescenzi, A. (2003). The complete nucleotide sequence of *Plum pox virus* isolates from sweet (PPV-SwC) and sour (PPV-SoC) cherry and their taxonomic relationships within the species. *Archives of Virology*, 148:2137–2153.

Faruqi, A. F., Hosono, S., Driscoll, M. D., Dean, F. B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., Du, Y., Kingsmore, S., Knott, T., and Lasken, R. S. (2001). High-throughput genotyping of single nucleotide polymorphisms with rolling circle amplification. *BMC Genomics*, 2(1):4.

Fire, A. and Xu, S. (1995). Rolling replication of short DNA circles. *Proceedings of the National Academy of Sciences U S A*, 92(10):4641–4645.

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., and Ishimoto, Y. (2003a). Detection of *Japanese yam mosaic virus* by RT-LAMP. *Archives of Virology*, 148(9):1713–1720.

Fukuta, S., Kato, S., Yoshida, K., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., and Ishimoto, Y. (2003b). Detection of *Tomato yellow leaf curl virus* by loop-mediated isothermal amplification reaction. *Journal of Virological Methods*, 112(1-2):35–40.

Fukuta, S., Mizukami, Y., Ishida, A., and Kanbe, M. (2006). Development of loop-mediated isothermal amplification (LAMP)-based SNP markers for

shelf-life in melon (*Cucumis melo* L.). *Journal of Applied Genetics*, 47(4):303–308.

Fukuta, S., Ohishi, K., Yoshida, K., Mizukami, Y., Ishida, A., and Kanbe, M. (2004). Development of immunocapture reverse transcription loop-mediated isothermal amplification for the detection of *Tomato spotted wilt virus* from chrysanthemum. *Journal of Virological Methods*, 121(1):49–55.

Gandelman, O. A., Church, V. L., Moore, C. A., Kiddle, G., Carne, C. A., Parmar, S., Jalal, H., Tisi, L. C., and Murray, J. A. H. (2010). Novel bioluminescent quantitative detection of nucleic acid amplification in real-time. *PLoS One*, 5(11):e14155.

Glasa, M. and Candresse, T. (2005). Partial sequence analysis of an atypical turkish isolate provides further information on the evolutionary history of *Plum pox virus* (PPV). *Virus Research*, 108:199–206.

Glasa, M., Kudela, O., Marie-Jeanne, V., and Quiot, J. (2001). Evidence of a naturally occurring recombinant isolate of *Plum pox virus* from Slovakia. *Plant Disease*, 85(8):920.

Glasa, M., Malinowski, T., Predajna, L., Pupola, N., Dekena, D., Michalczuk, L., and Candresse, T. (2011). Sequence variability, recombination analysis and specific detection of the W strain of *Plum pox virus*. *Phytopathology*, 101(11):980–985.

Glasa, M., Marie-Jeanne, V., Labonne, G., Subr, Z., Kúdela, O., and Quiot, J. B. (2002a). A natural population of recombinant *Plum pox virus* is viable and competitive under field conditions. *European Journal of Plant Pathology*, 108(9):843–853.

Glasa, M., Marie-Jeanne, V., Moury, B., Kúdela, and Quiot, J. B. (2002b). Molecular variability of the P3-6K1 genomic region among geographically and biologically distinct isolates of *Plum pox virus*. *Archives of Virology*, 147(3):563–575.

Glasa, M., Palkovics, L., Kominek, P., Labonne, G., Pittnerova, S., Kudela, O., Candresse, T., and Subr, Z. (2004). Geographically and temporally distant natural recombinant isolates of *Plum pox virus* (PPV) are genetically very similar and form a unique PPV subgroup. *Journal of General Virology*, 85(9):2671–2681.

Glasa, M., Paunovic, S., Jevremovic, D., Myrta, A., Pittnerová1, S., and Candresse, T. (2005). Analysis of recombinant *Plum pox virus* (PPV) isolates from Serbia confirms genetic homogeneity and supports a regional origin for the PPV-Rec subgroup. *Archives of Virology*, 150(10):2051–2060.

Glasa, M., Svanella, L., and Candresse, T. (2006). The complete nucleotide sequence of the *Plum pox virus* El Amar isolate. *Archives of Virology*, 151(8):1679–1682.

Goldmeyer, J., Kong, H., and Tang, W. (2007). Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection. *Journal of Molecular Diagnostics*, 9(5):639–644.

Goto, M., Honda, E., Ogura, A., Nomoto, A., and Hanaki, K.-I. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques*, 46(3):167–172.

Goto, M., Shimada, K., Sato, A., Takahashi, E., Fukasawa, T., Takahashi, T., Ohka, S., Taniguchi, T., Honda, E., Nomoto, A., Ogura, A., Kirikae, T., and Hanaki, K.-I. (2010). Rapid detection of *Pseudomonas aeruginosa* in mouse feces by colorimetric loop-mediated isothermal amplification. *Journal of Microbiological Methods*, 81(3):247–252.

Góra-Sochacka, A. (2004). Viroids: unusual small pathogenic RNAs. *Acta Biochimica Polonica*, 51(3):587–607.

Grüntzig, M., Ernst, I., Herzog, U., Kegler, H., Fischer, M., and Fuchs, E. (2002). Zum Verhalten von *Prunus armeniaca* L. und *P. domestica* L. gegenüber dem *Plum pox virus* (PPV). *Archives Of Phytopathology And Plant Protection*, 34(6):435–462.

Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., Barringer, K. J., Richman, D. D., and Gingeras, T. R. (1990). Isothermal, *in vitro* amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proceedings of the National Academy of Sciences U S A*, 87(5):1874–1878.

Gutierrez, C. (1999). Geminivirus DNA replication. *Cellular and Molecular Life Sciences*, 56(3-4):313–329.

Hadersdorfer, J., Neumüller, M., Treutter, D., and Fischer, T. (2011). Fast and reliable detection of *Plum pox virus* in woody host plants using the Blue LAMP protocol. *Annals of Applied Biology*, 159(3):456–466.

Hafner, G., Yang, I., Wolter, L., Stafford, M., and Giffard, P. (2001). Isothermal amplification and multimerization of DNA by *Bst* DNA polymerase. *Biotechniques*, 30(4):852–867.

Hamdorf, G. (1976). Further studies about the host range of Sharka (Plum pox) virus. *Mitteilungen aus der*

Biologischen Bundesanstalt für Land- und Forstwirtschaft, 170:57–63.

Hammond, J., Pühringer, H., da Câmara Machado, A., and da Câmara Machado, M. L. (1998). A broad-spectrum PCR assay combined with RFLP analysis for detection and differentiation of *Plum pox virus* isolates. *Acta Horticulturae*, 472(2):483–490.

Hardenbol, P., Banér, J., Jain, M., Nilsson, M., Namsaraev, E. A., Karlin-Neumann, G. A., Fakhrai-Rad, H., Ronaghi, M., Willis, T. D., Landegren, U., and Davis, R. W. (2003). Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nature Biotechnology*, 21(6):673–678.

Harper, S. J., Ward, L. I., and Clover, G. R. G. (2010). Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology*, 100(12):1282–1288.

Hart, J. L., Harris, Z. M., and Testa, S. M. (2010). Analyzing and predicting the thermodynamic effects of the metabolite trehalose on nucleic acids. *Biopolymers*, 93(12):1085–1092.

Hatano, B., Maki, T., Obara, T., Fukumoto, H., Hagisawa, K., Matsushita, Y., Okutani, A., Bazartseren, B., Inoue, S., Sata, T., and Katano, H. (2010). LAMP using a disposable pocket warmer for anthrax detection, a highly mobile and reliable method for anti-bioterrorism. *Japanese Journal of Infectious Diseases*, 63(1):36–40.

Hatch, A., Sano, T., Misasi, J., and Smith, C. L. (1999). Rolling circle amplification of DNA immobilized on solid surfaces and its application to multiplex mutation detection. *Genetic Analysis: Biomolecular Engineering*, 15(2):35–40.

Herranz, M., Sanchez-Navarro, J., Aparicio, F., and Pallás, V. (2005). Simultaneous detection of six stone fruit viruses by non-isotopic molecular hybridization using a unique riboprobe or 'polyprobe'. *Journal of Virological Methods*, 124(1-2):49–55.

Hilgert, I., Cikánek, D., Krištofová, H., Karešová, R., and Navrátil, M. (1993). Monoclonal antibodies suitable for *Plum pox virus* determination. *Hybridoma*, 12(2):215–220.

Holbrook, J. F., Stabley, D., and Sol-Church, K. (2005). Exploring whole genome amplification as a DNA recovery tool for molecular genetic studies. *Journal of Biomolecular Techniques*, 16(2):125–133.

Houseley, J. and Tollervey, D. (2010). Apparent non-canonical trans-splicing is generated by reverse transcriptase *in vitro*. *PLoS One*, 5(8):e12271.

Hsuih, T. C., Park, Y. N., Zaretsky, C., Wu, F., Tyagi, S., Kramer, F. R., Sperling, R., and Zhang, D. Y. (1996). Novel, ligation-dependent PCR assay for detection of hepatitis C in serum. *Journal of Clinical Microbiology*, 34(3):501–507.

Inoue, J., Shigemori, Y., and Mikawa, T. (2006). Improvements of rolling circle amplification (RCA) efficiency and accuracy using *Thermus thermophilus* SSB mutant protein. *Nucleic Acids Research*, 34(9):e69.

Inoue-Nagata, A. K., Albuquerque, L. C., Rocha, W. B., and Nagata, T. (2004). A simple method for cloning the complete begomovirus genome using the bacteriophage Phi29 DNA polymerase. *Journal of Virological Methods*, 116(2):209–211.

Iwamoto, T., Sonobe, T., and Hayashi, K. (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* Complex, *M. avium*, and *M. intracellulare* in sputum samples. *Journal of Clinical Microbiology*, 41(6):2616–2622.

James, D. and Varga, A. (2005). Nucleotide sequence analysis of *Plum pox virus* isolate W3174: Evidence of a new strain. *Virus Research*, 110:143–150.

James, D., Varga, A., Thompson, D., and Hayes, S. (2003). Detection of a new and unusual isolate of *Plum pox virus* in plum (*Prunus domestica*). *Plant Disease*, 87:1119–1124.

Jaroenram, W., Kiatpathomchai, W., and Flegel, T. W. (2008). Rapid and sensitive detection of *White spot syndrome virus* by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Molecular and Cellular Probes*, 23(2):65–70.

Jarošová, J. and Kundu, J. (2010). Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR. *Scientia Horticulturae*, 125(1):68–72.

Jensen, M. A., Fukushima, M., and Davis, R. W. (2010). DMSO and betaine greatly improve amplification of GC-rich constructs in *de novo* synthesis. *PLoS One*, 5(6):e11024.

Jonstrup, S. P., Koch, J., and Kjems, J. (2006). A microRNA detection system based on padlock probes and rolling circle amplification. *RNA*, 12(9):1747–1752.

Kalashyan, Y., Bilkey, N., Verderevskaya, T., and Rubina, E. (1994). *Plum pox potyvirus* on sour cherry in Moldova. *EPPO Bulletin*, 24(3):645–649.

Kaneko, H., Kawana, T., Fukushima, E., and Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods*, 70(3):499–501.

Kang, J., Lee, M. S., and Gorenstein, D. G. (2005). The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: application to *in vitro* combinatorial selection of aptamers. *Journal of Biochemical and Biophysical Methods*, 64(2):147–151.

Kaocharoen, S., Wang, B., Tsui, K. M., Trilles, L., Kong, F., and Meyer, W. (2008). Hyperbranched rolling circle amplification as a rapid and sensitive method for species identification within the *Cryptococcus* species complex. *Electrophoresis*, 29(15):3183–3191.

Karayiannis, I., Thomidis, T., Michailides, Z., and Audergon, J. M. (2010). Reaction of an F1 apricot hybrid population to artificial and natural inoculation of *Plum pox virus*. *New Zealand Journal of Crop and Horticultural Science*, 38(4):283–288.

Kegler, H., Bauer, E., Grüntzig, M., Fuchs, E., Verderevskaja, T. D., and Bivol, T. F. (1985). Nachweis unterschiedlicher Resistenztypen bei Pflaumen gegen das Scharka-Virus (*Plum pox virus*). *Archives of Phytopathology and Plant Protection*, 21:339–346.

Kegler, H., Fuchs, E., Grüntzig, M., and Schwarz, S. (1998). Some results of 50 years' research on resistance to *Plum pox potyvirus*. *Archives of Phytopathology and Plant Protection*, 31(6):479–506.

Kegler, H., Grüntzig, M., and Fuchs, E. (1994). A glasshouse test for detecting resistance of plum genotypes to *Plum pox virus*. *Acta Horticulturae*, 359:152–158.

Kerlan, C. and Dunez, J. (1979). Différenciation biologique et serologique de souches du virus de la Sharka. *Annales de phytopathologie*, 11:241–250.

Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S., and Flegel, T. W. (2008). Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of Virological Methods*, 153(2):214–217.

Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N., and Kanzaki, N. (2009). A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. *Phytopathology*, 99(12):1365–1369.

Kleppe, K., de Sande, J. H. V., and Khorana, H. G. (1970). Polynucleotide ligase-catalyzed joining of deoxyribo-oligonucleotides on ribopolynucleotide templates and of ribo-oligonucleotides on deoxyribopolynucleotide templates. *Proceedings of the National Academy of Sciences U S A*, 67(1):68–73.

Knapp, E., da Câmara Machado, A., Pühringer, H., Wang, Q., Hanzer, V., Weiss, H., Weiss, B., Katinger, H., and da Câmara Machado, M. L. (1995). Localization of fruit tree viruses by immuno-tissue printing in infected shoots of *Malus sp.* and *Prunus sp.* *Journal of Virological Methods*, 55(2):157–173.

Knott, T., Smith, C., Pickering, J., and Schwarz, T. (2002). Suppression of non-specific nucleic acid amplification. *Patent number: WO 2002/057487 A2*

Koide, Y., Maeda, H., Yamabe, K., Naruishi, K., Yamamoto, T., Kokeguchi, S., and Takashiba, S. (2010). Rapid detection of *mecA* and *spa* by the loop-mediated isothermal amplification (LAMP) method. *Letters in Applied Microbiology*, 50(4):386-392.

Kong, H., Kucera, R. B., and Jack, W. E. (1993). Characterization of a DNA polymerase from the hyperthermophile archaea *Thermococcus litoralis*. Vent DNA polymerase, steady state kinetics, thermal stability, processivity, strand displacement, and exonuclease activities. *Journal of Biological Chemistry*, 268(3):1965–1975.

Korschineck, I., Himmler, G., Sagl, R., Steinkellner, H., and Katinger, H. W. (1991). A PCR membrane spot assay for the detection of *Plum pox virus* RNA in bark of infected trees. *Journal of Virological Methods*, 31(2-3):139–145.

Kuan, C.-P., Wu, M.-T., Lu, Y.-L., and Huang, H.-C. (2010). Rapid detection of *Squash leaf curl virus* by loop-mediated isothermal amplification. *Journal of Virological Methods*, 169(1):61–65.

Kubo, T., Agoh, M., Mai, L. Q., Fukushima, K., Nishimura, H., Yamaguchi, A., Hirano, M., Yoshikawa, A., Hasebe, F., Kohno, S., and Morita, K. (2010). Development of reverse transcription-loop-mediated isothermal amplification assay for pandemic (H1N1) 2009 virus as a novel molecular based testing for pandemic influenza even in

resource limited settings. *Journal of Clinical Microbiology*, 48(3):728–735.

Kubota, R., Vine, B. G., Alvarez, A. M., and Jenkins, D. M. (2008). Detection of *Ralstonia solanacearum* by loop-mediated isothermal amplification. *Phytopathology*, 98(9):1045–1051.

Kuhn, H., Demidov, V. V., and Frank-Kamenetskii, M. D. (2002). Rolling-circle amplification under topological constraints. *Nucleic Acids Research*, 30(2):574–580.

Kuhn, H. and Frank-Kamenetskii, M. (2005). Template-independent ligation of single-stranded DNA by T4 DNA ligase. *FEBS Journal*, 272(23):5991–6000.

Kuhn, H., Hu, Y., Frank-Kamenetskii, M. D., and Demidov, V. V. (2003). Artificial site-specific DNA-nicking system based on common restriction enzyme assisted by PNA openers. *Biochemistry*, 42(17):4985–4992.

Kurn, N., Chen, P., Heath, J. D., Kopf-Sill, A., Stephens, K. M., and Wang, S. (2005). Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. *Chlinical Chemistry*, 51(10):1973–1981.

Kwoh, D. Y., Davis, G. R., Whitfield, K. M., Chappelle, H. L., DiMichele, L. J., and Gingeras, T. R. (1989). Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. *Proceedings of the National Academy of Sciences U S A*, 86(4):1173–1177.

Lage, J. M., Leamon, J. H., Pejovic, T., Hamann, S., Lacey, M., Dillon, D., Segraves, R., Vossbrinck, B., González, A., Pinkel, D., Albertson, D. G., Costa, J., and Lizardi, P. M. (2003). Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-cgh. *Genome Research*, 13(2):294–307.

Lagunavicius, A., Kiveryte, Z., Zimbaite-Ruskuliene, V., Radzvilavicius, T., and Janulaitis, A. (2008). Duality of polynucleotide substrates for Phi29 DNA polymerase: 3'->5' RNase activity of the enzyme. *RNA*, 14:503–513.

Lagunavicius, A., Merkiene, E., Kiveryte, Z., Savaneviciute, A., Zimbaite-Ruskuliene, V., Radzvilavicius, T., and Janulaitis, A. (2009). Novel application of Phi29 DNA polymerase: RNA detection and analysis *in vitro* and *in situ* by target RNA-primed RCA. *RNA*, 15(5):765–771.

Lain, S., Riechmann, J., and Garcia, J. (1989). The complete nucleotide sequence *of Plum pox potyvirus* RNA. *Virus Research*, 13(2):157–172.

Landegren, U., Kaiser, R., Sanders, J., and Hood, L. (1988). A ligase-mediated gene detection technique. *Science*, 241(4869):1077–1080.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23(21):2947–2948.

Larsson, C., Koch, J., Nygren, A., Janssen, G., Raap, A., Landegren, U., and Nilsson, M. (2004). *In situ* genotyping individual DNA molecules by target-primed rolling circle amplification of padlock probes. *Nature Methods*, 1(3):227–232.

Lasken, R. S. and Stockwell, T. B. (2007). Mechanism of chimera formation during the multiple displacement amplification reaction. *BMC Biotechnology*, 7:19.

Latorra, D., Arar, K., and Hurley, J. M. (2003a). Design considerations and effects of LNA in PCR primers. *Molecular and Cellular Probes*, 17(5):253–259.

Latorra, D., Campbell, K., Wolter, A., and Hurley, J. M. (2003b). Enhanced allele-specific PCR discrimination in SNP genotyping using 3' locked nucleic acid (LNA) primers. *Human Mutation*, 22(1):79–85.

Le, D. T., Netsu, O., Uehara-Ichiki, T., Shimizu, T., Choi, I.-R., Omura, T., and Sasaya, T. (2010). Molecular detection of nine rice viruses by a reverse-transcription loop-mediated isothermal amplification assay. *Journal of Virological Methods*, 170(1-2):90-93.

Lee, D., Mura, M. L., Allnutt, T., Powell, W., and Greenland, A. (2009). Isothermal amplification of genetically modified DNA sequences directly from plant tissues lowers the barriers to high-throughput and field-based genotyping. *Journal of Agricultural and Food Chemistry*, 57(20):9400–9402.

Lee, M.-S., Yang, M.-J., Hseu, Y.-C., Lai, G.-H., Chang, W.-T., Hsu, Y.-H., and Lin, M.-K. (2011). One-step reverse transcription loop-mediated isothermal amplification assay for rapid detection of *Cymbidium mosaic virus*. *Journal of Virological Methods*, 173(1):43–48.

Leone, G., van Schijndel, H., van Gemen, B., Kramer, F. R., and Schoen, C. D. (1998). Molecular beacon probes combined with amplification by NASBA

enable homogeneous, real-time detection of RNA. *Nucleic Acids Research*, 26(9):2150–2155.

Leontis, N. B., Kwok, W., and Newman, J. S. (1991). Stability and structure of three-way DNA junctions containing unpaired nucleotides. *Nucleic Acids Research*, 19(4):759–766.

Levy, L. and Hadidi, A. (1994). A simple and rapid method for processing tissue infected with *Plum pox potyvirus* for use with specific 3'non-coding region RT-PCR assays. *EPPO Bulletin*, 24(3):595–604.

Li, C., Li, Z., Jia, H., and Yan, J. (2011a). One-step ultrasensitive detection of microRNAs with loop-mediated isothermal amplification (LAMP). *Chemical Communications*, 47(9):2595–2597.

Li, J. and Zhong, W. (2007). Typing of multiple single-nucleotide polymorphisms by a microsphere-based rolling circle amplification assay. *Analytical Chemistry*, 79(23):9030–9038.

Li, N., Jablonowski, C., Jin, H., and Zhong, W. (2009a). Stand-alone rolling circle amplification combined with capillary electrophoresis for specific detection of small RNA. *Analytical Chemistry*, 81(12):4906–4913.

Li, S., Fang, M., Zhou, B., Ni, H., Shen, Q., Zhang, H., Han, Y., Yin, J., Chang, W., Xu, G., and Cao, G. (2011b). Simultaneous detection and differentiation of *dengue virus* serotypes 1-4, *Japanese encephalitis virus*, and *West nile virus* by a combined reverse-transcription loop-mediated isothermal amplification assay. *Virology Journal*, 8:360.

Li, T., Xie, Z., Wang, Y., Li, B., Zhang, Y., and An, L. (2008). A new combination of RNA-mediated DNA ligation and on-chip elongation for detecting viral RNA. *Diagnostic Microbiology and Infectious Disease*, 62(1):26-33

Li, X., Nie, J., Ward, L., Madani, M., Hsiang, T., Zhao, Y., and Boer, S. H. D. (2009b). Comparative genomics-guided loop-mediated isothermal amplification for characterization of *Pseudomonas syringae* pv. phaseolicola. *Journal of Applied Microbiology*, 107(3):717–726.

Liang, X., Jensen, K., and Frank-Kamenetskii, M. D. (2004). Very efficient template/primer-independent DNA synthesis by thermophilic DNA polymerase in the presence of a thermophilic restriction endonuclease. *Biochemistry*, 43(42):13459–13466.

Liu, D., Daubendiek, S., Zillman, M., Ryan, K., and Kool, E. (1996). Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerases. *Journal of the American Chemical Society*, 118(7):1587–1594.

Liu, M., Luo, Y., Tao, R., He, R., Jiang, K., Wang, B., and Wang, L. (2009a). Sensitive and rapid detection of genetic modified soybean (Roundup ready) by loop-mediated isothermal amplification. *Bioscience, Biotechnology and Biochemistry*, 73(11):2365–2369.

Liu, Y., Chuang, C.-K., and Chen, W.-J. (2009b). *In situ* reverse-transcription loop-mediated isothermal amplification (*in situ* RT-LAMP) for detection of Japanese encephalitis viral RNA in host cells. *Journal of Clinical Virology*, 46(1):49-54.

Liu, Y., Wang, Z., Qian, Y., Mu, J., Shen, L., Wang, F., and Yang, J. (2010). Rapid detection of *Tobacco mosaic virus* using the reverse transcription loop-mediated isothermal amplification method. *Archives of Virology*, 155(10):1681–1685.

Liu, Z., Teng, Y., Xie, X., Li, H., Lv, J., Gao, L., Tian, F., Jiang, Y., Chu, Z., Xie, C., and Liu, H. (2008). Development and evaluation of a one-step loop-mediated isothermal amplification for detection of spring viraemia of carp virus. *Journal of Applied Microbiology*, 105(4):1220–1226.

Lizardi, P., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D., and Ward, D. (1998). Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nature Genetics*, 19:225–232.

Lohmann, J. S., Stougaard, M., and Koch, J. (2007). A new enzymatic route for production of long 5'-phosphorylated oligonucleotides using suicide cassettes and rolling circle DNA synthesis. *BMC Biotechnology*, 7:49.

López-Moya, J., Valli, A., and Garcia, J. (2009). *Potyviridae*. *Encyclopedia of Life Sciences*.

Luo, J., Bergstrom, D., and Barany, F. (1996). Improving the fidelity of *Thermus thermophilus* DNA ligase. *Nucleic Acids Research*, 24(15):3071–3078.

Luo, L., Nie, K., Yang, M.-J., Wang, M., Li, J., Zhang, C., Liu, H.-T., and Ma, X.-J. (2011). Visual detection of high-risk human papillomavirus genotypes 16, 18, 45, 52, and 58 by loop-mediated isothermal amplification with hydroxynaphthol blue dye. *Journal of Clinical Microbiology*, 49(10):3545–3550.

Lutz, S., Weber, P., Focke, M., Faltin, B., Hoffmann, J., Müller, C., Mark, D., Roth, G., Munday, P., Armes, N., Piepenburg, O., Zengerle, R., and von Stetten, F. (2010). Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal

recombinase polymerase amplification (RPA). *Lab Chip*, 10(7):887–893.

Ma, X.-J., Shu, Y.-L., Nie, K., Qin, M., Wang, D.-Y., Gao, R.-B., Wang, M., Wen, L.-Y., Han, F., Zhou, S.-M., Zhao, X., Cheng, Y.-H., Li, D.-X., and Dong, X.-P. (2010). Visual detection of pandemic influenza a H1N1 virus 2009 by reverse-transcription loop-mediated isothermal amplification with hydroxynaphthol blue dye. *Journal of Virological Methods*, 167(2):214–217.

Maiss, E., Casper, R., Deborré, G., and Jelkmann, W. (1995). Complete nucleotide sequence of a *Plum pox potyvirus* isolate (PPV-SC) deriving from sour cherries and influence of a coat protein sequence motif on aphid transmission. *Acta Horticulturae*, 386:340–345.

Maiss, E., Timpe, U., Brisske, A., Jelkmann, W., Casper, R., Himmler, G., Mattanovich, D., and Katinger, H. W. D. (1989). The complete nucleotide sequence of *Plum pox virus* RNA. *Journal of General Virology*, 70(3):513–524.

Marciniak, J., Kummel, A., Esener, S., Heller, M., and Messmer, B. (2008). Coupled rolling circle amplification loop-mediated amplification for rapid detection of short DNA sequences. *Biotechniques*, 45(3):275–280.

Mekata, T., Sudhakaran, R., Kono, T., Supamattaya, K., Linh, N. T. H., Sakai, M., and Itami, T. (2008). Real-time quantitative loop-mediated isothermal amplification as a simple method for detecting *White spot syndrome virus*. *Letters in Applied Microbiology*, 48(1):25-32.

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. *Journal of Virological Methods*, 99(1-2):81–92.

Mikawa, T., Inoue, J., and Shigemori, Y. (2009). Single-stranded DNA binding protein facilitates specific enrichment of circular DNA molecules using rolling circle amplification. *Analytical Biochemistry*, 391:81–84.

Millard, P. J., Bickerstaff, L. E., LaPatra, S. E., and Kim, C. H. (2006). Detection of infectious *Haematopoietic necrosis virus* and infectious *Salmon anaemia virus* by molecular padlock amplification. *Journal of Fish Diseases*, 29(4):201–213.

Mitani, Y., Lezhava, A., Kawai, Y., Kikuchi, T., Oguchi-Katayama, A., Kogo, Y., Itoh, M., Miyagi, T., Takakura, H., Hoshi, K., Kato, C., Arakawa, T., Shibata, K., Fukui, K., Masui, R., Kuramitsu, S., Kiyotani, K., Chalk, A., Tsunekawa, K., Murakami, M., Kamataki, T., Oka, T., Shimada, H., Cizdziel, P. E., and Hayashizaki, Y. (2007). Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. *Nature Methods*, 4(3):257–262.

Mizuta, R., Mizuta, M., and Kitamura, D. (2003). Atomic force microscopy analysis of rolling circle amplification of plasmid DNA. *Archives of Histology and Cytology*, 66(2):175–181.

Mori, Y., Hirano, T., and Notomi, T. (2006). Sequence specific visual detection of LAMP reactions by addition of cationic polymers. *BMC Biotechnology*, 6:3.

Mori, Y., Kitao, M., Tomita, N., and Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, 59(2):145–157.

Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications*, 289(1):150–154.

Mori, Y. and Notomi, T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy*, 15(2):62–69.

Morvan, G. and Castelain, C. (1976). Localisation of Sharka virus in peach trees in reference to sanitary checking. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft*, 170:65–72.

Motré, A., Li, Y., and Kong, H. (2008). Enhancing helicase-dependent amplification by fusing the helicase with the DNA polymerase. *Gene*, 420(1):17–22.

Mullis, K. B. and Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 155:335–350.

Murakami, T., Sumaoka, J., and Komiyama, M. (2009). Sensitive isothermal detection of nucleic-acid sequence by primer generation-rolling circle amplification. *Nucleic Acids Research*, 37(3):e19.

Murakami, T., Sumaoka, J., and Komiyama, M. (2012). Sensitive RNA detection by combining three-way junction formation and primer generation-rolling circle amplification. *Nucleic Acids Research* 40(3):e22.

Myrta, A., Potere, O., Crescenzi, A., Nuzzaci, M., and Boscia, D. (2000). Production of two monoclonal antibodies specific to cherry strain of *Plum pox virus* (PPV-C). *Journal of Plant Pathology*, 82:95–103.

Myrta, A., Terlizzi, B. D., Boscia, D., Caglayan, K., Gavriel, I., Ghanem, G., Varveri, C., and Savino, V. (1998). Detection and serotyping of mediterranean *Plum pox virus* isolates by means of strain-specific monoclonal antibodies. *Acta Virologica*, 42(4):251–253.

Myrta, A., Varga, A., and James, D. (2006). The complete genome sequence of an El Amar isolate of *Plum pox virus* (PPV) and its phylogenetic relationship to other PPV strains. *Archives of Virology*, 151:1189–1198.

Nagai, M., Yoshida, A., and Sato, N. (1998). Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. *Biochemistry & Molecular Biology International*, 44(1):157–163.

Nagamine, K., Hase, T., and Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, 16(3):223–229.

Najafzadeh, M. J., Sun, J., Vicente, V. A., and de Hoog, G. S. (2011). Rapid identification of fungal pathogens by rolling circle amplification using *Fonsecaea* as a model. *Mycoses*, 54(5):e577–e582.

Nakahara, K., Hataya, T., and Uyeda, I. (1999). A simple, rapid method of nucleic acid extraction without tissue homogenization for detecting viroids by hybridization and RT-PCR. *Journal of Virological Methods*, 77(1):47–58.

Nallur, G., Luo, C., Fang, L., Cooley, S., Dave, V., Lambert, J., Kukanskis, K., Kingsmore, S., Lasken, R., and Schweitzer, B. (2001). Signal amplification by rolling circle amplification on DNA microarrays. *Nucleic Acids Research*, 29(23):e118.

Nazarenko, I. A., Bhatnagar, S. K., and Hohman, R. J. (1997). A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Research*, 25(12):2516–2521.

Nelson, J. R., Cai, Y. C., Giesler, T. L., Farchaus, J. W., Sundaram, S. T., Ortiz-Rivera, M., Hosta, L. P., Hewitt, P. L., Mamone, J. A., Palaniappan, C., and Fuller, C. W. (2002). TempliPhi, Phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. *Biotechniques*, 32:S44–47.

Nemchinov, L. and Hadidi, A. (1996). Characterization of the sour cherry strain of *Plum pox virus*. *Phytopathology*, 86(6):575–580.

Nemchinov, L. and Hadidi, A. (1998). Specific oligonucleotide primers for the direct detection of *Plum pox potyvirus*-cherry subgroup. *Journal of Virological Methods*, 70(2):231–234.

Nemchinov, L., Hadidi, A., Maiss, E., Cambra, M., Candresse, T., and Damsteegt, V. (1996). Sour cherry strain of *Plum pox potyvirus* (PPV): Molecular and serological evidence for a new subgroup of PPV strain. *Phytopathology*, 86:1215–1221.

Nemeth, M. (1994). History and importance of Plum pox in stone-fruit production. *EPPO Bulletin*, 24:525–536.

Nemoto, M., Imagawa, H., Tsujimura, K., Yamanaka, T., Kondo, T., and Matsumura, T. (2010). Detection of *equine rotavirus* by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Journal of Veterinary Medical Science*, 72(6):823-826.

Neumüller, M. (2005). *Die Hypersensibilität der Europäischen Pflaume (Prunus domestica L.) gegenüber dem Scharkavirus (Plum pox virus)*. PhD thesis, Universität Hohenheim.

Nie, K., Zhang, Y., Luo, L., Yang, M.-J., Hu, X.-M., Wang, M., Zhu, S.-L., Han, F., Xu, W.-B., and Ma, X.-J. (2011). Visual detection of human enterovirus 71 subgenotype C4 and coxsackievirus a16 by reverse transcription loop-mediated isothermal amplification with the hydroxynaphthol blue dye. *Journal of Virological Methods*, 175(2):283–286.

Nie, X. (2005). Reverse transcription loop-mediated isothermal amplification of DNA for detection of *Potato virus Y*. *Plant Disease*, 89(6):605–610.

Niessen, L. and Vogel, R. F. (2010). Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology*, 140(2-3):183–191.

Niesters, H. G. (2001). Quantitation of viral load using real-time amplification techniques. *Methods*, 25(4):419–429.

Nilsson, M. Barbany, G., Antson, D., Gertow, K., and Landegren, U. (2000). Enhanced detection and distinction of RNA by enzymatic probe ligation. *Nature Biotechnology*, 18:791–793.

Nilsson, M., Antson, D.-O., Barbany, G., and Landegren, U. (2001). RNA-templated DNA ligation for

transcript analysis. *Nucleic Acids Research*, 29(2):578–581.

Nilsson, M., Krejci, K., Koch, J., Kwiatkowski, M., Gustavsson, P., and Landegren, U. (1997). Padlock probes reveal single-nucleotide differences, parent of origin and *in situ* distribution of centromeric sequences in human chromosomes 13 and 21. *Nature Genetics*, 16:252–255.

Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B., and Landegren, U. (1994). Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science*, 265(5181):2085–2088.

Nimitphak, T., Kiatpathomchai, W., and Flegel, T. W. (2008). Shrimp hepatopancreatic parvovirus detection by combining loop-mediated isothermal amplification with a lateral flow dipstick. *Journal of Virological Methods*, 154:56–60.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12):e63.

Novick, R. P. (1998). Contrasting lifestyles of rolling-circle phages and plasmids. *Trends in Biochemical Sciences*, 23(11):434–438.

Obura, E., Masiga, D., Wachira, F., Gurja, B., and Khan, Z. R. (2011). Detection of phytoplasma by loop-mediated isothermal amplification of DNA (LAMP). *Journal of Microbiological Methods*, 84(2):312–316.

Ogata, N. and Miura, T. (1997). Genetic information 'created' by archaebacterial DNA polymerase. *Biochemical Journal*, 324 (Pt 2):667–671.

Olmos, A., Bertolini, E., and Cambra, M. (2002). Simultaneous and co-operational amplification (co-PCR): a new concept for detection of plant viruses. *Journal of Virological Methods*, 106(1):51–59.

Olmos, A., Bertolini, E., and Cambra, M. (2007). Isothermal amplification coupled with rapid flow-through hybridisation for sensitive diagnosis of *Plum pox virus*. *Journal of Virological Methods*, 139(1):111–115.

Olmos, A., Bertolini, E., Gil, M., and Cambra, M. (2004). Real-time RT-PCR for quantitative detection of *Plum pox virus*. *Acta Horticulturae*, 657:149–153.

Olmos, A., Bertolini, E., Gil, M., and Cambra, M. (2005). Real-time assay for quantitative detection of non-persistently transmitted *Plum pox virus* RNA targets in single aphids. *Journal of Virological Methods*, 128(1-2):151–155.

Olmos, A., Cambra, M., Dasi, M., Candresse, T., Esteban, O., Gorris, M., and Asensio, M. (1997). Simultaneous detection and typing of *Plum pox potyvirus* (PPV) isolates by heminested-PCR and PCR-ELISA. *Journal of Virological Methods*, 68(2):127–137.

Owor, B. E., Shepherd, D. N., Taylor, N. J., Edema, R., Monjane, A. L., Thomson, J. A., Martin, D. P., and Varsani, A. (2007). Successful application of FTA classic card technology and use of bacteriophage Phi29 DNA polymerase for large-scale field sampling and cloning of complete *Maize streak virus* genomes. *Journal of Virological Methods*, 140(1-2):100–105.

Palkovics, L., Burgyán, J., and Balázs, E. (1993). Comparative sequence analysis of four complete primary structures of *Plum pox virus* strains. *Virus Genes*, 7(4):339–347.

Palkovics, L., Burgyán, J., and Balázs, E. (1994). Sensitive non-radioactive nucleic acid hybridization assay for *Plum pox virus* detection. *Research in Virology*, 145(6):387–392.

Parida, M., Posadas, G., Inoue, S., Hasebe, F., and Morita, K. (2004). Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of *West nile virus*. *Journal of Clinical Microbiology*, 42(1):257–263.

Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V. L., and Morita, K. (2008). Loop-mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, 18(6):407–421.

Pasquini, G., Barba, M., Hadidi, A., Faggioli, F., Negri, R., Sobol, I., Tiberini, A., Caglayan, K., Mazyad, H., Anfoka, G., Ghanim, M., Zeidan, M., and Czosnek, H. (2008). Oligonucleotide microarray-based detection and genotyping of *Plum pox virus*. *Journal of Virological Methods*, 147(1):118–126.

Perera, N., Aonuma, H., Yoshimura, A., Teramoto, T., Iseki, H., Nelson, B., Igarashi, I., Yagi, T., Fukumoto, S., and Kanuka, H. (2008). Rapid identification of virus-carrying mosquitoes using reverse transcription-loop-mediated isothermal amplification. *Journal of Virological Methods*, 156(1-2):32-36.

Pickering, J., Bamford, A., Godbole, V., Briggs, J., Scozzafava, G., Roe, P., Wheeler, C., Ghouze, F., and Cuss, S. (2002). Integration of DNA ligation

and rolling circle amplification for the homogeneous, end-point detection of single nucleotide polymorphisms. *Nucleic Acids Research*, 30(12):e60.

Piepenburg, O., Williams, C. H., Stemple, D. L., and Armes, N. A. (2006). DNA detection using recombination proteins. *PLoS Biology*, 4(7):e204.

Qi, X., Bakht, S., Devos, K. M., Gale, M. D., and Osbourn, A. (2001). L-RCA (ligation-rolling circle amplification): a general method for genotyping of single nucleotide polymorphisms (SNPs). *Nucleic Acids Research*, 29(22):e116.

Rees, W. A., Yager, T. D., Korte, J., and von Hippel, P. H. (1993). Betaine can eliminate the base pair composition dependence of DNA melting. *Biochemistry*, 32(1):137–144.

Rigano, L. A., Marano, M. R., Castagnaro, A. P., Amaral, A. M. D., and Vojnov, A. A. (2010). Rapid and sensitive detection of Citrus bacterial canker by loop-mediated isothermal amplification combined with simple visual evaluation methods. *BMC Microbiology*, 10:176.

Roux, K. H. (2009). Optimization and troubleshooting in PCR. *Cold Spring Harbor Protocols*, 4(4):pdb.ip66.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230(4732):1350–1354.

Saitou, Y., Kobayashi, Y., Hirano, S., Mochizuki, N., Itou, T., Ito, F. H., and Sakai, T. (2010). A method for simultaneous detection and identification of Brazilian dog- and vampire bat-related rabies virus by reverse transcription loop-mediated isothermal amplification assay. *Journal of Virological Methods*, 168(1-2):13-17.

Saleh, M. (2009). *Entwicklung von Testsystemen auf der Basis der "Loop-mediated Isothermal Amplification (LAMP)" Methode zum Nachweis von Yersinia ruckeri, dem Erreger der Rotmaulseuche (ERM) und von Renibacterium salmoninarum, dem Erreger der bakteriellen Nierenkrankheit (BKD) der Salmoniden.* PhD thesis, Ludwig-Maximilians-Universität München.

Salvador, B., Delgadillo, M. O., Sáenz, P., García, J. A., and Simón-Mateo, C. (2008). Identification of *Plum pox virus* pathogenicity determinants in herbaceous and woody hosts. *Molecular Plant-Microbe Interactions*, 21(1):20–29.

Salvador, B., Garcia, J. A., and Simon-Mateo, C. (2006). Causal agent of Sharka disease: *Plum pox virus* genome and function of gene products. *EPPO Bulletin*, 36(2):229–238.

Santoro, M. M., Liu, Y., Khan, S. M., Hou, L. X., and Bolen, D. W. (1992). Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochemistry*, 31(23):5278–5283.

Schneider, W. L., Sherman, D. J., Stone, A. L., Damsteegt, V. D., and Frederick, R. D. (2004). Specific detection and quantification of *Plum pox virus* by real-time fluorescent reverse transcription-PCR. *Journal of Virological Methods*, 120(1):97–105.

Scholthof, K.-B. G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C., and Foster, G. D. (2011). Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology*, 12(9):938–954.

Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I., Zhou, Z., Tchernev, V. T., Christiansen, J., Velleca, M., and Kingsmore, S. F. (2002). Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nature Biotechnology*, 20(4):359–365.

Schweitzer, B., Wiltshire, S., Lambert, J., O'Malley, S., Kukanskis, K., Zhu, Z., Kingsmore, S. F., Lizardi, P. M., and Ward, D. C. (2000). Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. *Proceedings of the National Academy of Sciences U S A*, 97(18):10113–10119.

Serçe, C. U., Candresse, T., Svanella-Dumas, L., Krizbai, L., Gazel, M., and Çaglayan, K. (2009). Further characterization of a new recombinant group of *Plum pox virus* isolates, PPV-T, found in orchards in the Ankara province of Turkey. *Virus Research*, 142(1-2):121–126.

Singh, S., Koshkin, A., Wengel, J., and Nielsen, P. (1998). LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. *Chemical Communications*, 4:455–456.

Sánchez-Navarro, J., Aparicio, F., Herranz, M., Minafra, A., Myrta, A., and Pallás, V. (2005). Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. *European Journal of Plant Pathology*, 111(1):77–84.

Spargo, C. A., Fraiser, M. S., Cleve, M. V., Wright, D. J., Nycz, C. M., Spears, P. A., and Walker, G. T. (1996). Detection of *M. tuberculosis* DNA using

thermophilic strand displacement amplification. *Molecular and Cellular Probes*, 10(4):247–256.

Spiess, A.-N., Mueller, N., and Ivell, R. (2004). Trehalose is a potent PCR enhancer: lowering of DNA melting temperature and thermal stabilization of *Taq* polymerase by the disaccharide trehalose. *Clinical Chemistry*, 50(7):1256–1259.

Steain, M. C., Dwyer, D. E., Hurt, A. C., Kol, C., Saksena, N. K., Cunningham, A. L., and Wang, B. (2009). Detection of influenza a H1N1 and H3N2 mutations conferring resistance to oseltamivir using rolling circle amplification. *Antiviral Research*, 84(3):242-248.

Stougaard, M., Lohmann, J. S., Zajac, M., Hamilton-Dutoit, S., and Koch, J. (2007). In situ detection of non-polyadenylated RNA molecules using turtle probes and target primed rolling circle prins. *BMC Biotechnology*, 7:69.

Stump, M. D., Cherry, J. L., and Weiss, R. B. (1999). The use of modified primers to eliminate cycle sequencing artifacts. *Nucleic Acids Research*, 27(23):4642–4648.

Sun, Z.-F., Hu, C.-Q., Ren, C.-H., and Shen, Q. (2006). Sensitive and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimps by loop-mediated isothermal amplification. *Journal of Virological Methods*, 131(1):41–46.

Szemes, M., Bonants, P., de Weerdt, M., Baner, J., Landegren, U., and Schoen, C. (2005). Diagnostic application of padlock probes–multiplex detection of plant pathogens using universal microarrays. *Nucleic Acids Research*, 33(8):e70.

Szemes, M., Kálmán, M., Myrta, A., Boscia, D., Németh, M., Kölber, M., and Dorgai, L. (2001). Integrated RT–PCR:nested PCR diagnosis for differentiating between subgroups of *Plum pox virus*. *Journal of Virological Methods*, 92:165–175.

Takahashi, H., Matsumoto, A., Sugiyama, S., and Kobori, T. (2010). Direct detection of green fluorescent protein messenger RNA expressed in *Escherichia coli* by rolling circle amplification. *Analytical Biochemistry*, 401(2):242–249.

Takahashi, H., Yamamoto, K., Ohtani, T., and Sugiyama, S. (2009). Cell-free cloning using multiply-primed rolling circle amplification with modified RNA primers. *Biotechniques*, 47(1):609–615.

Tan, E., Erwin, B., Dames, S., Ferguson, T., Buechel, M., Irvine, B., Voelkerding, K., and Niemz, A. (2008). Specific versus nonspecific isothermal DNA amplification through thermophilic polymerase and nicking enzyme activities. *Biochemistry*, 47(38):9987–9999.

Tang, H., Yang, X., Wang, K., Tan, W., Li, H., He, L., and Liu, B. (2008). RNA-templated single-base mutation detection based on T4 DNA ligase and reverse molecular beacon. *Talanta*, 75(5):1388–1393.

Teycheney, P., Tavert, G., Delbos, R., Ravelonandro, M., and Dunez, J. (1989). The complete nucleotide sequence of *Plum pox virus* RNA (strain D). *Nucleic Acids Research*, 17(23):10115–10116.

Thekisoe, O. M. M., Bazie, R. S. B., Coronel-Servian, A. M., Sugimoto, C., Kawazu, S.-I., and Inoue, N. (2009). Stability of loop-mediated isothermal amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. *Journal of Veterinary Medical Science*, 71(4):471–475.

Thomas, D., Nardone, G., and Randall, S. (1999). Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction. *Archives of Pathology and Laboratory Medicine*, 123(12):1170–1176.

Tomita, N., Mori, Y., Kanda, H., and Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, 3(5):877–882.

Tomlinson, J., Boonham, N., and Dickinson, M. (2010a). Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathology*, 59(3):465–471.

Tomlinson, J. A., Barker, I., and Boonham, N. (2007). Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. *Applied and Environmental Microbiology*, 73(12):4040–4047.

Tomlinson, J. A., Dickinson, M., Hobden, E., Robinson, S., Giltrap, P. M., and Boonham, N. (2010b). A five-minute DNA extraction method for expedited detection of *Phytophthora ramorum* following prescreening using *Phytophthora spp.* lateral flow devices. *Journal of Microbiological Methods*, 81(2):116–120.

Tomlinson, J. A., Dickinson, M. J., and Boonham, N. (2010c). Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. *Letters in Applied Microbiology*, 51(6):650–657.

Tomlinson, J. A., Dickinson, M. J., and Boonham, N. (2010d). Rapid detection of *Phytophthora*

ramorum and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology*, 100(2):143–149.

Tong, Z., Kong, F., Wang, B., Zeng, X., and Gilbert, G. L. (2007). A practical method for subtyping of *Streptococcus agalactiae* serotype III, of human origin, using rolling circle amplification. *Journal of Microbiological Methods*, 70(1):39–44.

Tsutsumi, N., Yanagisawa, H., Fujiwara, Y., and Ohara, T. (2010). Detection of *Potato spindle tuber viroid* by reverse transcription loop-mediated isothermal amplification. *Research Bulletin of the Plant Protection Service Japan*, 46:61–67.

Tyagi, S. and Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology*, 14(3):303–308.

Šubr, Z. and Glasa, M. (2008). *Plum pox virus* variability detected by the advanced analytical methods. *Acta Virologica*, 52(2):75–89.

Šubr, Z., Pittnerova, S., and M., G. (2004). A simplified RT-PCR-based detection of recombinant *Plum pox virus* isolates. *Acta Virologica*, 48(3):173–176.

Šubr, Z. W., Kamencayová, M., Nováková, S., Nagyová, A., Nosek, J., and Glasa, M. (2010). A single amino acid mutation alters the capsid protein electrophoretic double-band phenotype of the *Plum pox virus* strain PPV-Rec. *Archives of Virology*, 155:1151–1155.

Varadaraj, K. and Skinner, D. M. (1994). Denaturants or cosolvents improve the specificity of PCR amplification of a G + C-rich DNA using genetically engineered DNA polymerases. *Gene*, 140(1):1–5.

Varani, G. (1995). Exceptionally stable nucleic acid hairpins. *Annual Review of Biophysics & Biomolecular Structure* , 24:379–404.

Varga, A. and James, D. (2005). Detection and differentiation of *Plum pox virus* using real-time multiplex PCR with SYBR green and melting curve analysis: a rapid method for strain typing. *Journal of Virological Methods*, 123(2):213–220.

Varga, A. and James, D. (2006a). Real-time RT-PCR and SYBR green I melting curve analysis for the identification of *Plum pox virus* strains C, EA, and W: Effect of amplicon size, melt rate, and dye translocation. *Journal of Virological Methods*, 1-2:146–153.

Varga, A. and James, D. (2006b). Use of reverse transcription loop-mediated isothermal amplification for the detection of *Plum pox virus*. *Journal of Virological Methods*, 138:184–190.

Varveri, C., Candresse, T., Cugusi, M., Ravelonandro, M., and Dunez, J. (1988). Use of a ^{32}P-labeled transcribed RNA probe for dot hybridization detection of *Plum pox virus*. *Phytopathology*, (10):1280–1283.

Varveri, C., Ravelonandro, M., and Dunez, J. (1987). Construction and use of a cloned cDNA probe for the detection of *Plum pox virus* in plants. *Phytopathology*, 77:1221–1224.

Viguera, E., Canceill, D., and Ehrlich, S. D. (2001). In vitro replication slippage by DNA polymerases from thermophilic organisms. *Journal of Molecular Biology*, 312(2):323–333.

Vincent, M., Xu, Y., and Kong, H. (2004). Helicase-dependent isothermal DNA amplification. *EMBO Reports*, 5(8):795–800.

Walker, G. T., Fraiser, M. S., Schram, J. L., Little, M. C., Nadeau, J. G., and Malinowski, D. P. (1992a). Strand displacement amplification–an isothermal, *in vitro* DNA amplification technique. *Nucleic Acids Research*, 20(7):1691–1696.

Walker, G. T., Little, M. C., Nadeau, J. G., and Shank, D. D. (1992b). Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proceedings of the National Academy of Sciences U S A*, 89(1):392–396.

Wang, B., Dwyer, D. E., Blyth, C. C., Soedjono, M., Shi, H., Kesson, A., Ratnamohan, M., McPhie, K., Cunningham, A. L., and Saksena, N. K. (2010). Detection of the rapid emergence of the H275Y mutation associated with oseltamivir resistance in severe pandemic influenza virus A/H1N1 09 infections. *Antiviral Research*, 87(1):16–21.

Wang, B., Dwyer, D. E., Chew, C. B., Kol, C., He, Z. P., Joshi, H., Stein, M. C., Cunningham, A. L., and Saksena, N. K. (2009). Sensitive detection of the K103N non-nucleoside reverse transcriptase inhibitor resistance mutation in treatment-naïve HIV-1 infected individuals by rolling circle amplification. *Journal of Virological Methods*, 161(1):128–135.

Wang, B., Potter, S. J., Lin, Y., Cunningham, A. L., Dwyer, D. E., Su, Y., Ma, X., Hou, Y., and Saksena, N. K. (2005). Rapid and sensitive detection of severe acute respiratory syndrome coronavirus by rolling circle amplification. *Journal of Clinical Microbiology*, 43(5):2339–2344.

Wang, X., Zhu, J.-P., Zhang, Q., Xu, Z.-G., Zhang, F., Zhao, Z.-H., Zheng, W.-Z., and Zheng, L.-S. (2011).

Detection of enterovirus 71 using reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Journal of Virological Methods*, 179(2):330-334.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., and Dunez, J. (1992). A highly sensitive immunocapture polymerase chain reaction method for *Plum pox potyvirus* detection. *Journal of Virological Methods*, 39:21–37.

Wetzel, T., Candresse, T., Ravelonandro, M., Delbos, R. P., Mazyad, H., Aboul-Ata, A. E., and Dunez, J. (1991a). Nucleotide sequence of the 3'-terminal region of the RNA of the E1 Amar strain of *Plum pox potyvirus*. *Journal of General Virology*, 72(7):1741–1746.

Wetzel, T., Candresse, T., Ravelonandro, M., and Dunez, J. (1991b). A polymerase chain reaction assay adapted to *Plum pox potyvirus* detection. *Journal of Virological Methods*, 33:355–365.

Wetzel, T., Tavert, G., Teycheney, P. Y., Ravelonandro, M., Candresse, T., and Dunez, J. (1990). Dot hybridization detection of *Plum pox virus* using ^{32}P-labeled RNA probes representing non-structural viral protein genes. *Journal of Virological Methods*, 30(2):161–171.

Wharam, S. D., Marsh, P., Lloyd, J. S., Ray, T. D., Mock, G. A., Assenberg, R., McPhee, J. E., Brown, P., Weston, A., and Cardy, D. L. (2001). Specific detection of DNA and RNA targets using a novel isothermal nucleic acid amplification assay based on the formation of a three-way junction structure. *Nucleic Acids Research*, 29(11):e54.

Yin, S., Shang, Y., Zhou, G., Tian, H., Liu, Y., Cai, X., and Liu, X. (2010). Development and evaluation of rapid detection of classical *Swine fever virus* by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Journal of Biotechnology*, 146(4):147–150.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I., and Tsukamoto, T. (2007). Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. *Journal of Medical Virology*, 79(3):326–334.

Zaphiropoulos, P. G. (1998). Non-homologous recombination mediated by *Thermus aquaticus* DNA polymerase I. evidence supporting a copy choice mechanism. *Nucleic Acids Research*, 26(12):2843–2848.

Zhang, D., Brandwein, M., Hsuih, T., and Li, H. (1998). Amplification of target-specific, ligation-dependent circular probe. *Gene*, 211:277–285.

Zhang, D., Brandwein, M., Hsuih, T., and Li, H. (2001a). Ramification amplification: A novel isothermal DNA amplification method. *Molecular Diagnosis*, 6(2):141–150.

Zhang, D., Zhang, W., Li, X., and Konomi, Y. (2001b). Detection of rare DNA targets by isothermal ramification amplification. *Gene*, 1(2):209–216.

Zhang, S., Wu, Z., Shen, G., and Yu, R. (2009). A label-free strategy for SNP detection with high fidelity and sensitivity based on ligation-rolling circle amplification and intercalating of methylene blue. *Biosensors and Bioelectronics*, 24(11):3201–3207.

Zhang, Z.-P., Zhang, Y., Liu, J.-P., Zhang, J.-T., An, Z.-X., Quan, F.-S., Zhang, L., Cai, X., and Pu, S. W. J. (2008). Codeposition of dNTPs detection for rapid LAMP-based sexing of bovine embryos. *Reproduction in Domestic Animals*, 44(1):116–121.

Zhao, K., Liu, Y., and Wang, X. (2010). Reverse transcription loop-mediated isothermal amplification of DNA for detection of *Barley yellow dwarf viruses* in China. *Journal of Virological Methods*, 169(1):211–214.

Zyrina, N. V., Zheleznaya, L. A., Dvoretsky, E. V., Vasiliev, V. D., Chernov, A., and Matvienko, N. I. (2007). N.BspD6I DNA nickase strongly stimulates template-independent synthesis of non-palindromic repetitive DNA by *Bst* DNA polymerase. *Biological Chemistry*, 388(4):367–372.

Publications emerged from this work

Symposium contributions:

*XI*th *International Symposium on Plum and Prune Genetics, Breeding and Pomology*, Palermo (Italy), March 16-19, 2008:
Establishing of a PPV isolate collection as a prerequisite for an effective selection process in breeding plums for durable resistance against Sharka.

*XXI*st *International Conference on Virus other Graft Transmissible Diseases of Fruit Crops*, Neustadt/Weinstraße (Germany), July 5-10, 2009:
Preliminary studies on the use of the cascade rolling circle amplification technique for *Plum pox virus* detection.

*II*nd *EUFRIN Plum and Prune Working Group Meeting on Present Constraints of Plum Growing in Europe*, Craiova (Romania), July 20-22, 2010:
Blue LAMP: A fast and reliable PPV detection system.

*X*th *International Symposium on Plum and Prune Genetics, Breeding and Pomology*, Davis (USA), May 20-25, 2012:
Blue LAMP supports the selection of *Prunus domestica* genotypes with hypersensitivity resistance to the *Plum pox virus.*

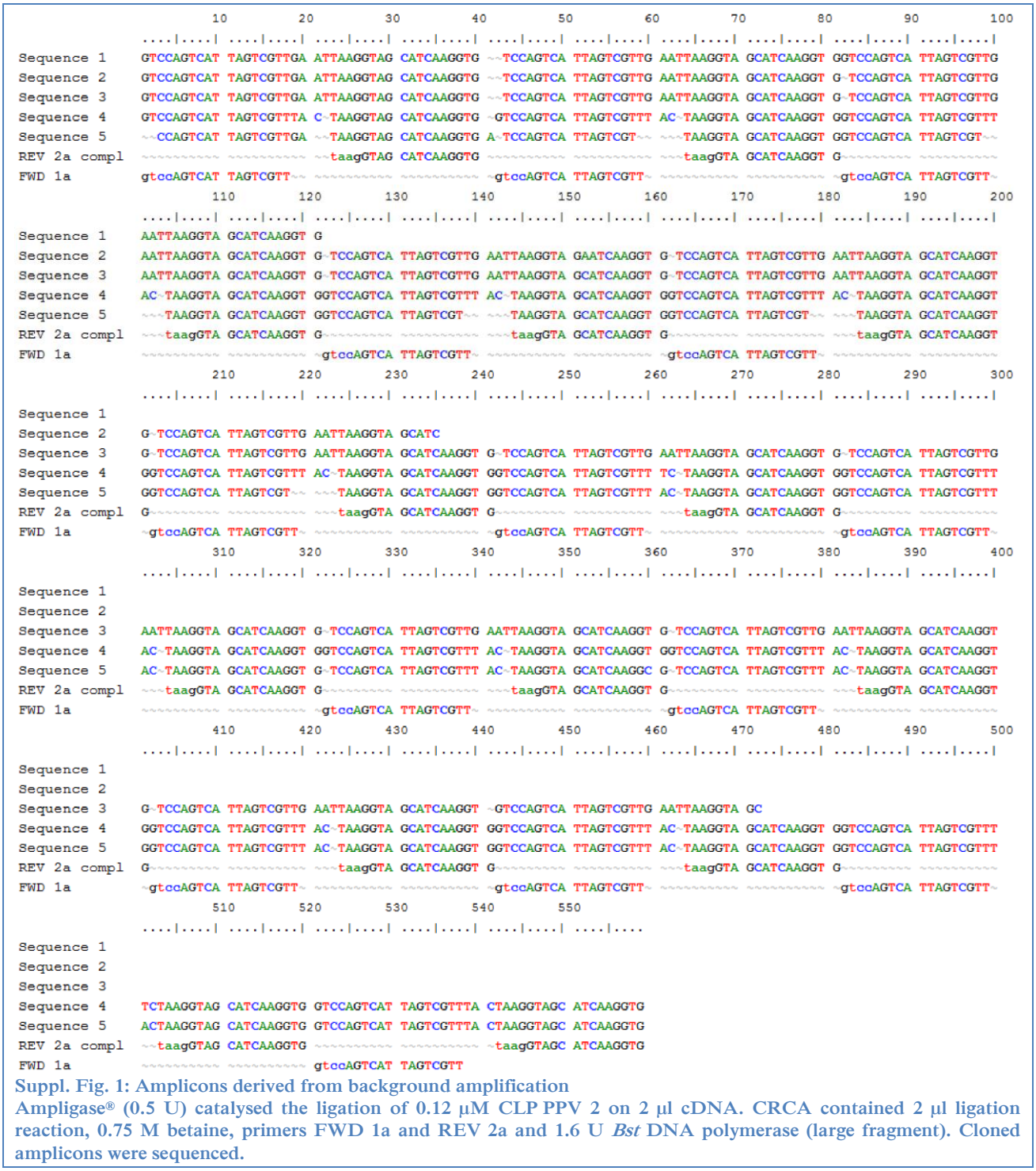
Journal article:

Hadersdorfer, J., Neumüller, M., Treutter, D., Fischer, T.C. (2011). Fast and reliable detection of *Plum pox virus* in woody host plants using the Blue LAMP protocol. *Annals of Applied Biology*, 159:456-466

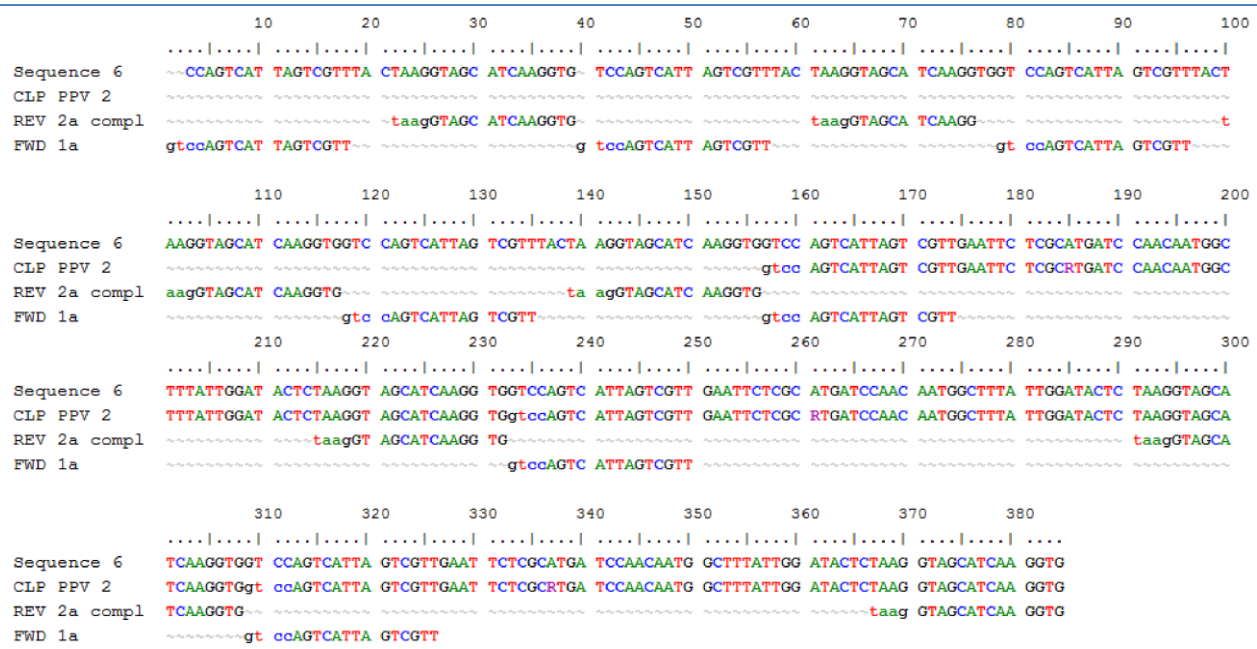
Supplementary information

Suppl. Tab. 1: Isolates of the Weihenstephan PPV collection assigned to the PPV subgroups
RNA was extracted from the samples derived from isolate collection and applied to cDNA synthesis followed by PCR. C - RT-PCR according to Wetzel et al. (1991b), D - RT-PCR according to Szemes et al. (2001), E - RT-PCR according to Šubr et al. (2004)

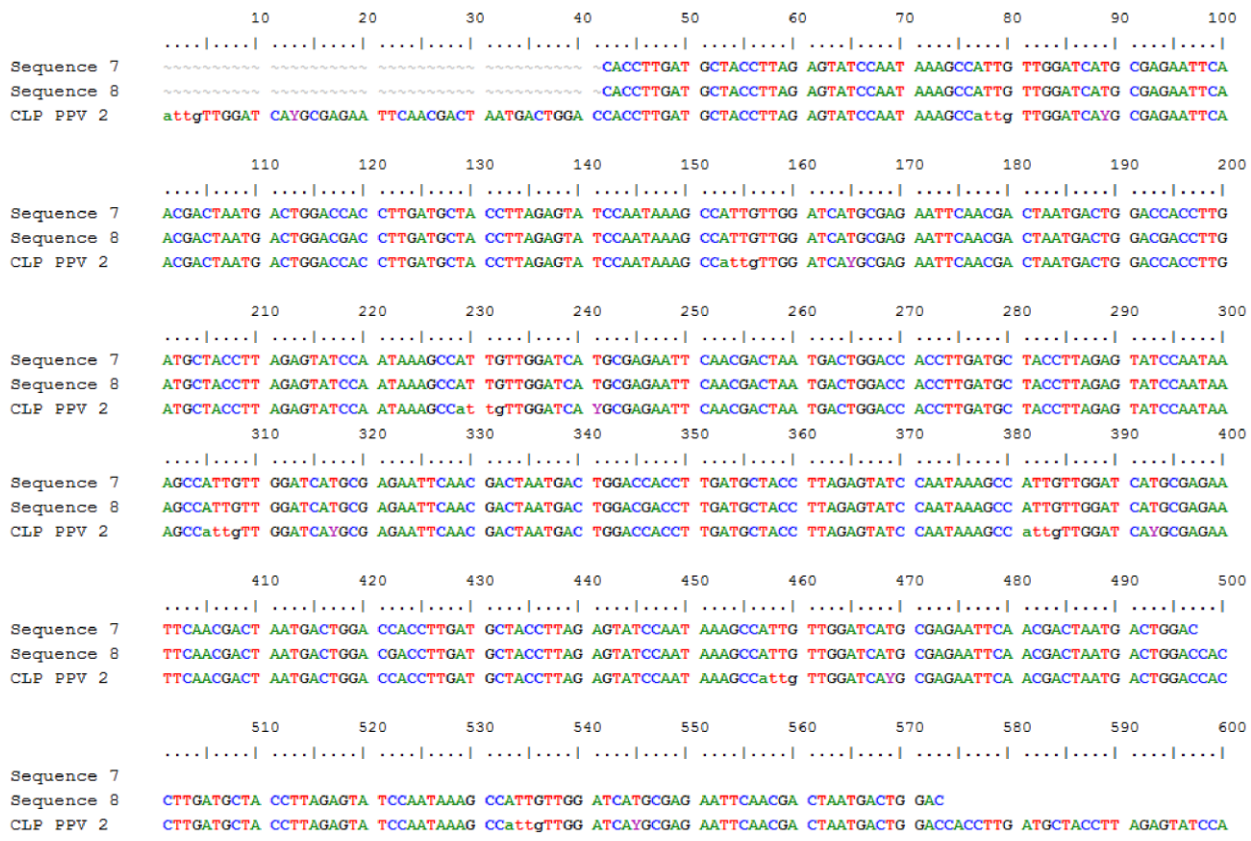
Isolate	Origin	C	D						E	
		PPV	PPV	D	M	C	EA	W	M	Rec
PPV 1	Ortenberg, Germany	+	+	+	-	-	-	-	-	-
PPV 2	Aschersleben, Germany	+	+	-	+	-	-	-	-	+
PPV 3		+	+	-	+	-	-	-	-	+
PPV 4	Cech Republik	+	-	-	+	-	-	-	+	-
PPV 5	Romania	+	+	+	-	-	-	-	-	-
PPV 6	Cech Republik	+	+	-	+	-	-	-	-	+
PPV 7	Cacak, Serbia	+	-	+	-	-	-	-	-	-
PPV 8	Cacak, Serbia	+	+	-	+	-	-	-	+	-
PPV 9	Skierniewice, Poland	+	+	+	-	-	-	-	-	-
PPV 10	Cech Republik	+	+	+	-	-	-	-	-	-
PPV 11	El Amar, Egypt	+	+	-	-	-	+	-	-	-
PPV 12	Canada	+	-	+	+	-	-	+	-	-
PPV 13	Slovakia	+	+	+	-	-	-	-	-	-
PPV 14	Aschersleben, Germany	+	+	-	+	-	-	-	+	-
PPV 15	Linkenheim/Hochstetten, Germany	+	-	-	+	-	-	-	?	?
PPV 16		+	+	+	-	-	-	-	-	-
PPV 17	Ortenberg, Germany	+	+	+	-	-	-	-	-	-
PPV 18	Ortenberg, Germany	+	+	?	?	-	-	-	?	?
PPV 19	Marbach, Germany	+	+	?	?	-	-	-	?	?
PPV 20	Aschersleben, Germany	+	-	+	-	-	-	-	-	-
PPV 21		+	-	+	-	-	-	-	-	-
PPV 22	Herrenberg, Germany	+	+	+	-	-	-	-	-	-
PPV 23	Marbach, Germany	+	-	+	-	-	-	-	-	-
PPV 24	Obersasbach, Germany	+	+	+	-	-	-	-	-	-
PPV 25	Schriesheim, Germany	+	+	+	-	-	-	-	-	-
PPV 26	Bötzingen, Germany	+	+	+	-	-	-	-	-	-
PPV 27		+	-	+	-	-	-	-	-	-
PPV 28	Oberschopfheim, Germany	+	-	?	-	-	-	-	?	?
PPV 29	Italien	+	+	+	-	-	-	-	-	-
PPV 30	Aschersleben, Germany	+	+	+	-	-	-	-	-	-
PPV 31	Saarland, Germany	+	+	+	-	-	-	-	-	-
PPV 32	Dossenheim, Germany	+	+	+	-	-	-	-	-	-
PPV 33	Nebringen, Germany	+	+	+	-	-	-	-	-	-
PPV 34	Worms, Germany	+	-	+	-	-	-	-	-	-
PPV 35	Engelstadt, Germany	+	-	+	-	-	-	-	-	-
PPV 36	Wackernheim, Germany	+	+	+	-	-	-	-	-	-
PPV 37	Halle/Saale, Germany	+	+	+	-	-	-	-	-	-
PPV 38	Halle/Saale, Germany	+	+	+	-	-	-	-	-	-
PPV 39	Jork,Germany	+	+	+	-	-	-	-	-	-
PPV 40	Jork, Germany	+	-	+	-	-	-	-	-	-
PPV 41	Jork, Germany	+	+	+	-	-	-	-	-	-
PPV 42	Engelstadt, Germany	+	+	+	-	-	-	-	-	-
PPV 43	Wackernheim, Germany	+	+	+	-	-	-	-	-	-
PPV 44	Dienheim, Germany	+	+	+	-	-	-	-	-	-
PPV 45	Niederbüren, Switzerland	+	+	+	-	-	-	-	-	-
PPV 46	Freising, Germany	+	-	+	-	-	-	-	-	-
PPV 47	Freising, Germany	+	-	+	-	-	-	-	-	-
PPV 48	Burglengenfeld, Germany	+	-	+	-	-	-	-	-	-



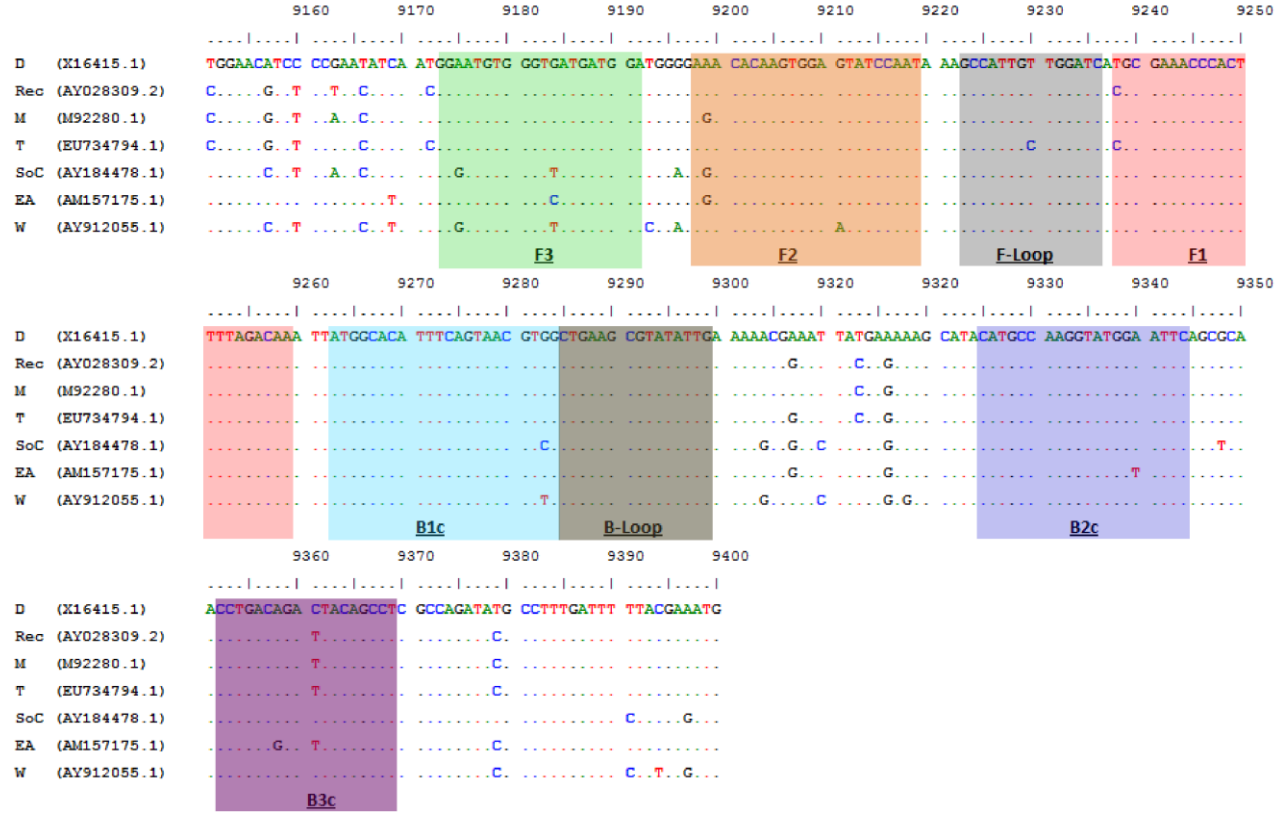
Suppl. Fig. 1: Amplicons derived from background amplification
Ampligase® (0.5 U) catalysed the ligation of 0.12 µM CLP PPV 2 on 2 µl cDNA. CRCA contained 2 µl ligation reaction, 0.75 M betaine, primers FWD 1a and REV 2a and 1.6 U *Bst* DNA polymerase (large fragment). Cloned amplicons were sequenced.



Suppl. Fig. 2: An amplicon containing specific and unspecific amplification products
CLP PPV 2 (0.12 µM) was ligated by Ampligase® (0.5 U) on 2 µl cDNA. The amplicon derived from CRCA (2 µl ligation reaction, 0.75 M betaine, primers FWD 1a and REV 2a, 1.6 U *Bst* DNA polymerase was sequenced.



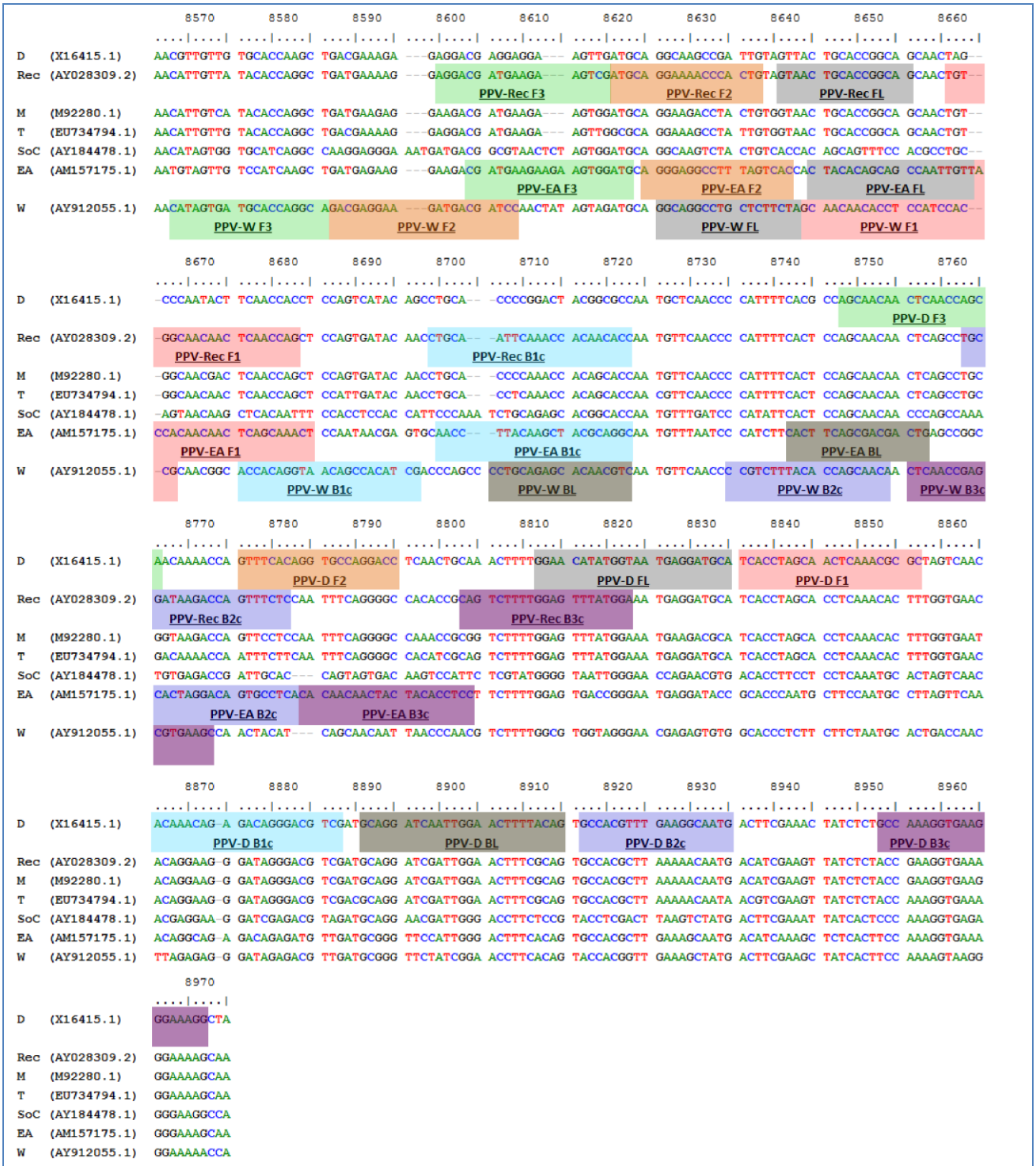
Suppl. Fig. 3: Sequences of amplicons of specific CRCA
Amplicons were derived from CRCA (2 µl ligation reaction, 0.75 M betaine, primers FWD 1a and REV 2a, 1.6 U *Bst* DNA polymerase). Previous ligation reaction contained 2 µl cDNA, 0.12 µM CLP PPV 2 and 0.5 U Ampligase®



Suppl. Fig. 4: Localisation of RT-LAMP primers within selected PPV sequences
The primers were developed by Varga and James (2006b). Each selected sequence represents an isolate belonging to one PPV strain. The accession number of each sequence is shown in the parathesis. Primer BIP contains the sequences B2 and B1c, primer FIP is made of F2 and F1c. D - PPV-D, Rec - PPV-rec, M - PPV-M, T - PPV-T, SoC - PPV-SoC, EA - PPV-EA, W - PPV-W.

Suppl. Tab. 2: Comparison of PPV detection protocols using template from diverse extraction techniques HNB-RT-LAMP, RT-PCR and one-step multiplex RT-PCR (Multiplex) were supplemented with virus suspension, RNA extracted from plant extract, conventionally extracted RNA or cDNA derived from the different extraction methods. NTC - no template control

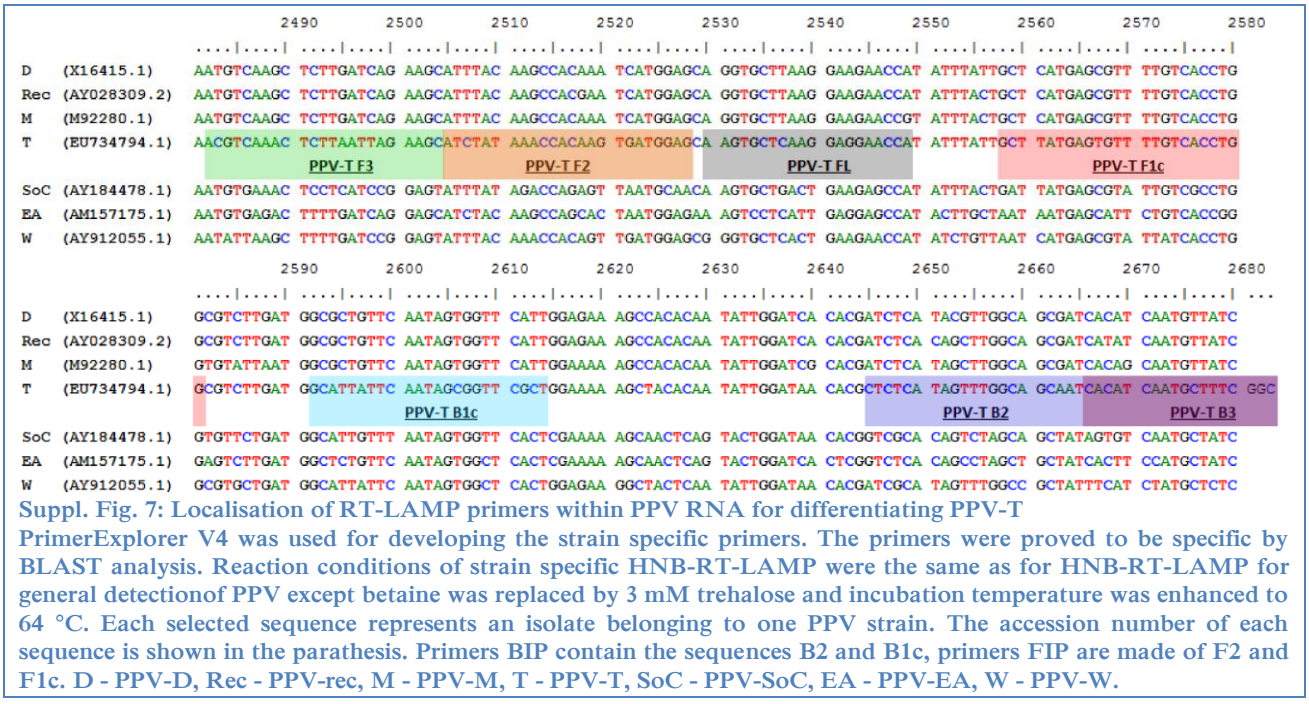
		Blue LAMP			RT-PCR			Multiplex					
		PPV			PPV			PPV			NAD		
Genotype	Leaf symptoms	Plant extract	RNA extracted from plant extract	conventional RNA extraction	cDNA (Plant extract)	cDNA (RNA extracted from plant extract)	cDNA (conventional RNA extraction)	Plant extract	RNA extracted from plant extract	conventional RNA extraction	Plant extract	RNA extracted from plant extract	conventional RNA extraction
NTC		-	-	-	-	-	-	-	-	-	-	-	-
Wei 1101	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 1962	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 1982	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 2008	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 1101	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 1962	-	-	+	-	-	-	-	-	-	-	+	+	+
Wei 1982	-	-	-	-	-	-	-	-	-	-	-	+	-
Wei 2008	-	-	-	-	-	-	-	-	-	-	-	+	+
NTC		-	-	-	-	-	-	-	-	-	-	-	-
Wei 2024	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2067	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2104	+	+	+	+	+	+	+	+	+	+	-	+	+
Wei 2118	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2120	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2024	-	-	+	-	-	-	-	-	-	-	+	+	+
Wei 2067	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 2104	-	-	-	-	-	-	-	-	-	-	-	+	+
Wei 2118	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 2120	-	-	-	-	-	-	-	-	-	-	-	+	+
NTC		-	-	-	-	-	-	-	-	-	-	-	-
Wei 2147	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2155	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 2186	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 2228	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2866	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 2147	-	-	-	-	-	-	-	-	-	-	-	+	+
Wei 2155	-	-	-	-	-	-	-	-	-	-	-	+	+
Wei 2186	-	-	-	-	-	-	-	-	-	-	-	+	+
Wei 2228	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 2866	-	-	-	-	-	-	-	-	-	-	-	+	+
NTC		-	-	-	-	-	-	-	-	-	-	-	-
Wei 3179	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 3408	+	+	+	+	+	+	+	+	+	+	-	+	+
Wei 3540	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 4134	+	+	+	+	+	+	+	+	+	+	-	+	+
Wei 4161	+	+	+	+	+	+	+	+	+	+	-	+	+
Wei 3179	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 3408	-	-	-	-	-	-	-	-	-	-	+	+	-
Wei 3540	-	-	-	-	-	-	-	-	-	-	-	+	+
Wei 4134	-	-	-	-	-	-	+	-	-	-	+	+	+
Wei 4161	-	-	-	-	-	-	-	-	-	-	+	+	+
NTC		-	-	-	-	-	-	-	-	-	-	-	-
Wei 4166	+	+	+	+	+	+	+	-	+	+	+	+	+
Wei 4199	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 4554	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 5270	+	+	+	+	+	+	+	-	+	+	-	+	+
Wei 5349	+	+	+	+	+	+	+	+	+	+	+	+	+
Wei 4166	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 4199	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 4554	-	-	-	+	-	-	-	-	-	-	-	+	+
Wei 5270	-	-	-	-	-	-	-	-	-	-	+	+	+
Wei 5349	-	-	-	-	-	-	+	-	-	-	-	+	+
Sensitivity		1,00	1,00	1,00	1,00	1,00	1,00	0,71	1,00	1,00			
Specificity		1,00	0,92	0,96	1,00	1,00	0,92	1,00	1,00	1,00			



Suppl. Fig. 5: Localisation of RT-LAMP primers for differentiating PPV-D, -Rec, -EA and -W
PrimerExplorer V4 was used for developing the strain specific primers. The primers were proved to be specific by BLAST analysis. Reaction conditions of strain specific HNB-RT-LAMP were the same as for HNB-RT-LAMP for general detectionof PPV except betaine was replaced by 3 mM trehalose and incubation temperature was enhanced to 64 °C. Each selected sequence represents an isolate belonging to one PPV strain. The accession number of each sequence is shown in the parathesis. Primers BIP contain the sequences B2 and B1c, primers FIP are made of F2 and F1c. D - PPV-D, Rec - PPV-rec, M - PPV-M, T - PPV-T, SoC - PPV-SoC, EA - PPV-EA, W - PPV-W.

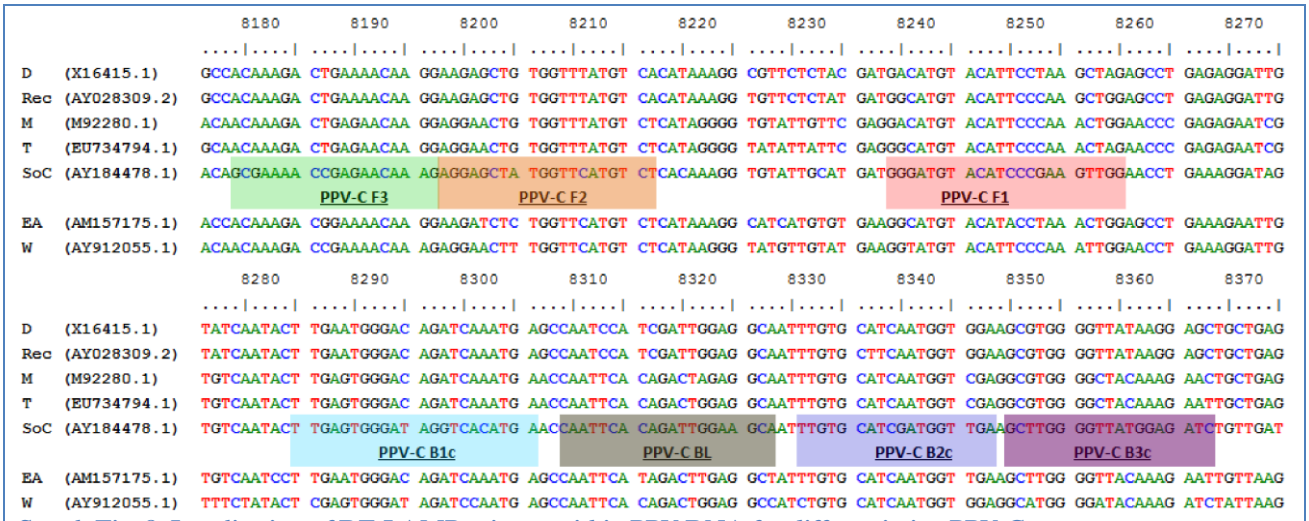
Suppl. Fig. 6: Localisation of RT-LAMP primers within PPV RNA for differentiating PPV-M

PrimerExplorer V4 was used for developing the strain specific primers. The primers were proved to be specific by BLAST analysis. Reaction conditions of strain specific HNB-RT-LAMP were the same as for HNB-RT-LAMP for general detectionof PPV except betaine was replaced by 3 mM trehalose and incubation temperature was enhanced to 64 °C. Each selected sequence represents an isolate belonging to one PPV strain. The accession number of each sequence is shown in the parathesis. Primers BIP contain the sequences B2 and B1c, primers FIP are made of F2 and F1c. D - PPV-D, Rec - PPV-rec, M - PPV-M, T - PPV-T, SoC - PPV-SoC, EA - PPV-EA, W - PPV-W.

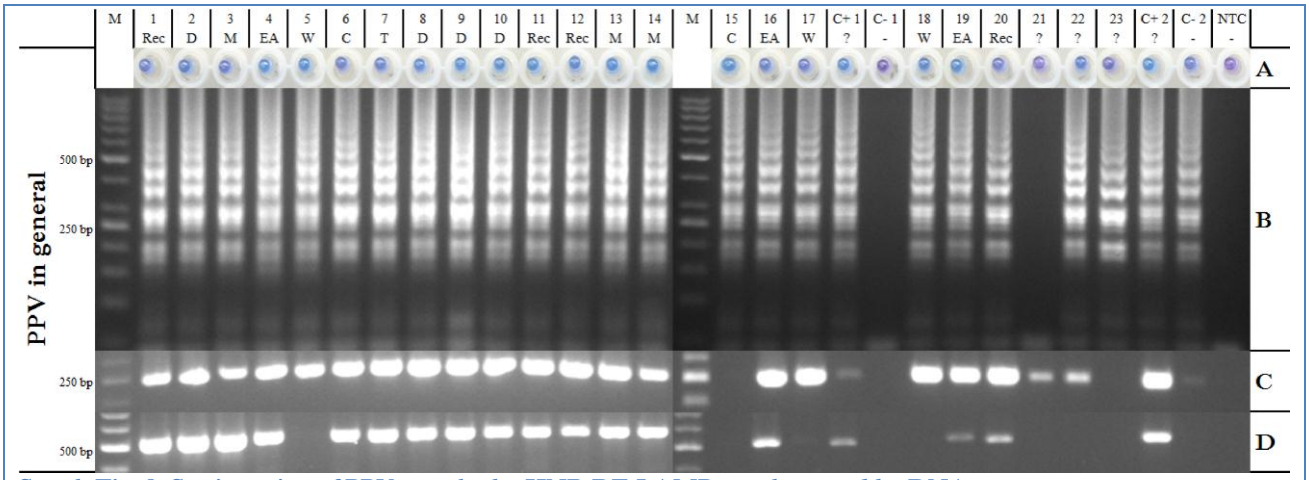


Suppl. Fig. 7: Localisation of RT-LAMP primers within PPV RNA for differentiating PPV-T

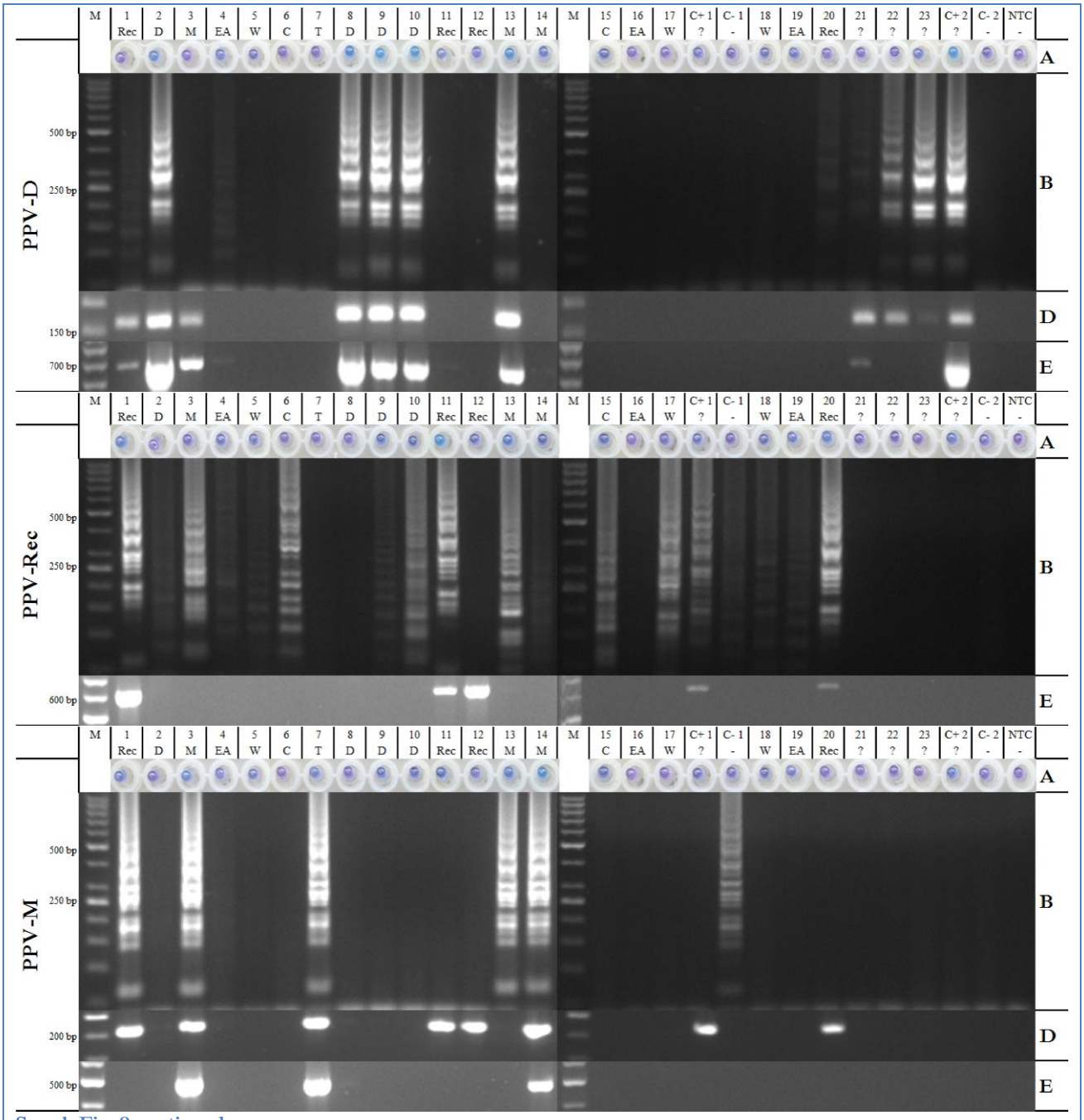
PrimerExplorer V4 was used for developing the strain specific primers. The primers were proved to be specific by BLAST analysis. Reaction conditions of strain specific HNB-RT-LAMP were the same as for HNB-RT-LAMP for general detectionof PPV except betaine was replaced by 3 mM trehalose and incubation temperature was enhanced to 64 °C. Each selected sequence represents an isolate belonging to one PPV strain. The accession number of each sequence is shown in the parathesis. Primers BIP contain the sequences B2 and B1c, primers FIP are made of F2 and F1c. D - PPV-D, Rec - PPV-rec, M - PPV-M, T - PPV-T, SoC - PPV-SoC, EA - PPV-EA, W - PPV-W.



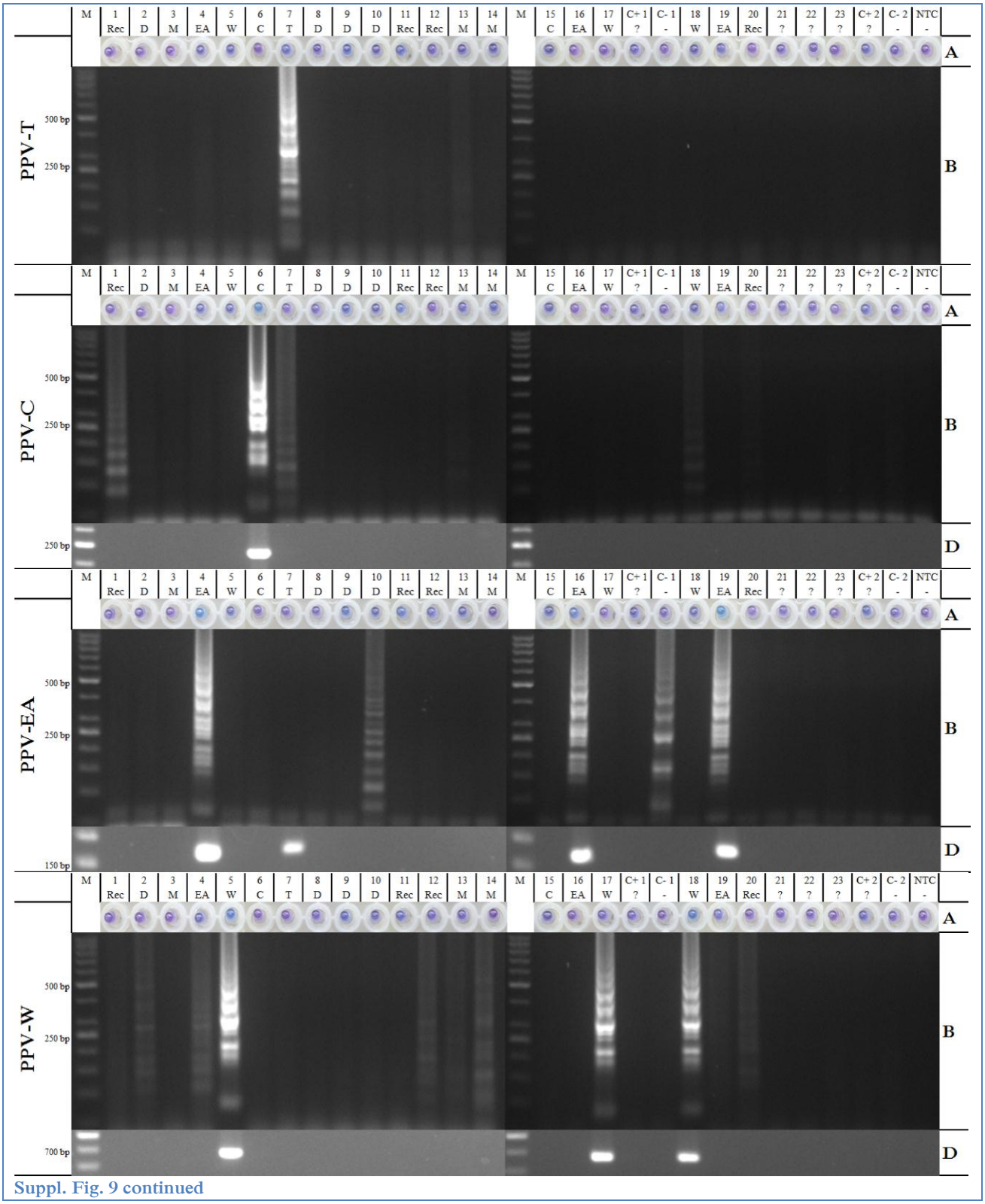
Suppl. Fig. 8: Localisation of RT-LAMP primers within PPV RNA for differentiating PPV-C
PrimerExplorer V4 was used for developing the strain specific primers. The primers were proved to be specific by BLAST analysis. Reaction conditions of strain specific HNB-RT-LAMP were the same as for HNB-RT-LAMP for general detectionof PPV except betaine was replaced by 3 mM trehalose and incubation temperature was enhanced to 64 °C. Each selected sequence represents an isolate belonging to one PPV strain. The accession number of each sequence is shown in the parathesis. Primers BIP contain the sequences B2 and B1c, primers FIP are made of F2 and F1c. D - PPV-D, Rec - PPV-rec, M - PPV-M, T - PPV-T, SoC - PPV-SoC, EA - PPV-EA, W - PPV-W.



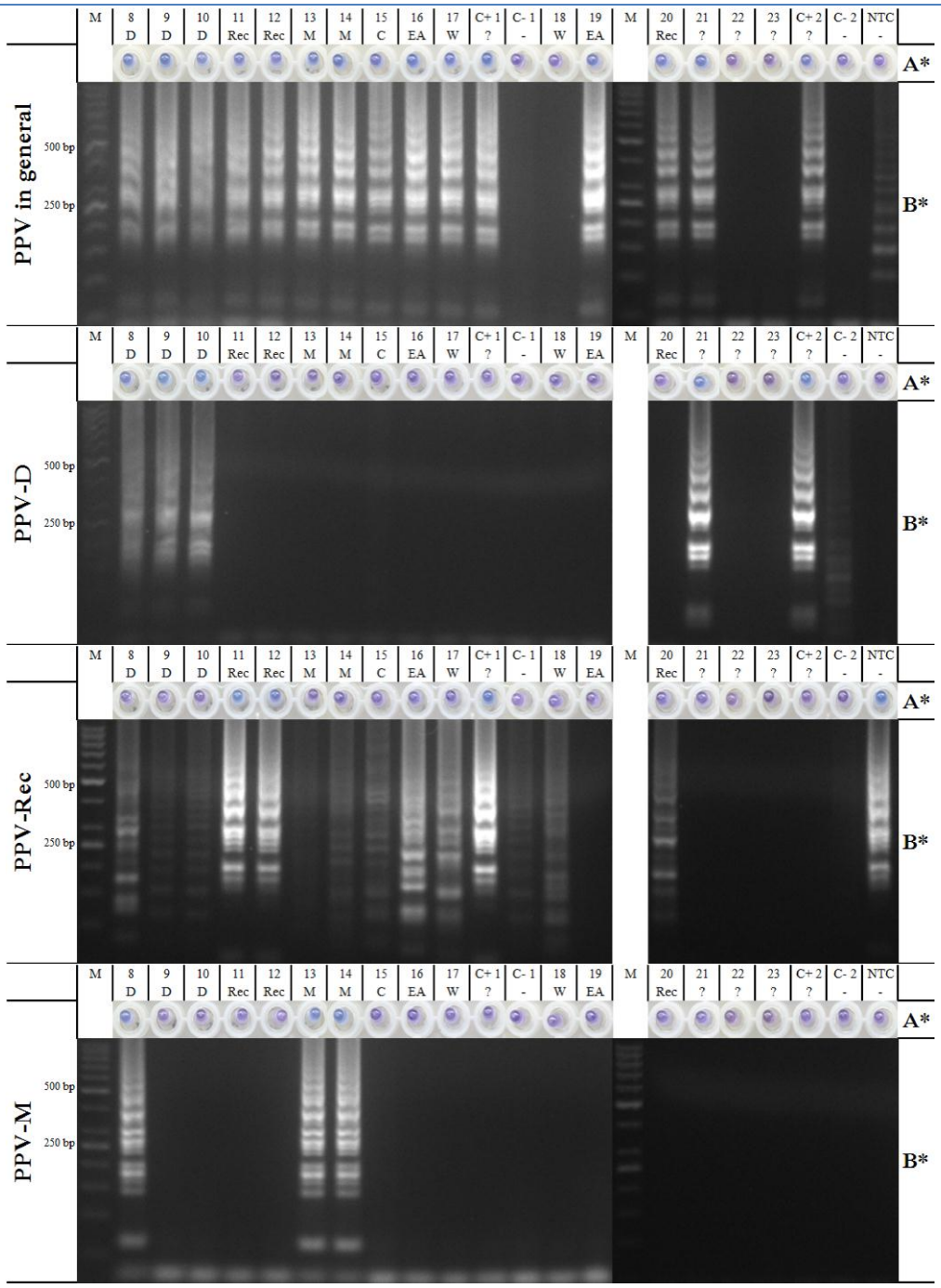
Suppl. Fig. 9: Strain typing of PPV samples by HNB-RT-LAMP supplemented by RNA
The seven primer sets developed for the specific detection of one of the PPV subgroups were examined in HNB-RT-LAMP using a modified HNB-RT-LAMP for the general detection of PPV (incubation at 64 °C for 40 min, 3 mM trehalose) and compared to strain typing by RT-PCR. A - colour change to blue by HNB-RT-LAMP, B - gel electrophoresis of HNB-RT-LAMP amplicons, C - RT-PCR according to Wetzel et al. (1991b), D - RT-PCR according to Szemes et al. (2001), E - RT-PCR according to Šubr et al. (2004); C+ - positive control, C- - negative control, NTC - no template control, ? - isolate not assigned to a PPV subgroup previously, M - size marker



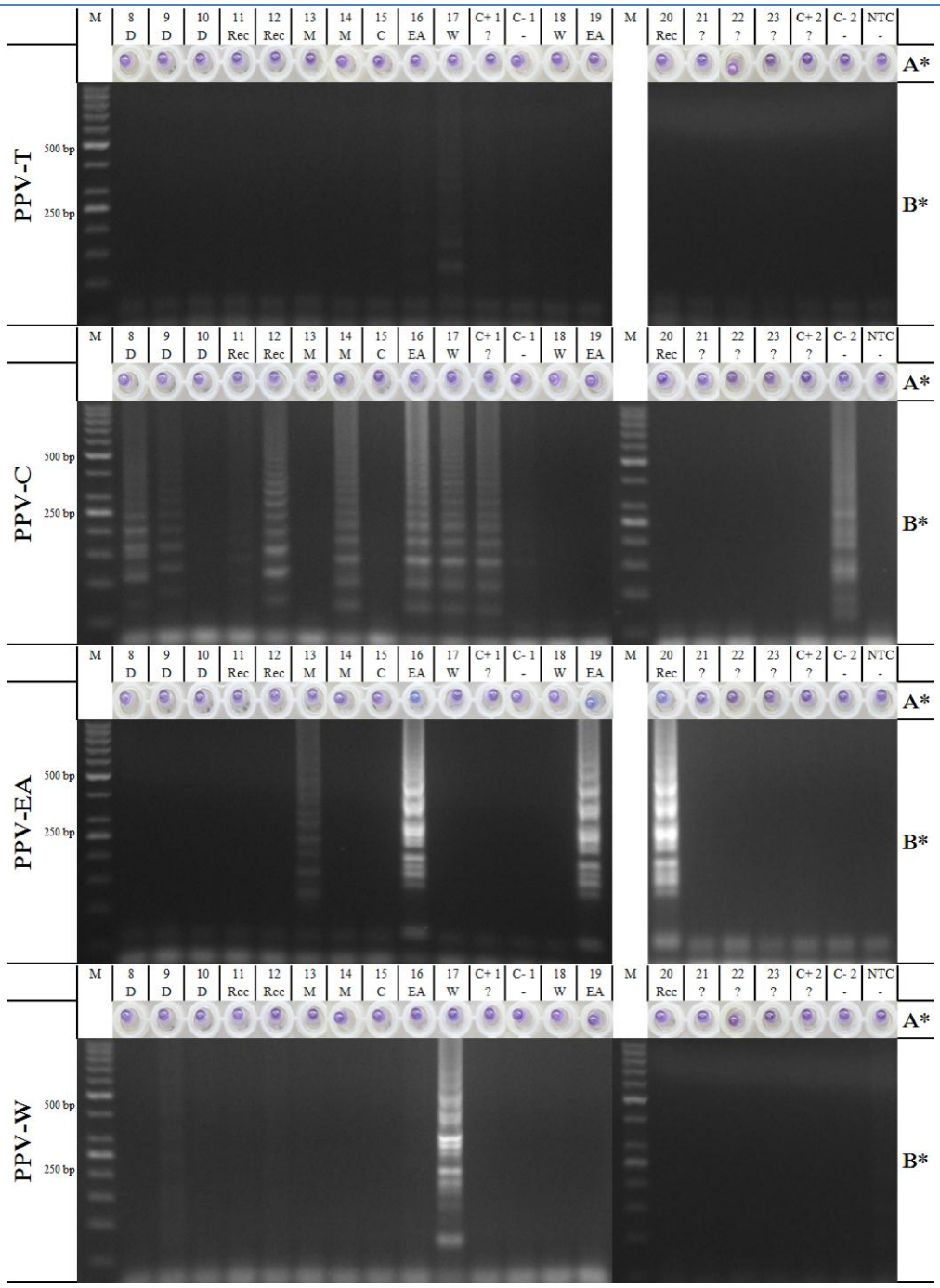
Suppl. Fig. 9 continued



Suppl. Fig. 9 continued



Suppl. Fig. 10: Strain typing of PPV samples by HNB-RT-LAMP supplemented by virus suspension
The seven primer sets developed for the specific detection of one of the PPV subgroups were examined in HNB-RT-LAMP using a modified HNB-RT-LAMP for the general detection of PPV (incubation at 64 °C for 40 min, 3 mM trehalose). A* - colour change to blue by HNB-RT-LAMP, B* - gel electrophoresis of HNB-RT-LAMP amplicons; C+ - positive control, C- - negative control, NTC - no template control, ? - isolate not assigned to a PPV subgroup previously, M - size marker



Suppl. Fig. 10 continued

Acknowledgement

The realization of this work was supported by numerous people whom I would like to express my sincere thanks:

Prof. Dr. Dieter Treutter for providing me with the opportunity to receive a doctorate and for the possibility to turn to him whenever a question arised regarding either the preparation of this doctoral thesis or general concerns.

Prof. Dr. Thilo Fischer for his guidance regarding all molecular biological aspects of my work despite the spatial distance and for reviewing my doctoral thesis.

Prof. Dr. Wilfried Schwab to chair the examination board as well as for enabling the participation in seminars held at the Unit Biotechnology of Natural Products and for discussion.

Dr. Michael Neumüller for his great advice within the project, for discussion, for motiviation and the posibility to talk to him whenever a problem occurred.

all former and actual members of the Unit of Fruit Science with whom I worked with for the familiar working environment.

Ludwig Lichtenegger, Ina Tittel and Laszlo Janvary who became good friends to me for all conversations, for the support and motivation and also for diversion.

my parents Johann Hadersdorfer who passed away far too early and Anneliese Hadersdorfer, my brother Andreas Hadersdorfer and his family and my sister Julia Hadersdorfer for their faith and support.

my friends Stephanie Schaider, Frederike Fritsch, Daniel Greiner, Roland and Verena Kremer for encouraging me in my work but also for leisure time activities.

Curriculum vitae

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