Rapid Detection of Grapevine Leafroll-associated virus Type 3 using the reverse transcription loop-mediated amplification method

By

Helen Ann Walsh

Submitted in partial fulfilment of the requirements for the degree of:
Master of Science

In the Faculty of Natural and Agricultural Science,
Department of Microbiology and Plant Pathology

University of Pretoria
Pretoria

Supervisor: Prof. Gerhard Pietersen

September 2013

Declaration of Originality

Full names of student: Helen Ann Walsh

Student number: 25105958

Topic of work: Rapid Detection of Grapevine Leafroll-associated virus Type 3 using the reverse transcription loop-mediated amplification method.

Declaration

I, Helen Ann Walsh, declare that this dissertation, which I hereby submit for the degree MSc Microbiology at the University of Pretoria, is my own work and has not been submitted previously by me for a degree at this or any other tertiary institution

Signature.....

Acknowledgements:

I would like to extend my most sincere gratitude to the following people:
First and foremost my Supervisor Professor Gerhard Pietersen, without your unwavering support and enthusiasm, this project would have never happened. Thank you for always having an open door.

Elise Jooste, for always being willing to help and to share her expertise and her grapevines and Marika van der Merwe, for her help with the ELISA tests.

Tobie Oosthuizen, Maritsa van Rensburg and Marietta Louw for their patience and advice.

My Lab mates (Lab 9-37) and friends in the Department of Microbiology and Plant Pathology for your help and support.

Winetech and the University of Pretoria for financial support for this project.

Finally, my friends and family for their understanding and their unwavering support and love.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

101-14	101-14 Mtjt
℃	Degrees centigrade
µl	Microliter
3SR	Self- sustained Sequence Replication
AMV	Avian Myeloblastosis Virus
B3	Backward Outer Primer
BIP	Backward Inner Primer
bps	Base pairs
BS	Black Spanish
BYSV	*Beet yellow stunt virus*
CP	Coat Protein
CTAB	Cetyl trimethylammonium bromide
CTV	Citrus Tristeza Virus
dNTP	dioxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme- linked immosorbent assay
F3	Forward Outer Primer
FIP	Forward Inner Primer
g	Gram
GFkV	Grapevine fleck virus
GLD	Grapevine Leafroll disease
GLRaV	Grapevine Leafroll-associated virus
GLRaV-3	Grapevine Leafroll-associated virus type 3
GVA	Grapevine virus A
h	Hour
HCL	Hydrochloric acid
HNB	hydroxy napthol Blue
ICAN	Isothermal and Chimeric primer initiated amplification of Nucleic acid

ISEM	Immunosorbent electron microscopy
LAMP	Loop mediated amplification of Nucleic acid
ME	Mercapto-ethanol
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
min	Minute
mpg	Months post grafting
NH_4	Ammonium
Nts	Nucleotides
OD	Optical Density
PCR	Polymerase chain reaction
Pfu	Plaque-forming unit
PVPP	Polyvinyl-pyrrolidone
qPCR	Real- Time PCR
RdRp	RNA-dependent RNA polymerase
RT-LAMP	Reverse transcription Loop mediated amplification of Nucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Real-time reverse transcription PCR
R99	Richter 99 RY 13C
R110	Richter 110 RQ28C
Rug	Ruggerie 140 RU 354 E
SC	Ramsey/ Salt Creek SC 18 AB
SDA	Strand displacement amplification
sec	Second
sgRNA	subgenomic ribonucleic acid
SNP	Single nucleotide polymorphisms
SSCP	Single strand conformation polymorphism
TAE	Tris-acetate- EDTA
TAS	Triple antibody sandwich
Tm	Melting Temperature
Tris	Tris(hydroxymethyl)aminomethane
V. vinifera	*Vitis vinifera*
V	Volts

Abstract

Grapevine Leafroll disease (GLD), one of the most destructive diseases of grapevines, has been found in every country where grapevines are grown. Grapevine Leafroll associated virus type 3 (GLRaV-3), one of several viruses associated with GLD globally, is the most prevalent virus in South African grapevines and therefore control of GLRaV-3 takes high priority in any strategy aimed at control of GLD. GLD can be controlled through the use of an integrated strategy which includes using certified plant material, controlling insect vectors through use of systemic insecticides and the removal of infected vines by roguing. Infected individuals are identified each autumn, using either symptom display (in red cultivars, where infected individuals display interveinal reddening and downward rolling of leaves) or ELISA (in symptomless white cultivars). ELISA is laborious, time consuming and relatively insensitivity compared to molecular techniques and a simpler, more rapid and more sensitive means of indentifying GLRaV-3 infected vines is required.

A simple RNA extraction procedure combined with a single-tube reverse transcriptase loop-mediated amplification (RT-LAMP) has been developed which allows for the rapid, simple detection of GLRaV-3. Using RT-LAMP, a viral target can be amplified in 2 hours under isothermal conditions. This GLRaV-3 specific RT-LAMP uses hydroxy napthol blue (HNB), a colourimetric indicator that changes from violet to sky blue only where a positive RT-LAMP reaction has occurred, making results quick and easy to interpret. The sensitivity of this technique was compared to ELISA and nested PCR by pooling samples at varying ratios of healthy to infected plants. Using nested PCR and RT-LAMP 1 infected sample could be detected amongst 50 healthy individuals while ELISA could only detect 1 amongst 30 infected making RT-LAMP more sensitive than ELISA. Further RT-LAMP could be performed in 2 hours compared to nested PCR and ELISA's 8 and 48 hours respectively. Based on these results, RT-LAMP is viable alternative for ELISA for the detection of GLRaV-3 in the field.

RT-LAMP was also tested for its ability to detect GLRaV-3 in grapevine rootstocks where, due to low viral titres and erratic distribution, it is notoriously difficult to detect. The rootstocks which were used for testing of GLRaV-3 had been tested in a previous study and it was found that only 28% of samples tested positive after 33 months (post inoculation). Using RT-LAMP, 78% of samples tested positive for GLRaV-3. Although further testing must be done, RT-LAMP may also be a viable alternative for testing grapevine rootstocks for GLRaV-3 infection.

CHAPTER 1: Literature Review

1. 1 Introduction

Grapevine leafroll disease (GLD) is one of the most economically important diseases of grapevines, occurring in every country where grapevines grow (Martelli, 1993) GLD affects grapevine crops, lowering the quality and yields of grapes and has a major impact on the wine industry (Over de Linden & Chamberlain 1970) . Symptoms are most obvious in red *Vitis vinifera* varieties where infected vines display downward rolling of leaves and interveinal reddening. However symptoms of GLD can vary significantly and are dependent on many factors including the grape variety, environmental conditions and the viral isolate (Martelli, 1993).

GLD is associated with a number Grapevine leafroll viruses (known as Grapevine leafroll- associated viruses (GLRaVs)) and includes; *Closterovirus* (GLRaV-2) and *Ampeloviruses* (GLRaV-1, GLRaV-3-6, GLRaV-9 and GLRaV-10) and the unclassified GLRaV-7 (Fuchs et al., 2009a). All GLRaVs are phloem limited and transmitted through plant propagation material however several GLRaV also have insect vectors including some species of mealybug (Hemiptera: Pseudococcidae) and soft scale insects (Hemiptera: Coccidae) (Fuchs et al., 2009a). Due to its widespread distribution, and the observation that GLD spreads readily in vineyards where GLRaV-3 is present (Ling et al., 1998) GLRaV-3 is considered the most important virus associated with GLD.

It is believed that GLRaV-3 is spread primarily through the use of infected propagation material and that spread within and between neighbouring vineyards occurs through its insect vectors (Turturo et al., 2005). Therefore the most effective mechanism of controlling GLD is an integrated strategy which controls all possible means of GLRaV-3 spread. A study conducted at Vergelegen Wine Farm (Somerset West, South Africa) showed that planting certified virus free planting material, controlling insect vectors through use of

systemic insecticides and roguing of infected plant material could effectively control GLD (Pietersen et al.,2009; Pietersen et al., 2013).

The first phase of controlling GLRaV-3 in vineyards involves the use of virus free nuclear material to establish mother blocks and foundation blocks. The foundation blocks, which are monitored to ensure they remain virus free, are then used to provide planting material for the industry. Once virus free material has been planted in a field, it is still susceptible to viral infection and must be monitored and infected plants must be removed by roguing to prevent secondary spread from infected vines to surrounding vines (Pietersen, 2004). Roguing of infected plant material has been shown to be a highly effective mechanism for controlling of GLD in red cultivars, where infected plants are identified on a plant for plant basis each autumn when GLD symptoms are most obvious (Pietersen and Walsh, 2012; Pietersen et al., 2013). Roguing of white cultivars is more problematic as they do not display symptoms and therefore each plant has to be tested individually before infected plants can be removed.

White cultivars can either be tested using the enzyme- linked immunosorbent assay (ELISA) or reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. ELISA is simple and can handle a large number of samples, however it is laborious and time consuming and has been shown to be insensitive compared to molecular techniques such as PCR (Arora et al., 2006; Mirelman et al., 1997). Molecular techniques, such as PCR and Real-time PCR (Q-PCR), which are currently available are fast and reliable and have high sensitivity, however they require specialised equipment (such as thermocyclers, Gel electrophoresis tanks or in the case of Q-PCR real-time thermocyclers) which are not normally readily available in the basic field labs found on most wine farms. Further the techniques are technical and usually require personal who are trained in molecular diagnostics (Francois et al., 2011) where as the majority of personnel who would test vines are wine makers and lack this training. What is required is a simple, rapid technique which can handle high through- put of samples but which does not require

specialised equipment or training and that preferably has a real-time monitoring system.

Loop-mediated amplification (LAMP) is simple, rapid means of amplifying nucleic acid and has been used in the detection of many pathogens (Nie, 2005; Tomlinson et al., 2010; Lee et al., 2011). LAMP relies on a strand displacing DNA polymerase in conjunction with 4 primers which target 6 regions on the target DNA to amplify target under isothermal conditions (Notomi et al., 2000).With the addition of reverse transcriptase, LAMP can also be used in the detection of RNA in a single tube reaction (Parida et al., 2004). Various methods have been developed to visualise LAMP products including agarose gel electrophoresis (Notomi et al., 2000), turbidity (Mori et al., 2001) and using various colourimetric indicators (Goto et al., 2009; Maeda et al., 2005; Yoda et al., 2007).

LAMP is a sensitive technique with a high amplification efficiency (Notomi et al., 2000; Parida et al., 2004), and has been shown to be able to detect very low levels of pathogen (Le Roux et al., 2009; Li et al., 2009; Martelli and Boudon-Padieu, 2006; Pandey et al., 2008; Wastling et al., 2010). Parida et al (2004) developed a RT-LAMP for the detection of West Nile virus which showed a 10- fold higher sensitivity than RT-PCR and was able to detect 0.1 PFU and Li et al. (2009) had similar results, where RT-LAMP sensitivity was 25- fold higher than the RT-PCR used to detect Newcastle disease. LAMP has also been shown to be more robust against substances that can inhibit PCR reactions which increases its usefulness as the target template can be extracted crudely with little effect on the test (Francois et al., 2011). Furthermore its high sensitivity means that its use may go beyond detecting of GLRaV-3 in scions and could have a use for the testing of *Vitis* rootstocks where testing has been problematic due to the low viral titres (Ioannou et al., 1999).

While LAMP has shown great potential as diagnostic tool, there are some inherent disadvantages associated with it. LAMP relies on either four or six primers which target either 6 or 8 regions on the target. In the case of small

genomes (as in the case of viruses) or genomes where little sequence data is available or little homology between isolates, designing primers can be difficult. Further, several of the visual detection methods have been shown to inhibit the reaction where the amount of starting template is low (Wastling et al., 2010) LAMP is highly sensitive method of detection which also makes it prone to contamination and more complicated, because of the nature of amplicons, little can be done to confirm that products are as a result of amplifying a specific target and not the results of non-specific amplification. While these hurdles have been indentified and every care is taken to avoid them, they should none the less be considered.

The aim of this project is to design a RT-LAMP for the detection for GLRaV-3 which can be used in the testing of white cultivars in basic field labs where minimal equipment is available, in place of currently used ELISA method. This may aid in the control of GLRaV-3 spread. Further, depending on the sensitivity of the GLRaV-3 RT-LAMP, it may be used in detection of GLRaV-3 in rootstocks in which the detection of viruses has shown to be problematic.

1.2 Grapevine Leafroll disease

Grapevine leafroll disease (GLD), associated with several viruses known as Grapevine Leafroll-associated viruses (GLRaVs), is the most economically destructive diseases of grapevines world-wide, accounting for 60% of the crop loss globally (Rayapati et al., 2008). GLD is associated with five serologically distinct, viruses designated Grapevine Leafroll-associated viruses (GLRaV) 1-4 and 7 (Fuchs et al., 2009; Martelli et al., 2002; Martelli et al., 2012). GLRaV-3, the type species for ampelovirus, is the most widely spread virus associated with GLD and as such is considered the most important virus associated with the disease.

GLRaVs belong to the family *Closteroviridae* where they fall into the families *Ampelovirus* and *Closterovirus.* Virus are grouped according to their genome organisation, conservation of key gene sequences and mode of transmission. *Closterovirus* are transmitted by aphids and have a positive- sense single-stranded RNA (ssRNA). *Ampelovirus* (type member GLRaV-3) are transmitted

by mealybugs (coccid or pseudococcid) and have a a positive- sense ssRNA with a larger coat protein (35–39 kDa) (Martelli et al., 2002). GLRaV classified into separate species where the variation in the genes for polymerase, HSP70h and CP is greater than 25% (Martelli et al., 2012)

1.2.1 Impact of Grapevine Leafroll Disease

GLD is a highly destructive disease and has a large impact on the global grape industry. GLD is highly detrimental to vine physiology, with advanced infection resulting in the degeneration of phloem cells in the fruit petioles, stems and leaves and the depression of photosynthetic mechanism of the vine (Over de Linden & Chamberlain 1970; Golino et al., 2008b). This has a large impact in the wine industry where the disease reduces both wine quality and quantity (Over de Linden and Chamberlain, 1970).GLD adversely effects vine growth, yield, fruit colour and sugar content (Over de Linden and Chamberlain, 1970) however the extent to which this will affect a single vine is dependent on a number of factors. These factors include; the scion cultivar, rootstock, vine age and environmental factors such as climate and soil nutrients (Over de Linden & Chamberlain, 1970; Cabaleiro & Segura, 1997b; Lee & Martin, 2009)

GLRaV-3 infection has been shown to dramatically reduce the net photosynthesis in infected grapevines due to its interaction of with several proteins on the thylakoid membrane (Bertamini et al., 2004). This can reduce the photosynthetic capability of the plant to between 25 and 60% depending on the cultivar (Charles et al., 2006; Golino et al., 2008b). Reduced photosynthesis results in a reduction in the supply of carbohydrates and sugar to the berries and impacts the vines early development at the beginning of each season. Over time this results in large loss of the productivity of the vine (Rayapati et al., 2008).

The reduction of net photosynthesis in a vine has a large impact on the growth and vigour of a grapevine. GLD infected vines have been shown to have fewer, smaller leaves (Sampol et al., 2003) as well as reduced cane weight and growth (Over de Linden & Chamberlain, 1970). This decrease can cause

a reduction in the lifespan and productivity of a vineyard (Cabaleiro et al., 2007), however the amount that growth is reduced seems to be affected by the grape variety and may also be influenced by the environment (Charles et al., 2006).

GLD has had a major impact on the wine industry where it affects the quality and quantity of berries. Due to the reduced photosynthetic ability and lower anthocyanin accumulation, infected vines produce smaller berries with lower sugar levels and poorer colour (Over de Linden and Chamberlain, 1970). Overall, GLD affects berry maturation increasing ripening times and reduces the quality of the berries (Over de Linden and Chamberlain, 1970) which is highly detrimental in the wine industry.

1.2.2 Symptoms

GLD occurs in all grape varieties; however the disease severity and symptom expression can vary significantly (Freeborough, 2008). Typically symptoms are most pronounced in red *Vitis vinifera* cultivars where GLD is characterised by downward rolling of leaves combined inter-veinal reddening with dark green veins (figure 1). White *Vitis* cultivars either remain asymptomatic or develop downward rolling leaves which are green- yellow colour with dark green veins (figure 1). These symptoms are best viewed in late summer and autumn.

Symptom expression can differ significantly between cultivars which can make identification of individual infected vines difficult. These variations are dependent on a number of factors including the variety, the age of the vine and environmental factors. Symptoms expression differs greatly between wine varieties, table grape varieties and rootstocks; with symptoms being less pronounced in table grape varieties and rootstocks remaining asymptomatic (Krake et al., 1999). Visual identification of GLD based on symptoms can also be problematic because the symptoms may also resemble those caused by mechanical damage to the canes and trunk, several other diseases of grapevines, arthropod damage and nutrient deficiencies such as low nitrogen levels (Lee et al., 2009).

White wine cultivars, rootstocks and table grapes must be tested using serological or molecular diagnosis methods in order to determine infected individuals.

1.2.3 Spread of Grapevine Leafroll disease

Grapevine Leafroll disease can be spread in a number of ways; infected propagative material, semi-persistent transmission by insect vectors and the use of incorrect farming practices (e.g movement of pruning equipment between vineyards, sharing of farm equipment which allows for spread of insect vectors). Once GLD has been introduced to a vineyard the spread can be very rapid, with reports of infection growing from 11% to 100% in 5 years within a vineyard (Petersen, 1997).

The ubiquitous global distribution of GLD is thought to be due to the use of infected material for propagation (Cabaleiro & Segura 1997b). Studies of genetic diversity of GLRaV-3 has shown that there is little variation between virus isolated from different geographical locations and it is hypothesised that the use of infected propagative material has had a large role in the long distance spread of GLRaVs (Turturo et al., 2005) and that insect vectors have only contributed to spread of the disease within and between neighbouring vineyards.

Once GLD is present within a vineyard, epidemiological studies have shown that it spreads along rows at a more rapid pace than between rows and that infected vines tend to cluster rather than be randomly spread throughout a vineyard (Cabaleiro & Segura, 1997a; Pietersen, 2004). This may indicate that infected plant material acts as a reservoir and that insect vectors contribute to the secondary spread of the virus. These vectors may also account for the spread of the disease between adjacent vineyards as they may be dispersed by wind or through the incidental dispersal by farm activities (Charles et al., 2009)

Two insect types have been identified as vectors for GLRaV; scale insects and mealybugs (Fuchs et al., 2009a). Mealybug contribution to the spread of

GLD may be dependent on the species present in a vineyard. It has been observed that some species (such as *Planococcus ficus)* have been shown to be highly efficient vectors, requiring just one infected individual to transmit the virus (Douglas and Krüger, 2008), while other species are less efficient (Grasswitz and James, 2008).

While the contribution of mealybugs and scale insects to spread of GLD within and between vineyards is not fully understood, the link has been shown in many separate studies (Cabaleiro and A Segura, 1997a; Golino et al., 2002; Walton, 2004). Therefore, in terms of control of GLD, control of vectors seems to play a vital role in the control of the disease.

1.2.4 Grapevine Leafroll-associated virus vectors

Grapevine Leafroll disease was initially only thought to spread through use of infected plant material, however in 1983 it was discovered that certain mealybugs (*Pseudococcidae*) were able to act as vectors for grapevine leafroll associated viruses (Rosciglione and Martelli, 1983; Engelbrecht and Kasdorf, 1990). Since this initial observation, several insects within the hemipteran family's *Pseudococcidae* (mealybugs) and *Coccidae* (soft scale) have been identified as vectors for GLRaV-3 (Engelbrecht and Kasdorf, 1990; Cabaleiro and Segura, 1997a; Petersen, 1997; Ling et al., 1998; Sforza et al., 2003; Zorloni et al., 2006; Golino et al., 2008a; Fuchs et al., 2009a; Tsai et al., 2011).

GLRaV-3 has been shown to be genetically diverse, with 5 (possibly 6) phylogenically distinct groups (Jooste et al., 2010). A study of soft scale insects *Parthenolecanium corni*, *Pulvarnia innumerabilis* and the mealybug *Pseudococcus maritimus* showed no preference for any particular phylogenetic group (Fuchs et al., 2009a). Based on these results it is thought that while mealybugs and soft scale insects are important for the spread of GLRaV-3, they have no effect on selective transmission of the virus (Fuchs et al., 2009a)

Citrus tristeza virus, another member of the *Closteroviridae* family, is vectored by aphids and it has been shown that there is selection between vector and

certain phylogenetic groups (Lin and Brlansky, 2002). While there is no evidence that the insect vectors of GLRaVs put any selective pressure on GLRaVs, there is evidence that the presence of various vectors will influence the efficiency with which the disease will be spread within a vineyard. Transmission efficiency has been studied in several Mealybug species and the efficiency rates have been shown to vary. *Planococcus ficus* and *Pseudococcus longispinus* are highly efficient vectors, requiring an acquisition access period of 1 hour and an inoculation period of 30 minutes in order for to transmit the virus (Douglas and Krüger, 2008). However, *Planococcus citri* Risso has been shown to be much less efficient with only 1/10 plant becoming infected when exposed to viruliferous mealybugs for 3 days (Cabaleiro and Segura, 1997b).

Transmission efficiency is also thought to be dependent on the developmental stage of the mealybug; Petersen (1997) studied the transmission of *P. longispinus* and *P. calceolariae* and found that only the first instar could transmit GLRaV-3 (however in this study the third instar may have been damaged during their transfer to healthy plants which could have affected the transmission of GLRaV-3), Mahfoudhi et al. (2009) observed that the juvenile stages of *P. ficus* and *P. citri* transmit GLRaV-3 more efficiently than adult females. After moulting, infective *P. ficus, P. longispinus* and *P. citri* were negative for GLRaV-3 (Cabaleiro & Segura 1997; Douglas & Krüger 2008) indicating that mealybugs may not retain GLRaV-3 during moulting. This is important for the control as the juvenile instars are more easily dispersed by wind (Charles et al., 2006).

Infected nuclear material is thought to be the most important source of GLD and therefore a large part of the control of the disease should be focused on the production of virus free planting material. However it is also clear that the insect vectors of GLRaVs play a large role in the spread of GLD between vines both in and between neighbouring vineyards and severity of GLD. Therefore any control strategy aimed at eradicating GLD should include control the vectors. In South Africa, a control strategy which includes the use of certified planting material, control of insect vectors through the use of

systemic insecticides and the removal of infected vines by roguing has proven an effective mechanism of control GLD (Pietersen and Walsh, 2012; Pietersen et al., 2013)

1.3 Grapevine Leafroll Disease in South Africa

1.3.1 Grapevine leafroll - associated virus type 3

GLD is an important disease of grapevines, where its impact is felt in the quality and yields of crops. Globally the disease is associated with several virus (GLRaV) however GLD does not spread readily in vineyards except where GLRaV-3 is present, therefore GLRaV-3 is most commonly associate with the disease and is considered to be the most important GLD associated virus (Ling et al., 1998; Cabaleiro & Segura 2006). GLRaV-3 is commonly associated with GLD in South African vineyards and is considered the most important virus associated with the disease in South Africa.

1.3.1.1 Genome organisation of GLRaV-3

Grapevine leafroll-associated virus type 3 (GLRaV-3) part of the *Closteroviridae* family, is the type species for the genus *Ampelovirus* (Ling et al., 2004). It is a single stranded, positive sense RNA virus with a linear and filamentous virion, between 1250 -2200nm in length (Martelli et al., 2002). The genome size ranges from 16.9- 19.5kb (Martelli et al., 2002) and is organised into 13 open reading frames (designated ORFs 1a, 1b, and 2–12) (figure 1) with untranslated regions of 158 and 277 nts at its 5' and 3' ends (Ling et al., 2004, 1998)

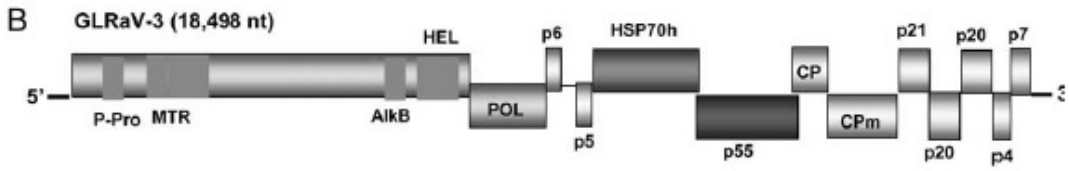

Figure 1: Schematic representation of the GLRaV-3 genome and positions of genes and ORFs. (modified from Martelli et al., 2012).

GLRaV-3 genome organisation is consistent with other typical monopartite closteroviruses (Dolja et al., 1994). (Figure 1)ORF1a and ORF1b contain the genes which code for proteins associated with replication and defence against

host proteins. ORF1a encodes a polyprotein with domains for leader protease (L-Pro) (Ling et al., 1998), AlkB domain (Engel et al.,2008; Maree et al.,2008), methyltransferase (MET) (Ling et al., 1998), and helicase (HEL) (Ling et al., 1998). ORF1b encodes for a RNA-dependent RNA polymerase (RdRp). Between ORF1b and ORF 2 there is a untranslated GC rich untranslated reading frame with extensive secondary structure (Karasev, 2000). The intergenic region of a comparable size to the ORF2 found in *Closteroviruses Beet yellow stunt virus*(BYSV) and CTV (Karasev, 2000). ORF2 encodes a small peptide which does not have any orthologs of a similar size in other closteroviruses (Karasev et al., 1995). The p6 protein is a conventional movement protein and is used in cell-to-cell transport (Dolja et al., 2006). ORF 3 encodes a small hydrophobic transmembrane protein. ORF4 encodes the Heat shock protein 70 (Hsp70) homologue protein which is unique to closterovirus family (Dolja et al.,1994). ORF5 encodes a 55K protein (Ling et al.,1998). ORF 6 encodes the coat protein (CP) gene. ORF7 encodes for the copy of CP (dCP) which another unique feature of the closteroviruses (Boyko et al., 1992). While the functions of ORFs 8-12 cannot be inferred by sequence analysis, however it has been suggested that ORF 8, 9 and 10 could be involved suppression of host defence (Lu et al., 2003; Reed et al., 2003; Chiba et al., 2006) and systemic movement proteins (Dolja et al., 2006)

A recent study investigating GLRaV-3 associated sub genomic ribonucleic acid (sgRNA) found that sgRNA is necessary for the expression of the 3' ORFs (3-12) in positive sense RNA viruses. It further predicted that at least seven 3' co-terminal positive sense sgRNA for the expression of these ORFs. Jarugula (2010), in a report on the gene expression and *cis*- acting elements used by GLRaV-3, showed that three sgRNAs (ORF7, 5, 3 and 4) were present in very low levels, two sgRNAs (ORF11 and 12) accumulated at intermediate levels and four putative 3' co-terminal sgRNAs (specific to ORF6, 8, 9 and 10) were present in higher levels (Jarugula et al., 2010). Based on the suggestion that 3' coterminal sgRNAs accumulate at variable amounts i.e have varying expression levels in infected grapevine tissues, It was suggested that ORF10-12 are likely to be translated from the same sgRNA (Maree et al., 2010).

1.3.1.2 Diversity of Grapevine Leafroll- associated Virus type 3

Due to the limited number of complete GLRaV-3 genomes, little is known about the genetic diversity within GLRaV-3 (Wang et al., 2011). Several techniques have been employed in order to identify population and genetic variability amongst GLRaV-3. including single-stranded conformation polymorphism (SSCP), sequence analysis of RdRp, HSP70h, CP and genome fragment analysis (Turturo et al., 2005; Fuchs et al., 2009b; Jooste et al.,2010; Wang et al., 2011). Using complete HSP70h sequences, Fuchs et al. (2009) separated GLRaV-3 into 5 phylogenetically distinct groups. Jooste et al. (2010) and Gouviea et al. (2011) conducted separate studies comparing the GLRaV-3 complete CP gene of 46 and 78 samples respectively and showed that the same 5 phylogenetically distinct groups of GLRaV-3 were found. It has been suggested that a sixth group may also exist with NZ-1 HSP70h isolate as the group representative, however further studies are needed to confirm this.

Table 1: Origins of *Grapevine leafroll associated* virus type 3 isolates

GLRaV-3 isolate	Origin	Reference
CI-766	Chile	Engel et al., 2008
NY-1	New York	Hu et al., 1990
621	South Africa	Jooste et al., 2010
623	South Africa	Jooste et al., 2010
GP18	South Africa	Maree et al., 2008
NZ-1	New Zealand	EF508151

Very few GLRAV-3 viral isolates have been fully sequenced, which limits the amount of known about the genetic diversity of GLRaV-3 (Turturo et al., 2005). However, based on single-stranded conformation polymorphism (SSCP) and sequence analysis of three different genomic regions: those encoding the RNA-dependent RNA polymerase (RdRp), HSP-70 and the coat protein (CP), 5 distinct phylogenetic groups of GLRaV-3 have been identified, (Turturo et al., 2005). These groups do not appear to be linked to the geographical location of the sample (Gouveia et al., 2011) and the majority of

samples which have been sequenced have clustered in either Group 1 (with NY-1, Cl-766, 621) or Group 2 (represented by GP18, 623). It has been speculated that this is due to empirical selection of infected plants which displayed less severe symptoms or due a difference in transmission efficiency of the various phylogenetic groups, however further studies need to be done in this area. A study of South African vineyards showed viral variants only cluster into groups 1, II and III, with the majority of the isolates clustering in group I and II (Jooste et al., 2010).

Information about the Grapevine leafroll genetic variation is limited by the amount of sequenced data available and the majority of genetic variability studies have focused on conserved regions of the GLRaV-3 genome including the RdRp, HSP70h, CP. Engel et al. (2004) found that the Czech isolates had 99% or greater homology with the NY-1 sequence as well very high homology with partial sequences from various areas of the world. There has also evidence of mixed infection, and in some cases recombination events, where Turturo et al. (2005) found 15% of CP genes and 10% of RdRp and HSP genes were made up of two or more variants. Analysis also suggested that the sequence variation in the CP gene was higher than that of the RdRp gene. This may be due to selective pressure on the RdRp gene (Turturo et al., 2005).

The genome sequence of isolate GP18 showed that 5' UTR end was 579 nucleotides longer than the previously sequenced NY-1 (Maree et al., 2008), Jooste el al (2010) confirmed this variation in the 5' UTR and showed that isolates 621, 623 and PL-20 5'UTR also had variation. This variation in the 5' UTR is low between members of the same phylogenetic group and higher between isolates of different groups. While the function of the 5' UTR is still unknown, it may be useful in identifying further variation in new isolates.

In order to design a reliable detection technique, it is important to understand the variation within in GLRaV-3. These results indicate that there is some degree of variation within the genome of GLRaV-3 and that this variation varies between different area of the genome. Detection techniques which rely

on sequence data, need to identify areas where there is high homology, in order to ensure that all variations of the virus will be reliably detected. In the case of LAMP, 4 primers detecting 6 different regions of the genome must have high enough homology to bind; this can only be achieved if enough is known about variation within GLRaV-3.

1.3.2 Control

Control of GLD in South Africa is mainly concerned with elimination of the GLD through the use of the S.A. certification scheme, which provides virus free material to the wine industry (Pietersen 2004). However once planted, vines are still susceptible to the disease and spread is common due the high prevalence of mealybugs in South African vineyards (Pietersen, 2004). In order to control GLD, an integrated approach of providing virus-free material, controlling of vectors through the use of systemic insecticides and the removal of infected vines through roguing has been shown to be effective (Pietersen and Walsh, 2012; Pietersen et al., 2013).

At Vergelegen Wine estate (Somerset West, South Africa) control of the disease was divided into three phases. In phase one, GLD was controlled in 34 (5 year old) vineyards which were planted on land where no vines had been planted previously. The vineyards were established by planting certified planting material which was then treated with systemic insecticide to control insect population. Following the establishment of the vineyards, the vines were monitored (using symptom display or ELISA) each year for infected vines and infected vines were removed by roguing. In phase 2, GLD was controlled in 29 (red cultivar) vineyards which had 100% infection. The vineyards were removed and once all vine remains and volunteer plants had been removed, new certified planting material was planted. Vineyards were monitored for infection and infected plants were removed by roguing. Using this method infection was reduced from 100% infection on 41.26 ha (111431 vines) in 2002, to 0.026% detected GLD infected vines on 77.84 ha (209626 vines) in 2012 (Pietersen and Walsh, 2012; Pietersen et al., 2013).

Phase 3 of GLD control at Vergelegen involves the control of GLD in White *Vitis* sp. by detection and replacement of infected vines using rouging. This phase is currently in progress, however it made more complicated by the lack of symptoms in white cultivars. Roguing of infected red cultivars, where symptoms are easily identified in the autumn months, has been shown to be a simple method of control. However, in order to achieve the same results in white cultivars each vine has to be tested before the infected vines can be rogued. This process has shown to be laborious and time-consuming and has made the control of GLD more difficult. One of the largest hurdles in the testing of white cultivars is that the methods currently available for the testing for GLRaV-3 are either too expensive or not suitable for large scale testing as in the case of PCR, or are slow and laborious as in the case of ELISA. A simple, rapid, sensitive method of testing a large quantity of samples without the need for expensive equipment or trained technicians is required.

1.3.4 Detection of GLRaV-3

1.3.4.1 Biological Indexing

Before the advent of ELISA and PCR, biological indexing was the only reliable method of testing for GLD was hardwood indexing on biological indicators (Maree et al., 2013). With this method, a small chip bud taken from the vine which is being tested, is grafted onto a indicator grapevine cultivar which is then left in the field for two seasons and is observed for disease symptoms (Rowhani et al., 1997). Indicator plants are chosen depending personal preference and/or climatic conditions however, the *V. vinifera* cultivars Cabernet Franc, Pinot noir and Cabernet Sauvignon are commonly used (Maree et al., 2013).

There are many disadvantages associated with Biological indexing. It is labour intensive and time consuming and requires successful inoculation of the indicator plant with the associated viruses (Weber et al., 2002). In the case of GLRaV-3, the low viral titers and erratic distribution of the virus make biological indexing more complicated. Further, biological indexing can be technical and usually requires a skilled virologist fro disease confirmation and relies on symptom expression which can be subjective (Maree et al., 2013)

1.3.4.2 ELISA

Currently wine farmers and basic diagnostics laboratories use ELISA to test for the presence of GLRaV-3 in vines (Golino et al., 2008). This system is a high through-put, simple technique which can be applied in basic labs without the need for highly trained technicians or expensive lab equipment. GLRaV-3 ELISA relies on the interaction between the viral antigen and viral specific antibodies to detect the presence of the virus. While different variations of ELISA have been developed, indirect double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) is the most commonly used test. It is a cheap and simple method of testing samples and with the purchase of a minimal amount of equipment can be used in very simple laboratories.

Although ELISA is simple to perform and can be used to test a large amount of sample relatively cheaply, it is time consuming and laborious. It is also less sensitive than molecular techniques such as PCR (Arora et al., 2006). For this reason, molecular based detection techniques have been developed which are more sensitive, faster and would allow for a greater number of samples to be tested at once as greater numbers of samples could be pooled together in a single test.

1.3.4.3 Polymerase chain reaction (PCR)

There are a number of problems associated with the detection of GLRaV-3 in vines. The virus is found in low concentrations in the plant, this, coupled with the fact that the viruses distribution is erratic and varies with season makes detection by ELISA methods difficult (with many scrapings or petioles required from each plant) and unreliable due to their low sensitivity (Dovas and Katis, 2003). Specific PCR (and RT-PCR in the case of RNA targets) have been shown to be more sensitive (10-100 fold more) and specific than diagnostics test based on serology and biological indexing (Pacifico et al., 2011).

PCR primers have been designed for regions of the Viral genome which are known to be highly conserved (e.g the RdRp regions (Ling et al., 2001)) increases their specificity over ELISA and biological indexing where cross-reactions can occur (Pacifico et al., 2011) Several RT-PCRs have been

developed for the detection of GLRaV-3 which have been able to detect the virus in Fentogram amounts (Notte & Minafra 1997; Nolasco, 2003). Also with the advent of real-time RT-PCR, detection of GLRaV-3 has become even more sensitive (where real-time RT-PCR has detected positive samples not detected by conventional RT-PCR) and because there is no need for gel electrophoresis the over-all time required for positive results and the risk of post incubation contamination is reduced (Osman et al., 2007).

PCR is very sensitive and therefore only a small amount of a target is required to observe a positive result. This is useful for detection of GLRaV-3, which is only found in low tires and is erratically in the grapevines (Monis and Bestwick, 1996). Further, this makes it possible to use crude nucleic acid extraction protocols with PCR which decreases both the time required to get a result (and therefore increases the number of samples which can be run in a day) as well as the over-all cost per sample.

While PCR is considered a standard diagnostic procedure for most science laboratories, the laboratories on wine farms tend to have very basic facilities which are not geared towards running these tests. ELISA has the advantage that it only requires very minimal equipment (incubators and multi-challenge pipettes) and therefore can be used in these setting. PCR requires thermocyclers, which although considered inexpensive for a diagnostics laboratory are not financially viable for most wine farms. Real-time thermocyclers are still considered as expensive, and cost considerably more than a basic thermocycler. Further PCR requires a basic knowledge of molecular biology which most wine farmers do not have. Also because results are visualised by gel electrophoresis the process can be laborious (although in real-time PCR this step has been made obsolete) and may increase the chance of false positives because of contamination. The sensitivity of the technique also makes it more prone to contamination than techniques such as ELISA.

Globally, there has been a movement towards virus-tested, healthy planting material and control of diseases within established vineyards, which has

increased the need for simple and sensitive detection mechanism (La Notte et al., 1997). In order to improve control of GLD in South Africa, there is a need for technique which is sensitive (comparative to PCR), rapid, simple and something which preferably has a real-time monitoring system but which does not require specialised equipment (such as thermocyclers) or trained technicians.

1.4 Isothermal amplification of Nucleic Acid:

PCR is one of the most widely used methods of amplifying nucleic acid. During a PCR reaction, two specific oligonucleotide primers hybridise to the 5' and 3' ends of a target sequence and DNA polymerase extends the sequence from the annealed primers by adding on deoxyribonucleoside-triphosphates (dNTPs) producing a double stranded product. Using a thermocycler which raises and lowers the temperature of the reaction, the two strands can then serve as template for the next round of amplification (Gill and Ghaemi, 2008). PCR has been widely used however need for a thermocycler has given it limited application in situation where very little laboratory specialised laboratory equipment is available (Gill and Ghaemi, 2008). Isothermal amplification techniques amplify nucleic acid without the need for a thermocycler (i.e at a single temperature) and therefore are being more widely applied in field situation where thermocyclers cannot be afforded or are not available.

Nucleic acid amplification is a vital diagnostic tool and as such several methods of amplification have been developed including; Isothermal and Chimeric primer initiated amplification of nucleic acid (ICAN), PCR, Strand displacement amplification, Self- sustained Sequence Replication (3SR) and Loop-mediated amplification of nucleic acid (LAMP). Each method uses a unique method of DNA synthesis initiation and re-initiation and has both advantages and disadvantages associated with it (Notomi et al., 2000).

In terms of developing an ‘in-field’ diagnostic technique, it is important to remember that the technique needs to be robust and must not need specialised equipment (such as thermocyclers). Isothermal amplification

techniques do not require large amounts of equipment and therefore lend themselves for use in labs where specialised equipment is not available.

1.4.1 Isothermal and chimeric primer initiated Amplification of Nucleic Acids (ICAN):

Isothermal and chimeric primer initiated Amplification of Nucleic acids (ICAN) is an alternate method of amplifying target DNA. It utilizes two chirmeric DNA-RNA primers, RNaseH and a strand displacing DNA polymerase to amplify target DNA under isothermal conditions (Mukai et al., 2007). ICAN amplifies the target using multi-priming, where multiple copies of the primer bind to a single copy of the target simultaneously (figure 2), and template switching (figure 3).

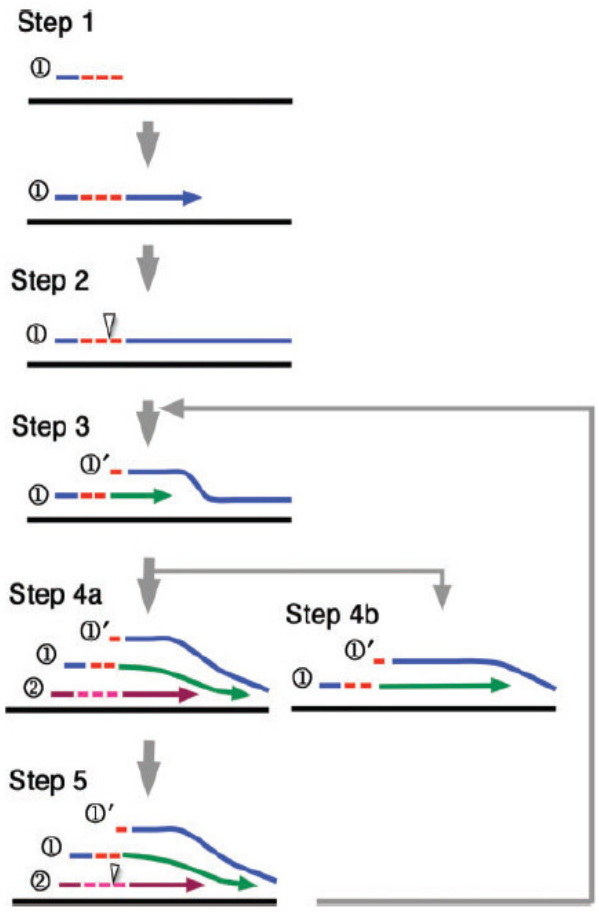

Figure 2: Multi-priming model for ICAN. This figure only depicts the reaction from one strand of a duplex DNA, and only the phase in which nicking occurs when three RNA residues remain intact is drawn (Modified from (Uemori et al., 2007)

During Multi-priming a chimeric primer binds to the template and is extended from the 3' end (step 1). RNaseH then introduces a nick into RNA portion of the primer (step 2). The strand-displacing DNA polymerase then extends the strand from the nick site (step 3). The new strand (extending from the nick site) displaces the pre-existing strand which then becomes available for binding from a primer in solution (step 4a). The new strand (primed at the nicked site) is extended

and displaces the pre-existing strand (4b). Strand-displacing DNA extension occurs, while at the same time RNAse introduces nicks at the RNA sites, resulting in multiple primers hybridising to a single strand of DNA simultaneously.

.

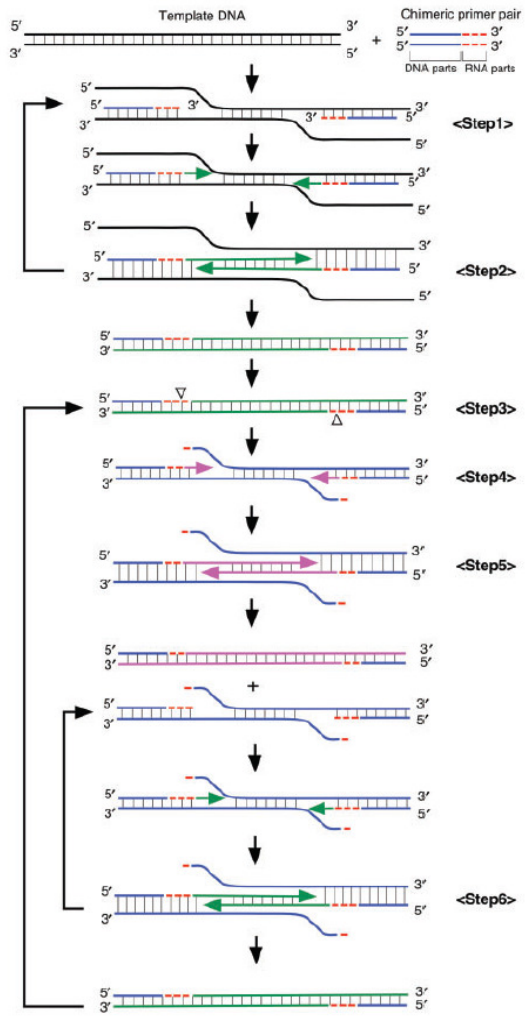

Figure 3: Model of Template switching during ICAN (Uemori et al., 2007)

In conjunction with multi-priming, amplification also occurs by template switching. During template switching, a primer pair hybridizes to a double stranded template and the DNA strand is extended by strand displacing DNA polymerase (step 1). The strands generated by the forwards and reverse primers separate from the original template and switch templates to the strands extended from the primers (step 2). RNaseH introduces a nick at the RNA site (step 3) and strand displacing reaction is initiated from the nicked site (step 4) and template switching reoccurs (step 5)

Mukai et al (2007) tested the parameters under which ICAN most efficiently amplified DNA and found that ICAN efficiency is highly dependent on both the target gene and the primer concentration. Using the target CSVd target sequence, ICAN produced a 7-fold increase in where primer concentration was increased from 0.2 to 2µM while PCR of the same target DNA produced a 2.8 fold increase. When the target was changed to the c-Ki-ras sequence and yield increased 77 times greater than a PCR of the same target (Uemori et al., 2007). This shows ICAN great efficiency as amplification method.

It has been used in the detection of several bacterial pathogens (Isogai et al., 2005; Urasaki et al., 2008; Inami et al., 2009) as well in the detection of Single nucleotide polymorphisms (SNPs) (Mukai et al., 2008)

ICAN can be monitored using gel electrophoresis as well through the use of a cycling probe labelled with a fluorescent molecule and a quencher molecule. When the probe is present in ICAN it is cleaved and fluoresces. The florescence is directly proportional to the amount of amplicon present (Mukai et al., 2007).

ICAN has a number of advantages. It produces a very high yield of DNA product which is useful when monitoring the reaction, it can also lower the amount of starting template needed and may make it more sensitive for diagnostics (Mukai et al., 2007).The volume of the reaction mixture can be increased without changing reactions conditions because it is an isothermal system which could improve yields and may be important for monitoring.

There are however disadvantage of this system. Firstly the use of probes and florescent dyes increases the cost of the system. These probes also require the use of UV (to excite the florescence) which increases the amount of equipment need and increases the risk of the system. Also the use of an RNase means that a single tube reaction for the detection of RNA targets is not feasible which increases the reaction time to detect a target RNA. The ideal system for the detection of GLRaV-3 is one which is simple and rapid. While ICAN is simple for detection of DNA targets, RNA targets add a level of complexity to the test which makes it unattractive as a means of detecting GLRaV-3.

1.4.2 Strand Displacement Amplification (SDA)

Strand displacement amplification (SDA) is an isothermal technique used for the rapid amplification of target DNA. Similar to ICAN, SDA relies on the introduction of a nick into a primer to initiate amplification. SDA uses 4 primers (S1, S2, B1 and B2) and two HincII and exo- Klenow DNA polymerases. Two of the primers, contain the target binding region at their

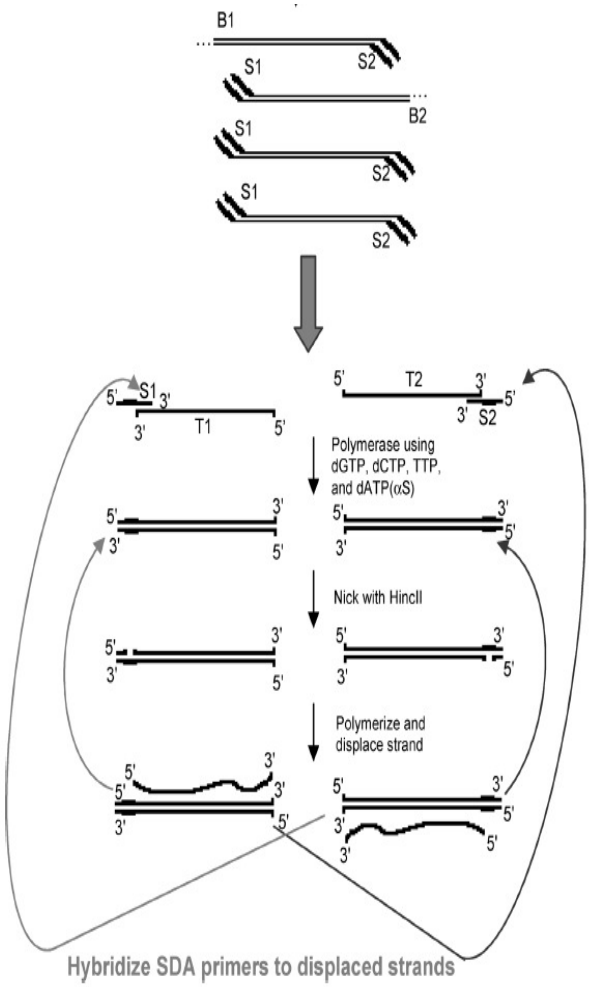

Figure 4: Amplification of nucleic acid using Strand displacing amplification (SDA) (modified from (Gill and Ghaemi, 2008)

3'end and HincII recognition sequence at their 5' end. The other two primers contain only the target binding regions (Walker et al., 1992). S1 and S2 bind to opposite strands (Figure 4) of target and B1 and B2 bind up stream of S1 and S2. Exo$^-$ Klenow then extends all four primers, B1 and B2 extensions cause the displacement of S1 and S2 extensions. S1 extension can then act as a template for S2 and B2 and vice versa for S2-extension. The extension and displacement results in 2 double stranded fragments with a HincII recognition at either one or both ends. HincII then has the ability to nick these sites and exo$^-$ Klenow extends the 3' end. This results in amplification of the target at an exponential rate. The amplified target can be detected using real-time detection (Little et al., 1999). SDA allows for the detection of target DNA when there are between 10 -50 initial target molecules (Walker and Fraiser, 1992)

This method has a number of advantages; target DNA can be either double or single stranded; the amplification is exponential and it has the ability to amplify low target numbers at low temperatures which is not possible in methods such as PCR (without very stringent conditions). However, there are a number of problems associated with SDA. SDA is affected by the amount of non-target DNA present in a sample as background amplifications can compete with specific amplification, which means that target DNA has to be pure and crude extraction methods may not be suitable for use with SDA. The target for

amplification with SDA must be between 50 – 200 nucleotides with every increase of 50 nucleotides over this size resulting in a 10 fold decrease in amplification which has implication for designing primers in targets which have few conserved areas (Walker & Fraiser, 1992). Optimisation of the process is also more complicated than other techniques due to the fact the organic solvent (e.g 1-methyl 2-pyrrolidinone, glycerol and formamide) used in the process affects the efficiency of the amplification. This means that the usefulness and efficiency of the system is affected by the target and means of extraction.

1.4.3 Self- sustained Sequence Replication (3SR)

Self-sustained sequence replication (3SR), based on the replication system of retroviruses, is a self-sustained sequence replication system which uses avian myeloblastosis virus (AMV) reverse transcriptase, RNase H and T7 RNA polymerase to amplify target sequences (Fahy et al., 1991). In this system, an RNA template is replicated using a continuous cycle of reverse transcription and translation with the use of a cDNA intermediate. Oligonucleotide primers, with a T7 polymerase binding site, bind to an RNA template allowing T7 to bind and create cDNA. RNase H degrades the resultant RNA-DNA hybrid allowing the complete synthesis of the cDNA. The cDNA and RNA products then act as templates from which RNA is produced repeating the cycle (Fahy et al., 1991). This exponential replication of the target can lead to 90-fold increase of the target sequence in 105 minutes (Guatelli et al., 1990). 3SR has been shown to be able to detect (when combined with bead-based sandwich capture assay and a rare metal chelated labelled probe) less than 12 copies of purified HIV- 1 RNA and in field tests was shown to detect all cases of HIV, demonstrating its high level of sensitivity (Bush et al., 1992). The system can be manipulated to detect DNA targets with the inclusion of a denaturation step (Guatelli et al., 1990)

As with other isothermal techniques, 3SR has the advantage that it uses a single temperature. It can create a large amount of template rapidly and if combined with a real-time detection system would allow for rapid detection system. Its major advantage over methods such as Loop mediated

amplification of Nucleic acid (LAMP) is that is produces a single stranded product which makes downstream use of the product (such as in direct sequencing) easier (Taylor & Gill, 2008).

The disadvantages associated with this system are firstly that it requires 3 enzymes which effects optimisation of the procedure and increase the overall cost. One of the greatest disadvantages of the system is the low temperature, which lower the anneal temperature of the primers and ultimately the specificity of the reaction (Fahy et al., 1991)

All of these methods have high sensitivity; however their low specificity (in the case of SDA and 3SR) or sensitivity to biological components means that they may not be ideal as a high through-put diagnostic test.

1.4.4 Loop- Mediated isothermal amplification of Nucleic Acid (LAMP)

LAMP is a means of amplifying nucleic acid using a strand displacing DNA polymerase and 4 or 6 primers targeting 6 or 8 target regions (Notomi et al., 2000) under isothermal conditions. This method is sensitive, specific, rapid and does not require expensive equipment which makes it ideal for in-field diagnostics.

LAMP relies on an auto-cycling strand displacement which is achieved by through the use of a DNA polymerase with strand displacing activity and 2 inner primers and two outer primers (Notomi et al., 2000). Amplification rate and specificity can also be increase with the use of a third set of loop primers (Nagamine et al., 2002).

1.4.4.1 LAMP mechanism

1.4..4.1.1 LAMP primers

The LAMP mechanism is heavily reliant on the design of the inner and outer primers. Figure 2 shows the design of the LAMP primers. The Inner primers (consisting of a Forward inner primer (FIP) and Backwards Inner Primer (BIP)) contain two distinct sequences which correspond to the sense and antisense sequences of the target linked with a –TTTT- region. For the purposes of

explaining the LAMP mechanism the Inner primers have been broken into two regions; the Inner regions (F2 and B2) and the Outer regions (F1c and B1c). F2 sequence is complementary to F2c on the positive sense strand of target DNA and B2 region is complementary to the B2c region on the anti-sense strand of target DNA. F1c is complementary to the F1 region on the anti-sense strand and B1c is complementary to the B1 region on the sense strand. The Outer primers are designated F3 and B3 and are complementary to the F3c and B3c regions respectively.

In addition to designing primers to a conserved area of the genomes, everal other parameters must be considered when designing LAMP primers. The GC content of the primers should be between 50–60% in the case of GC rich sequences or 40–50% for AT rich sequences (Parida et al., 2008). Further the primers should be designed so as to avoid the formation of secondary structures. Further the 3' end sequence of the primers not be complementary to any of the other primers and should not be AT rich. Lastly the distance for the loop forming region should be vary between 40–60bp. It order to confirm the amplified products, restriction enzyme site on the target sequence can be used too.

1.4.4.1.2 Loop-mediated amplification

LAMP can be separated into two stages; an initial stage where the inner and outer primers create a dumbbell structure that is required for the cycling stage and a cycling stage where the inner primers are used to create the long, concatenated products, characteristic of LAMP.

1. **Initial stage**

In the initial stage (Figure 6), FIP binds to F2c and complementary strand synthesis is initiated. Subsequently F3 (which is shorter and at a lower concentration than FIP) binds to the F3c region, initiating strand displacing DNA synthesis and releasing the FIP-linked complementary strand (Figure 6 step 2). Once the FIP-linked strand is released, the F1c segment of the primer binds to the F1 sequence of the target, forming a single sided loop (Figure 6 step 4). The BIP binds to the FIP-linked strand at B2c initiating DNA

synthesis, after which the B3 primer binds to B3c initiating strand displacing DNA synthesis, releasing the FIP and BIP bound target sequence which forms a dumbbell-like structure (Figure 6 step 6) which is then used in the cycling amplification stage (Notomi et al., 2000).

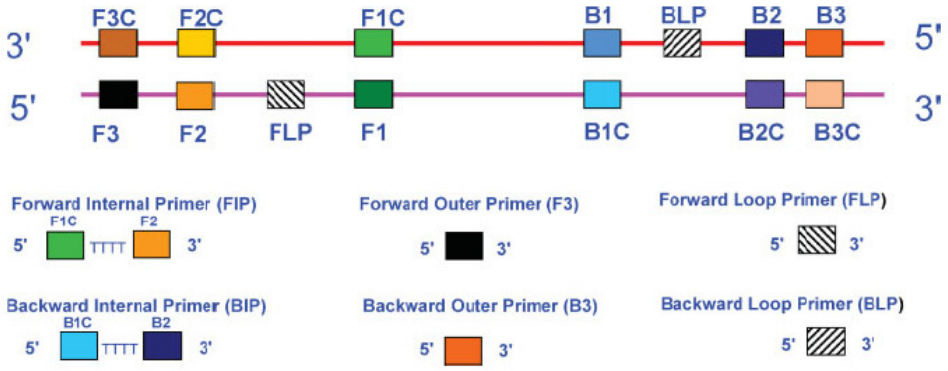

Figure 5: Schematic representation of LAMP primers (Modified from Parida et al. 2008)

2. **Cycling stage**

The Cycling stage creates large amounts of concatenate inverse repeats of target sequence which vary in size (Notomi et al., 2000). The LAMP cycling stage is initiated when the FIP binds to the FIP-linked loop on the Dumbbell-like structure created in the Initial stage (Step 8, Figure 6 (B)). This binding initiates strand displacement synthesis and creates a stem-loop DNA structure with an additional copy of the target sequence (inverted) and a loop (from the BIP sequence) at the 5' end. Subsequent self-priming creates an additional dumbbell structure and a stem loop structure which acts as the template for BIP-primed strand displacement synthesis. The process of elongation and recycling results in 3x amplification of the target sequence every half cycle (Notomi et al., 2000).

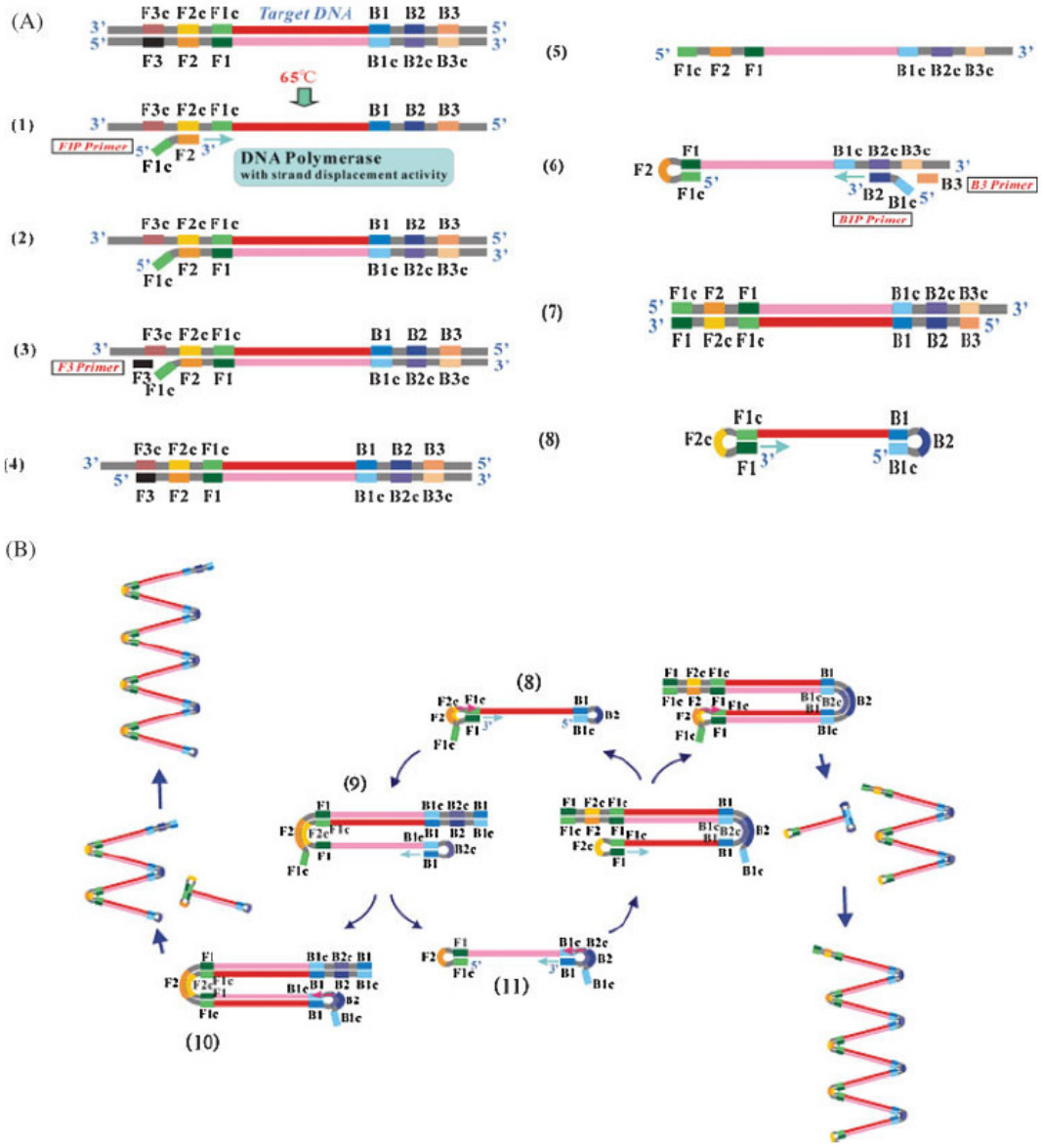

Figure 6: Principles of LAMP mechanism. Initial Stage (A) (1-8): Generation of stem-loop dumbbell structure that enters the cyclic step. Cycling stage (B) (8- 11) Exponential amplification of Dumbbell structure producing cauliflower- like concatenate products of varying sizes (Eiken Ltd.)

1.4.4.2 RT-LAMP

In the original protocol by Notomi et al. (2000) LAMP was used to amplify a DNA template in under an hour. However, with the addition of a thermostable reverse transcriptase, LAMP can also be used to detect RNA in a one-step protocol (RT-LAMP) (Parida et al., 2005). Originally developed for the detection of West Nile virus, RT-LAMP has been used in the detection of positive sense and negative sense single stranded RNA viruses from animal,

human and plant hosts (Soliman & El-Matbouli, 2006; Teng et al., 2007; Li et al., 2009; Cardoso et al., 2010; Liu et al., 2010). While both MULV and AMV have been tested for use with LAMP, AMV is more efficient and has improved sensitivity when compared with MULV (Parida et al., 2005). RT-LAMP has further been shown to have higher sensitivity and rapidity in comparison to RT-PCR (Parida et al., 2005)

1.4.4.3 Monitoring LAMP

There are several methods of monitoring LAMP reactions; conventional detection using gel electrophoresis, the visual inspection of turbidity (Mori et al., 2001), the addition of intercalating dyes (Nagamine et al., 2001) as well as the use of colourimetric indicators (Tomita et al., 2008; Goto et al., 2009)

Gel electrophoresis

Gel Electrophoresis is the most sensitive method of visualising LAMP amplicons (Tomlinson et al., 2010; Wastling et al., 2010). LAMP produces cauliflower- like concatenate products which can be visualised as a ladder on a 2% agarose gel. However this method has a number of disadvantages; it is impractical where large numbers of samples must are processed; basic laboratories are unlikely to own gel electrophoresis tanks and running of gels is complicated, adding to the technical skill required to use LAMP. Lastly LAMP is a very sensitive technique which produces a large amount of product and as such is prone to contamination (Tomita et al., 2008) therefore it is strongly advised by many authors that the tubes containing LAMP reactions should never be opened once the LAMP reaction has been run. For this reason other simpler, more time efficient ways of monitoring LAMP have been developed.

Turbidity

Mori et al. (2001) observed that when DNA was amplified using LAMP the reaction became turbid (figure 7); further studies showed that this was caused by the amount of magnesium pyrophosphate ions released during DNA amplification. This is the simplest method of monitoring a LAMP reaction. The turbidity is caused by the release of pyrophosphate ions as by-product, which bind with magnesium ions in the solution forming magnesium pyrophosphate which creates a white insoluble precipitate during nucleic acid amplification (Mori et al., 2001). Magnesium pyrophosphate may also be observed as a white precipitate when turbid tubes are spun down briefly in a centrifuge.

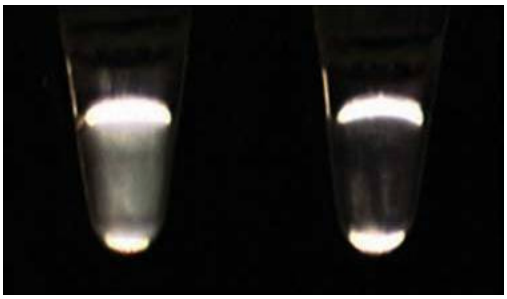

Figure 7: Visualisation of LAMP products using turbidity. Left: Positive Sample, Right: Negative (Image modified from

Turbidity has been used in determining presence of a target (Maeda et al., 2005; Higashimoto et al., 2008; Wastling et al., 2010) as well as in the quantification (Mori et al., 2004). Turbidity can be observed using the naked eye or confirmed using a spectrophotometer where a negative sample can be compared to the sample and the values compared, in order to determine quantity of target present (Mori et al., 2001). Real-time turbidity meters designed specifically to measure turbidity in LAMP reaction have been developed which are also able to quantify the amount of starting template (Mori et al., 2004)

In a study done on detection limits on Human African Trypanosomiasis it was shown that the detection limit (i.e. the end point at which turbidity could still be visualised) was the same as that of gel electrophoresis (Wastling et al., 2010). However, instances have been found where turbidity has not been observed in samples which are known to be positive. This is problematic when developing a diagnostic test for in field testing where it is not possible to

confirm every results using a method such as gel electrophoresis (Wastling et al., 2010). Also, interpreting results takes a certain amount of skill and can produce varying results depending on the user, most importantly leading to false positives and negatives (Paris et al., 2007) therefore other methods which rely on colour changes and/or florescence have also been developed (Goto et al., 2009)

Intercalating Dyes

Several intercalating dyes have been used in conjunction with LAMP for the detection of pathogens including ethidium bromide (EtBr) (Nagamine et al., 2001), SyBR green I (Iwamoto et al., 2003) and Quant-iT PicoGreen (Wastling et al., 2010), Goldview staining (Shi et al., 2011). These dyes are added post-incubation and bind to the product, eliciting a response (such as a colour change, or fluoresce under UV light, which can be monitored). While EtBR was originally used in LAMP, SYBR green I is the most commonly used intercalating dye because of its ability to change colour in natural light.

SYBR green I is an intercalating dye which is added to the LAMP post-incubation and will, in the presence of amplified product, change from orange to green (Iwamoto et al., 2003). This change is easily seen naked eye and removes the need for equipment to monitor the LAMP reaction. Iwamoto et al., (2003) showed that this method of detection is slightly less sensitive than gel electrophoresis but has the advantage that it is more convenient than gel electrophoresis. The main disadvantage of this method is that it is added post-incubation and this increases the risk of contamination of the test.

GoldView staining has also been used to evaluate LAMP reaction, and has been specifically used in evaluating reactions for RNA extraction. Samples with florescence change from orange to red under UV light and differ from other intercalating dyes in that Goldview staining can emit green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA.

Calcein

Figure 8: Visualisation of LAMP products using Calcein, Left: negative, Right: Positive (Modified from image at http://loopamp.eiken.co.jp)

Calcein is a metal indicator which, when bound to divalent metallic ions, emits a strong florescence (Tomita et al., 2008) At physiological pH's, Calcein fluorescence can be quenched by a number of ions including; Fe3+, Co2+, Cu2+ and Mn2+, while it is unaffected by Mg2+ and Ca2- (Morgan, 2000). Calcein (quenched by manganese) is added to a LAMP reaction (pH 8.8) at the beginning of the reaction. During the LAMP reaction large amounts of pyrophosphate, which has a higher affinity for Mn^{2+} than the Calcein molecules, are released and the Calcein, no longer quenched by the Mn^{2+}, fluoresces (figure 8). This fluorescence is further enhanced by its binding to the free Mg^{2+} ions (Tomita et al., 2008). Calcein emits its fluorescence at a wavelength of 515nm that can be seen when the LAMP reaction tube is placed under a UV lamp. Calcein has an advantage over other fluorescent dyes such as SYBR green and EtBr in that it can be added at the beginning of the test which reduces the risk of contamination which is so pervasive with the LAMP method.

Calcein has been used in conjunction with LAMP for the detection of several pathogens (Parida et al., 2005; Yoda et al., 2007; Gu et al., 2010; Wastling et al., 2010) . The use of calcein as an indicator has had varying results, with some studies showing that LAMPs which include calcein are as sensitive as the routine diagnostic RT-PCR (Yoda et al., 2007) while other reports have suggested that the addition of calcein lowers the sensitivity of the test and that other indicators are better suited for use with LAMP (Wastling et al., 2010). Wastling et al. (2010) also found that the fluorescence levels varied and that it was difficult to distinguish between some positive and negative samples. It is important that results be unequivocal when developing a diagnostic test which is going to be used outside of diagnostic labs where there are no other available methods of confirming results.

Hydroxy napthol blue

First reported by Goto et al., (2009), hydroxy napthol blue (HNB), a metal indicator used in measuring of Mg^{2+} ions, has been used as an indicator in a number of LAMPs used to detect bacteria and viruses (Harper et al., 2010; Tomlinson et al., 2010; Wastling et al., 2010). The production of magnesium pyrophosphate during LAMP results in an overall reduction in the amount of Mg^{2+} in solution. HNB can be used as a colourimetric indicator of this reduction as in the presence of high concentration of Mg^{2+} HNB is violet in colour and as Mg^{2+} concentration decreases HNB changes to sky blue (Goto et al., 2009) (figure 9). This colour change which occurs during a LAMP reaction, is easily discernible and it has been found to be easier to identify than other indicator methods (Goto et al., 2009; Wastling et al., 2010) HNB is added to a LAMP reaction at the beginning of the reaction and therefore is aids in the prevention of contamination of LAMP reactions as the tubes do not need to be opened post amplification.

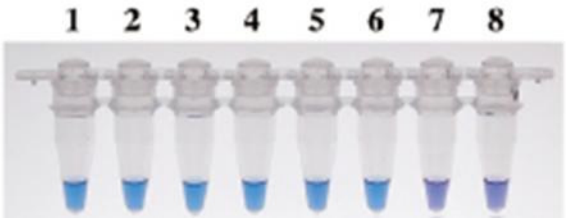

Figure 9: Visualisation of LAMP products using Hydroxy napthol blue. Tube 1- 6: Positive reaction, Tube 7-8: Negative reaction. (Modified from Goto et al. (2009))

LAMP is a highly sensitive, highly specific technique but some of the indicators have been shown to either inhibit amplification of target at low concentrations, or do not display a positive reaction where gel electrophoresis shows that amplification has occurred (Wastling et al., 2010). The use of HNB has shown mixed results with some experiments suggesting that HNB is not suitable for use where the target is at a low concentration (Harper et al., 2010) while others suggest that HNB is as sensitive as gel electrophoresis (Tomlinson et al., 2010). Tomlinson et al., (2010) also showed that in ambiguous cases the colour distinction can be increased by freezing the tube, and hence it is an ideal candidate as an indicator where only minimal lab equipment (i.e. in a field test situation) is available.

1.4.4.4 Optimised conditions for LAMP

LAMP specificity and efficiency is dependent on primer design. In order for LAMP to function most efficiently the T_m (melting temperature) of the Inner Primers should be higher than that of the Outer primers and should fall within the 60-65°C range (within the optimum range for Bst DNA polymerase) (Notomi et al., 2000). Further, the T_m of the F1c and B1c sequence should be slightly higher than F2 and B2 to ensure that the stem-loop structure forms immediately (Notomi et al., 2000). Primer concentrations also affect the efficiency of LAMP and the ratio of Outer: Inner primer should be kept at a 1:4 – 1:10 (Notomi et al., 2000).

Due to the strand displacing synthesis employed by LAMP, the size of DNA target will affect the efficiency rate of LAMP (Notomi et al., 2000). LAMP target size can range between 130 and 500bps however for the most efficient amplification targets should be between 130 and 300 bps (Nie, 2005; Notomi et al., 2000).

Chemicals which destabilize the DNA helix have been found to improve the amplification efficiency in LAMP as well as increase specificity (Notomi et al., 2000). Both betaine (N,N,N-trimethylglycine), an iso-stabilising agent which reduces the effect of high GC content in the target sequence (Frackman et al., 1998) and L-proline, which reduces based stacking (Notomi et al., 2000) have been shown to increase LAMP efficiency however the effect of Betaine on efficiency is greater than L-proline (Notomi et al., 2000).

Magnesium ion (Mg^{2+}) concentration plays a role in functioning and efficiency of LAMP reactions (Nie 2005; Gunimaladevi et al., 2005; Teng et al., 2007; Liu et al., 2010). Nie et al. (2005) studied the effects of Mg^{2+} on a RT-LAMP for Potato Y virus and found that if Mg^{2+} concentration is less than 4mM, LAMP will fail to amplify the target, this result has been confirmed in other studies which found that there is a minimum concentration below which LAMP will not occur (Nie 2005; Gunimaladevi et al., 2005; Teng et al., 2007; Liu et al., 2010) however this varies depending on the specific primers. Further, Nie et al., (2005) found that Mg^{2+} can also adversely LAMP were concentrations

become too high (Liu et al., 2010). Mg^{2+} concentration is known to affect primer annealing and enzyme activity and as LAMP is so highly dependent on primer design and annealing temperatures, optimisation of Mg^{2+} concentration is very important to when designing a efficient, specific diagnostic test.

LAMP reactions should also be optimised for temperature as the incubation temperature will affect the efficiency of the DNA polymerase. Bst DNA polymerase can amplify products between 50ºC and 65ºC however has been shown to work most efficiently between the 60-65ºC (Liu et al., 2010; Mori et al., 2001).

The incubation time for LAMP have been shown to vary; with some groups showing results in less than 30 minutes (Nagamine et al., 2002) while others only observed a reaction after 45 minutes- 1 hour (Soliman and El-Matbouli, 2006). Reaction time can be significantly decreased with the use of Loop - primers, however these primers add to the cost of the over-all reaction and are not necessary as LAMP reaction times is still very short. Gunimaladevi et al.,(2005) showed that while product can be observed after 45 minutes, 60 minutes produced significantly more product which is more easily monitored using visual detection systems such as turbidity.

While the effects of deoxynucleotide (dNTP) concentration have been studied in PCR, little study has been done on the effect of differing concentration of dNTPs in LAMP reactions. Notomi et al (2000) used a concentration of 1.4 mM dNTPs and most LAMP reactions follow this guide. Nie (2005) studied the effect of varying concentrations of dNTP on a LAMP for detection *Potato virus* Y and found that at a concentration of 0.2 mM for each individual dNTP, visible ladder-like DNA fragments were seen could be seen on 2% agarose gel and that the amount of product increased until 0.4 mM after which no further increases were seen until 0.6mM where the efficiency of the LAMP began to decrease.

1.4.4.5 Sensitivity and Specificity of LAMP

The specificity of LAMP can be directly attributed to the fact that LAMP uses either 4 or six primers (depending on whether loop primers are also used) which bind to either 6 or 8 specific regions on the target (Mori & Notomi 2009). All primers are needed to bind for amplification. In general, because it is not possible to determine that LAMP products are correct using gel electrophoresis, it is assumed that because LAMP requires all primers to bind, amplicon produced are the correct product. However, most groups use restriction enzyme digestion to confirm LAMP has amplified the correct product (Enosawa et al., 2003; Saitou et al., 2010). Further, the product of the restriction digest can be sequenced which confirm the results based on size, or alternatively the product can be sequenced (Boldbaatar et al., 2009; Saitou et al., 2010).

Alternatively, LAMP products can be analysed using melting- curve analysis (Uemura et al., 2008). LAMP products can be characterised by melting curve analysis because double-stranded DNA have a specific melting temperature (defined as temperature at which 50% of DNA will become single-stranded). which is determined using both base sequence and GC content. Primer-dimers and non-specific products will be observed as different peaks with to LAMP products.

The sensitivity of LAMP is dependent on primer design and optimisation. However, in general it has been shown that LAMP is at least as sensitive as PCR (with detection limits of between 0.01-10 pfu of virus) (Parida et al., 2008).

1.4.4.6 Detection of Plant Pathogens using LAMP

LAMP is a highly robust technique and has been used in the detection of a number of plant pathogens included viruses and bacteria (Nie, 2005; Tomlinson et al., 2010). It has been shown to be a highly efficient method of detection, comparable with that of RT-PCR and more sensitive and more efficient than ELISA (Nie, 2005; Liu et al., 2010).

Nie (2005) developed a RT-LAMP for the detection of Potato Virus Y and showed that it was more robust than the standard ELISA and had results comparable to that of RT-PCR. However the RT-LAMP did not detect all tubers known to be infected. This was attributed to the fact that Potato Y virus is known to be found in low titres in the tuber and therefore can be difficult to detect. The authors stressed the importance of the sampling method. GLRaV-3 is also found in low titres and it is important that this is taken into consideration when developing a RT-LAMP for its detection.

1.5 Crude extraction of Nucleic acid

At the moment, diagnostics for grape vines are carried out in very basic field laboratories. These laboratories have electricity, water baths or incubators and fridges to maintain samples, however have very little in the way of specialised equipment. In order for a LAMP to be useful in such a basic setting where the majority of the staff have little training in diagnostics, it is important that the LAMP testing protocol is simple. This includes the extraction of nucleic acid, which needs to be simple and efficient but still produce good quality nucleic acid. LAMP has been shown to be less sensitive to biological inhibitors than PCR and therefore crude DNA extraction methods can more readily be used with this system, making it more time efficient and lowering the cost of the system (Kaneko and Kawana, 2007).

In existing LAMP tests, several methods of crude nucleic acid extraction have been used including; the Cetyl trimethylammonium bromide (CTAB) method and with lateral flow devices, where samples are ground with simple extraction buffers and a small piece of this membrane is simply added to the LAMP reaction (Tomlinson et al., 2010). These simple methods of extraction increase the amount of samples which can be process in a day and increase the efficiency with which large batch samples can be processed at a relatively low cost. In order for GLRaV-3 RT-LAMP to be cost effective and for it to be able to compete with ELISA, it is essential that the sample preparation be as simple as ELISA. With crude RNA extraction, this may be possible.

1.6 Advantages of LAMP

LAMP is a highly specific, highly sensitive technique which, because of its isothermal nature, does not require any sophisticated equipment. It has a very low detection limit which makes its sensitivity comparable to PCR (Notomi et al., 2000) and (in some cases) qPCR (Francois et al., 2011). This sensitivity is less effected by the presence of non-target DNA than other techniques with the same sensitivity (Notomi et al., 2000) . Visualisation of results can be done in real time through a number of means including; turbidity, intercalating dyes and colourimetric indicators which mean that results are quickly available and easy to interpret. Through the addition of loop primers the time to a positive result can be lower and the specificity of LAMP can be increased (Nagamine et al., 2002). LAMP has been shown to be more robust than other diagnostic technique in circumstances where samples are crudely extracted or where inhibitory substances are present, which makes the test ideal for field testing where quick, simple processing of samples would be preferred (Francois et al., 2011). LAMP is versatile and can be used for the detection DNA and with the addition of reverse transcriptase can detect RNA in a single tube reaction.

1.7 Disadvantages of LAMP

LAMP has a number of disadvantages associated with it. The first disadvantage associated with LAMP primers. Firstly LAMP primer design can be complicated (despite the availability of software designed specifically for the design LAMP primers). This is due to the number of primers which are required in conjunction with link between efficiency and product size. Depending on the target sequence and the amount of sequence information which is available, it can be challenging to find six conserved sequences in a small area (Parida et al., 2008). Secondly, the amount of primers need for LAMP can increase the cost of test per sample.

A second disadvantage ironically lies in the strong points of the technique viz. that LAMP is a highly specific, highly sensitive technique which produces a large amount product in a very short amount of time. The high sensitivity of LAMP in conjunction with the production of high amounts of product, leads to a high risk of cross sample contamination. Many authors suggested that

LAMP tubes should never be opened once the reaction has been initiated. This can create problems, especially in a situation where further work has to be done of the products, where a negative result has to be confirmed (for example where no turbidity is observed) or where positive results are being confirmed using agarose gel electrophoresis.

CHAPTER 2: Rapid Detection of Grapevine Leafroll-associated virus Type 3 using a reverse transcription loop-mediated amplification (RT-LAMP) method.

(This chapter has been submitted and accepted as a manuscript for publication to the Journal of Virological Methods, and has been formatted in accordance with that Journal's specifications)

2.1 Introduction

Grapevine leafroll disease (GLD) is the most important disease of grapevines, occurring in every grape-growing country (Martelli, 1993). GLD affects grapevines adversely, where it delays the maturation of the berries, decreases the accumulation of sugars and affects the overall yield and quality of the fruit (Over de Linden and Chamberlain, 1970). This negatively impacts on the wine industry, where it affects the quality and colour of the wines, and the table grapes industry, where yields are affected (Golino et al., 2002).

Globally, five serologically distinct, phloem limited viruses designated Grapevine Leafroll-associated viruses (GLRaV) 1-4 and 7, are associated with GLD (Fuchs et al., 2009a; Martelli et al., 2012, 2002) and of these, GLRaV-3 is the most common (Cabaleiro et al., 2007). GLRaV- 3 is part of the Closteroviridae family, where it is type species for the *Ampelovirus* (Martelli et al., 2002). Studies of the genetic diversity of GLRaV-3 has shown that GLRaV-3 can be separated into five phylogenetic groups(Turturo et al., 2005; Jooste et al., 2010; Gouveia et al., 2011;). These groups do not seem to be geographically isolated and Group 1 isolates seem to be the most prevalent (Turturo et al., 2005). Initially it was thought that GLRaV-3 was only spread through the use of infected plant propagation material; however it is now known that several species of mealybugs and scale insects act as semi-persistent vectors for the virus with varying efficiencies (Cabaleiro and Segura, 1997a; Fuchs et al., 2009a; Golino et al., 2002; Walton, 2004).

GLD is the most important disease of grapevines in South Africa and, as it is the most prevalent virus associated with the disease, GLRaV-3 is considered the most important virus associated with GLD (Pietersen, 2004). A study of the variation of GLRaV-3 isolates in South Africa showed that three genetic variants of GLRaV-3; Group 1, 2 and 3; are present in South African Vineyards (Jooste et al., 2011). Several insects are known to vector the virus in South Africa, however the mealybug *Planococcus ficus* is considered the most important (Douglas and Krüger, 2008).

GLD occurs in all grape varieties, however symptom expression can vary greatly (Over de Linden and Chamberlain, 1970). In red wine cultivars, symptoms are usually expressed as interveinal reddening and down rolling of the leaves and are most distinct in autumn. However white wine cultivars tend to be asymptomatic (with the exception of a few varieties). GLD is usually also symptomless in most American wild *Vitis* sp. used as rootstocks and their hybrids (Krake et al., 1999). This variation in symptoms complicates control of the disease as asymptomatic infected individuals can only be detected using either serological (e.g. ELISA) or molecular (e.g. PCR) methods before they can be removed.

In 2002, a study was conducted at a commercial wine farm to prove that GLD could be controlled using an integrated control strategy (Pietersen et al., 2013). The strategy involved the planting of certified material, control of the vector through the use of systemic insecticide and the removal of infected vine material by rogueing (Pietersen and Walsh, 2012; Pietersen et al., 2013). This has been shown to be highly effective for the control of GLD in red cultivars, where symptomatic plants are identified on a vine- for- vine basis at the beginning of autumn each year (Pietersen and Walsh, 2012; Pietersen et al., 2013). However in white cultivars control is more problematic, due to the lack of symptom expression in the majority of varieties.

Currently each season infected white cultivars are identified using Enzyme-linked immunosorbent assay (ELISA) (Ling et al., 2000) before rogueing (Pietersen et al., 2013; Pietersen and Walsh, 2012). ELISA tests are (usually)

performed by cell technicians on the wine farms which have basic facilities such as water baths and fridges but do not have specialised equipment (e.g thermocyclers) which makes diagnostic test such as RT-PCR unfeasible. ELISA is simple and inexpensive (as it requires very little equipment) and can be used for a large number of samples. However, it is less sensitive than molecular techniques (Arora et al., 2006) and is time consuming. Reverse transcriptase polymerase chain reaction (RT-PCR) is sensitive and is less time intensive than ELISA but requires specialised equipment and is more complex than ELISA, usually being performed by trained technicians. The ideal detection technique needs to simple, rapid and specific and would ideally give results in real-time but would not require specialised equipment.

Loop-mediated Amplification of Nucleic acid (LAMP), a rapid, specific simple means of amplifying nucleic acid, has emerged as a powerful diagnostic technique. (Parida et al., 2008). LAMP relies on the a strand displacing DNA polymerase in conjunction with 4 primers (which target 6 specific areas on the target) to amplify target nucleic acid under isothermal conditions within a short period of time (Notomi et al., 2000).

LAMP can be monitored in a number of ways; LAMP amplicons can be viewed using gel electrophoresis; through visual inspection by inspecting turbidity (Mori et al., 2001), colourimetric indicators (Goto et al., 2009; Iwamoto et al., 2003) or intercalating dyes (Maeda et al., 2005).

LAMP has shown to be a highly versatile diagnostic technique and has been used in the detection of a wide variety of pathogens (Parida et al., 2008). There are a number of advantages associated with LAMP; it is isothermal so does not require specialised thermocycling equipment and tests can be heated in a simple heating block or a water bath. The system is highly efficient with no time lost for cycling between temperatures. Secondly because it requires at least 4 primers, it is highly specific. LAMP has also been shown to be more robust than other molecular based techniques and is less affected by biological substances which have been shown to be inhibitory in other techniques (Francois et al., 2011)

This paper reports on the development of a rapid detection technique for GLRaV-3 through the combination of a crude nucleic acid extraction protocol with RT-LAMP and colourimetric assay. This technique has been shown to be rapid, efficient and can reduce the time needed to test a sample from two days by ELISA to two hours with sensitivity comparable to that of nested RT-PCR. This technique may provide an alternative to ELISA and contribute the control of GLD in white cultivars.

2.2 Materials and Methods

2.2.1 Plant Material and Nucleic Acid

Petioles were collected from grapevines infected with GLRaV-3 kept in the glasshouses at the University of Pretoria Experimental Farm, Pretoria (01-2839, 01-0257, 01-2639); as well as from the glasshouses at the Agricultural Research Centre (ARC) Plant Protection Research Institute (PPRI), Roodeplaat (623, 621, PL-20, GH 30, 74/2/56, 37/71/84, 74/02/02, 93/0904/ 74/7/56, 92/1027/ 74/2/56). Bark scrapings infected with GLRaV-3 were obtained from dormant material from PPRI (623, 621, PL-20). GLRaV-3 strain PL-20 plasmids (pGEM plasmid, Promega) containing the target area (nucleotide positions 5876-8286 on PL-20 genome GQ352633) for the LAMP primers (designated F1, F8 and F9) were obtained from Elize Jooste at the PPRI (Jooste et al., 2010), and were used to assess the LAMP reaction, separately from the reverse transcriptase step.

2.2.2 LAMP Primer Design

Primers for LAMP on GLRaV-3 were designed using the 6 available GLRaV-3 whole genomes; GP18 (EU259806) (Maree et al., 2008), 621 , 623, PL-20 (GQ352631, GQ352632 and GQ352633) (Jooste et al., 2010), Cl- 766 (EU344893) (Engel et al., 2008) and NY-1 (AF037268) (Ling et al. 1998). The genomes were aligned ClustalW in Bioedit (Version 7.0.8, Ibis Bioscience, Carlsbad) and areas with high similarity (> 90%) were used as targets. The gene region for RdRp (RNA dependent RNA polymerase) was found to have the highest similarity and was used to design primers. LAMP primers were

then designed using using Primer Explore V4 (http://primerexplorer.jp/elamp4.0.0/index.html). Possible primers were then compared to available GLRaV-3 genomes (as well as GenBank database) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and primers with greatest specificity were selected (> 98% homology for all primers) (Table 2) and synthesised by IDT (Iowa, USA)

Table 2: Primer sequences for a reverse transcriptase loop-mediated amplification (RT-LAMP) of *Grapevine leafroll -associated virus* type 3 (GLRaV-3)

Primer Name	Type	Position*	Sequence (5'-3')
F3	Forward Outer Primer	7728 - 7747	GAAGTGTAACCTCGTCACGT
B3	Backward Outer Primer	7941-7958	GCCCGCTTGAGAGACTTG
FIP	Forward Inner Primer (F1c – TTTT- F2)	F1c: 7793 - 7813 F2: 7752 - 7771	CATGCGCCACAGAGTCGTCACTTTT-AAATGGGAATTTCAACGCCG
BIP	Backward Inner Primer (B1c- TTTT-B2)	B1c: 7851 – 7871 B2: 7909- 7928	GCTCGTTTAGCAGAGGTGACGGTTTT-GCCCTTTTGTCCAACCAATC

* Genome position according to GLRaV-3 strain 621 complete genome sequence (GenBank accession number GQ352631)

2.2.3 RT-LAMP

The final LAMP reaction mixture (25ul total volume in a 0.2ml tube) was made up as follows; 1.6µM FIP and BIP, 0.2µM F3 and B3, 8U Bst (Lucigen, Middleton, WI) and 1x Bst Buffer B (20mM Tris-HCl (pH 8.8), 10mM $(NH_4)_2SO_4$, 10mM KCl, 2mM $MgSO_4$ and 0.1% Triton X-100) (Lucigen), 1M Betaine (Sigma- Aldrich, St Louis, MO, USA), 1.4mM dNTPs (Promega, Madison, WI USA), 7mM $MgCl_2$ (Fermentas, Vilnuis, Lithuania), 120µM Hydroxy naphthol blue (HNB) (Acros Organics, Geel, Belgium), 10U AMV

reverse transcriptase (Roche, Palo Alto, CA USA) and 2µl of RNA. The mixture was then incubated at 60℃ using a heating block (Eppendorf Thermostat Plus 3130, Hamburg, Germany) for 1 hour followed by heating to 80℃ for 10 minutes to terminate the reaction. Results were analysed by a visual comparison of the colour change of samples to a either a healthy control or a negative (water) control. In order to prevent contamination LAMP mixtures were prepared in a separate laboratory from sample processing and post- LAMP visualisation.

2.2.4 Optimisation of LAMP

The LAMP reactions were optimized by assessing different incubation temperatures as well as the concentration and ratio of inner: outer primers and Mg^{2+} (4mM- 10mM). The temperature optimisation (using the optimum primer concentration) was carried out 60, 61, 62, 63, 64 and 65˚C for 1 hour and results were analysed using turbidity and confirmed using 2% agarose gel electrophoresis. All the optimisation reactions included negative controls; where no DNA template was present (water control) and the pGEM plasmid without the target region insert.

2.2.5 Visualisation of LAMP products

Products of LAMP were visualised using three different methods: 1) agarose gel electrophoresis where 5µl of sample was loaded per well of a 2% Agarose gel pre-stained with ethidium bromide at 5ug/ml and electrophoresed for 35 min at 100V in 1 x TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.2). Results were viewed using a UV-transilluminator (310nm) , A wide-range molecular weight DNA marker (100-bp ladder) (Inqaba Biotechnological Industries, South Africa) was used on each gel as the standard; 2) Turbidity; where the turbidity of positive samples were visually compared with two negative controls which contained either no template or pGEM plasmid without the target region. These LAMP reactions were also centrifuged for 5 minutes at 10 000g (Eppendorf 5804 centrifuge, Hamburg Germany) and each tube examined for a white pellet; and 3) the addition of HNB, prepared according to Goto et al. (2009), with the results visually analysed through

comparison of the samples to the negative control. HNB results were initially confirmed using 2% agarose gel electrophoresis.

2.2.6 Crude RNA extraction

Several methods were evaluated to select a reliable procedure to prepare crude RNA. For each method petioles were selected from a sample with a high viral titre (01-0257), a sample with a low viral titre (01- 2639), and a healthy negative control (01-2839) were included.

The optimal method assessed was; extraction 0.2g of sample homogenized in 5ml of extraction buffer (PBS, pH 7.2, supplemented with 2% (w/v) polyvinylpyrrolidone (PVP-10) and 0.2% (w/v) sodium diethyl dithiocarbamate) (Bertolini et al., 2001) in filter separated plastic bags (Bioreba, Reinach Switzerland), using a Homex 6 homogenizer (Bioreba), 30µl of this sample was added to nitrocellulose membrane (Sigma-Aldrich) which was dried for between 30 minutes. A $3mm^2$ disc was punched out using paper punch (Roche, Germany) which was added to 100µl of GES buffer (Osman and Rowhani 2006) and incubated at 95℃ for 10 minutes. 2µl of extract was added to RT-LAMP. This method gave the most reliable results and was used in all further testing.

Other methods tested included ; 1) the print capture procedure according to La Notte (1997), and several methods of RNA extraction including; 2) a simple extraction in an alkaline buffer (50mM NaOH, 2.5mM EDTA) (Turturo et al., 1998) according to Singh et al. (2006); 3) viral immobilization on nylon membrane using extraction buffer (Osman and Rowhani, 2006); 4) use of petioles used instead of midrib tissue in a modification of the method of Habili et al. (1997) ; 5) extraction of 0.5g of sample, ground in extraction buffer (50mM EDTA, 100mM Tris-HCl, 500mM NaCl and 10 mM β-mercaptoethanol) (Al-Musa et al., 2008); 6) maceration of 0.5g of sample in GES buffer (Osman and Rowhani 2006);and 7) grinding of 1g of sample in grapevine ELISA extraction buffer (0.1M Tris-HCl pH 7.6, 0.01M $MgSO_4$, 4% polyvinylpolypyrrolidone (PvPP (insoluble), 0.2% ß-Mercapto-ethanol (ME), 2% Triton – X100) (Ling et al., 2001) after which it was centrifuged

(Eppendorf) for 5 minutes at 10 000g and 1µl of sample was added directly to the RT-LAMP.

2.2.7 Pooling of Samples

LAMP samples were pooled by processing individual samples according to the optimal method above and pooling 10µl of each GES extract. Alternative methods tested included; pooling petioles by combining 6 petioles from each sample and adding 0.2g of combined petioles to Extraction buffer and; pooling extracted plant saps (100ul of plant in extraction buffer) before adding to 30ul of combined saps to nitrocelluose membrane. Pooling of GES extracts had greatest consistency (results not shown) and was used for all further testing.

In order to pool the samples being tested in parallel with ELISA, these were processed according to ELISA protocol and 100ul of each sample was pooled together for use in LAMP.

2.2.8 Nested PCR

Nested PCR was carried out according to Ling et al. (2001) on petioles collected and macerated in liquid nitrogen, after which 0.1g of each sample was suspended in grapevine ELISA extraction buffer (Ling et al., 2001).

A PCR reaction mixture (to a total volume of 50ul in 0.2ml tubes) (2% Triton X-100, 1x NH_4 Biotaq buffer (Bioline, London England), 3.5mM dNTP (promega), 1µM each of primer 93-110 and 92-98 (IDT) (Ling et al., 2001) 10mM dithiothreitol (DTT) (Sigma-Aldrich), 1.5mM $MgCl_2$ (Fermentas), 18U Protector RNase inhibitor (Roche), 8U Avian myeloblastis virus (AMV) reverse transcriptase (Roche) and 0.5U Biotaq DNA polymerase (Bioline)) and 0.5µl of crude RNA extract was made up. A healthy plant control (Black spanish) and a water (no template control) were included for all PCR tests. The reaction temperature profile was of reverse transcription at 37ºC for 45min; denaturation at 94ºC for 2min; 35 cycles of 94ºC for 60sec, 50ºC for 1 min, 72ºC for 2min, and a final elongation step of 72ºC for 10min using a Biorad (Hercules, California) T100™ thermocycler.

The product of the first round RT- PCR (0.5µl) was added to 50 µl of PCR reaction mixture (1 x Biotaq NH_4 buffer (Bioline); 175µM dNTP ((Promega), 1µM primer 93-23, 1µM primer 93-40 (IDT), 1.5mM $MgCl_2$ (Fermentas, Vilnuis, Lithuania) and 0.5U Biotaq (Bioline)). The cycling profile was denaturation at 94ºC for 2min; 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 2min and a final elongation of 72ºC for 10min. Products of the reaction were analysed by electrophoresis as above.

2.2.9 ELISA

Six petioles were collected from each vine and extracted at a 1/10 (weight / volume) ratio in ELISA extraction buffer (0.1M Tris/HCl, pH 7.6 buffer with 0.01M $MgSO_4$, 4% PVPP, and 2% Triton X-100) in filter separated plastic bags (Bioreba), using a Homex 6 homogenizer (Bioreba AG, Switzerland). A triple antibody sandwich (TAS) ELISA, capable of detecting *Grapevine leafroll-associated virus*1 (GLRaV-1), -2 (GLRaV-2) and GLRaV-3 separately or simultaneously (Goszczynski et al., 1997, 1996; Goszczyński et al., 1995) was then used to detect GLRaV-3. A Healthy Control (Black Spanish) and a buffer control were included as negative controls and a known positive extract (623) was included as a positive control. Virus specific antibodies, developed using electrophoretically-separated coat proteins of the respective viruses (Goszczynski et al. 1996, 1997, 1998) were used. Commercial goat-anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was used for sero-reaction detection. Positive results were detected as those individuals that yielded an absorbance value (405nm) of 0.122. In the case of sensitivity testing of ELISA, vines were pooled into groups of 10, 20, 30 and 50 respectively. The average absorbance of healthy control plants plus three standard deviations was used as a positive/negative threshold for ELISA.

2.3 Results

2.3.1 LAMP

In the initial tests using GLRaV-3 specific primers. LAMP was tested against plasmids containing GLRaV-3 inserts of the target area (F2, F8 and F9) as well as a plasmid without the target region and a negative control where water was added in place of template. The LAMP products were analysed using 2% agarose gel electrophoresis. LAMP amplified the target in all three plasmids, with typical ladder-like pattern displayed for all positive samples (Figure 10) while no amplification was observed in either of the negative controls. The ladder-like pattern is due to the formation of stem-loop concantenate products (of varying sizes) produced by LAMP.

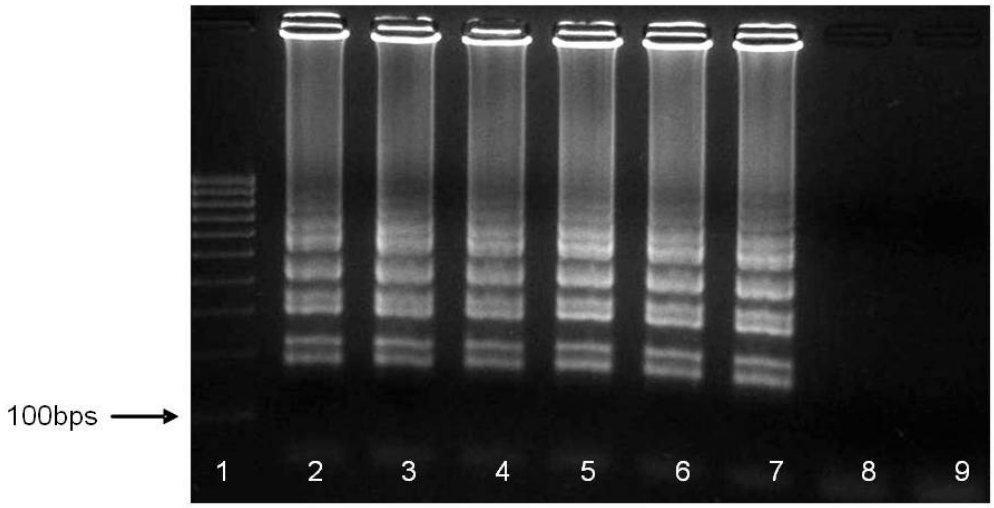

Figure 10: Loop-mediated amplification (LAMP) on 3 plasmids (F2, F8 and F9) containing *Grapevine leafroll -associated virus* type 3 specific sequence plasmids. Lane 1: Molecular marker (100bp ladder); Lane 2-3: Plasmid F2; Lane 4-5: Plasmid F8; Lane 6-7: Plasmid F9; Lane 8: Negative control: Plasmid only; Lane 9: Negative control: Water.

2.3.2 Optimisation of LAMP

Plasmid F2, F8 and F9 were used for the optimisation of the LAMP system. The relative detection limit for LAMP (under the conditions of Notomi et al, 2000) was determined using dilutions of plasmids F2, F8 and F9 at 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10 000. Each dilution was tested in triplicate and positive controls were included of each clone at a dilution of

1:100. Negative controls, where template was substituted for water or non-transformed plasmid, were also used. The relative detection limit of LAMP was established at 1:2000 after which LAMP was optimised for use with GLRaV-3 specific primers.

The concentration and the ratio of inner: outer primers was optimised by varying the concentration of outer primer (0.1µM- 0.5µM) and the ratio of outer: inner primer to 1:4, 1:6, 1:8, and 1:10. At each variation, LAMP was run against clones F2, F8 and F9 diluted at 1:2000, 1:5000 and 1: 10 000 and results were analysed using turbidity and confirmed using agarose gel electrophoresis. Results of the manipulation showed that where the concentration of outer primers is at 0.2µM or above and the ratio of inner: outer primer is 1:8 or higher, the sensitivity of LAMP sensitivity increased to detect plasmid diluted to 1:5000.

In order to determine of the optimal temperature, LAMP reactions were incubated at 60, 61, 62, 63, 64 and 65℃ (range selected because of primer constraints) for 60 min and efficiency was monitored by determining the percentage increase in DNA concentration (ng/µl) from before and after amplification in 1 hour using a Nanodrop as a increase in the amount of product will lead to an increase in the amount of magnesium pyrophosphate produced. Results of the temperature optimisation showed that LAMP performs at similar levels between 60- 62 and performs optimally at 63℃ (Table 3). However as AMV has a maximum temperature 60℃, and the difference between performance between 60 and 63℃ is not that different, LAMP reactions were routinely incubated at 60℃.

Mg^{2+} concentration was varied between 4mM and 10mM. While a positive control of 6mM was included. Results were analysed using turbidity and confirmed using gel electrophoresis. The results showed that a minimum Mg^{2+} concentration of 5mM is required for LAMP reaction to take place and that LAMP performs optimally at Mg^{2+} concentration of 7mM. At 9mM and above amplification either decreased (9mM) or no amplification was observed (10mM).

Table 3: Optimisation of incubation temperature of a Reverse transcriptase loop-mediated amplification (RT-LAMP) for detection of *Grapevine leafroll-associated virus* type 3

Temperature	DNA concentration Before amplification (ng/ul)	DNA concentration after amplification(ng/ul)	Average Percentage increase
60	153.67	2769.3	90.57
61	242.57	3838.76	86.78
62	241.67	2763.6	88.93
63	100.7	3176.33	94.19
64	524.4	3390.6	76.97
65	119.93	3833.83	93.74

Average percentage increase = (DNA concentration after amplification/ DNA concentration before amplification)/ Total DNA concentration)

2.3.3 Visualisation of LAMP products

LAMP product were initially visualised using agarose gel electrophoresis (**Error! Reference source not found.**B), however this method was laborious and contributed to a large amount of contamination. Positive results were then judged based on either turbidity (**Error! Reference source not found.** C) or colourimetrically (**Error! Reference source not found.**A) using HNB. In the case of turbidity, tubes became visually turbid and a white precipitate formed at the bottom of the reaction tube when the tubes were centrifuged only where amplification occurred. This was confirmed using agarose gel electrophoresis.

To test HNB, clones F2, F8 and F9 were amplified in LAMP reaction containing HNB (Goto et al., 2009) and tubes were visually monitored for a colour change from violet to sky blue. Positive results were seen for all clones, with an easily discernible colour change from violet to sky blue. Neither negative control (untransformed plasmid and water) displayed any colour change, showing that the colour change is specific to positive samples. HNB results were the most easily discernible and HNB was used as indicator in all further tests.

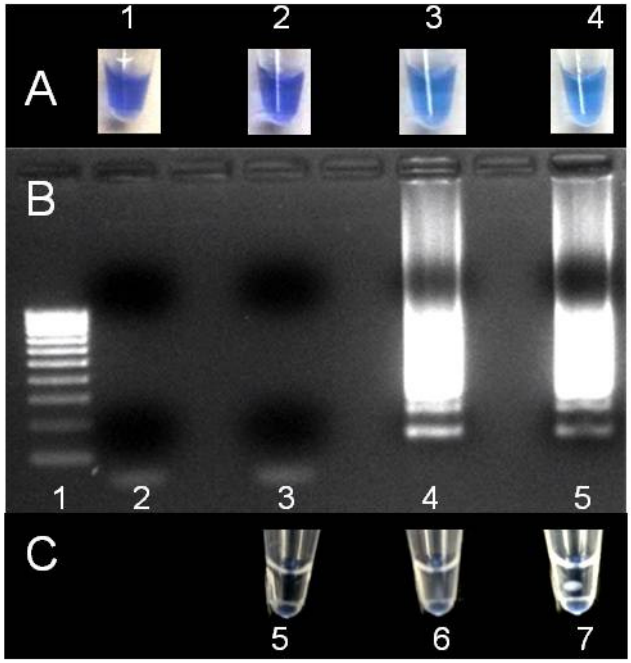

Figure 11: Comparison of three techniques used to monitor Loop-mediated amplification of nucleic acid (LAMP) detection technique. A) visualisation of LAMP product using HNB. Tube 1,2: Negative Control HNB reactions (Untransformed plasmid, water respectively) (Violet); Tube 3,4: Clone F2 (Positive HNB reaction) (Blue). B) visualisation of LAMP product using agarose gel electrophoresis. Lane 1: Ladder (100bps); Lane 2, 3: Negative Controls (Untransformed Plasmid, water control respectively); Lane 4, 5: Clone F2. C) visualisation of LAMP product using turbidity. Tube 5: Negative Control Turbidity reaction (clear); Tube 6: Clone F2 (Positive Turbidity reaction); Tube 7: Clone F2 (Positive Turbidity reaction, precipitate).

2.3.4 RT-LAMP

RNA was extracted from Bark scrapings of three different GLRaV-3 strains infected grapevines (623, 621 and PL-20). RNA was added to RT-LAMP reaction and incubated for 1 hour. Results were judged based on the colour change of HNB from violet to sky blue. A positive control (11-3014) and a healthy plant control (01- 2839) were included. All samples, known to be positive for GLRaV-3 displayed the desired colour change from Violet to Sky blue while the negative controls remained Violet (

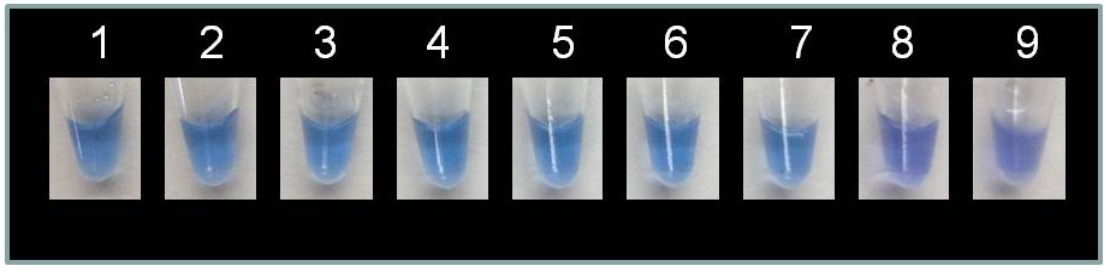

Figure 12).

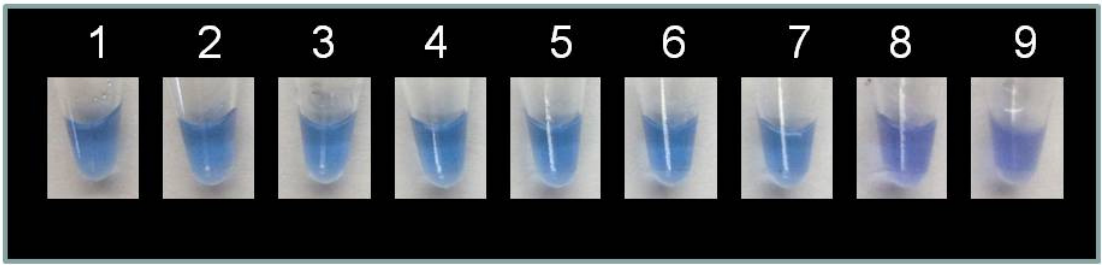

Figure 12: Visualisation of reverse transcriptase loop- mediated amplification (RT-LAMP) on three strains of *Grapevine leafroll-associated virus* type 3 using hydroxy napthol blue . Tube 1-2 PL-20; Tube 3-4 621; Tube 5-6 623; Tube 7: Positive Control (01-3014); Tube 8: Healthy plant (01/2839); Control Tube 9: Negative control (water)

2.3.5 Crude extraction protocol

Several crude nucleic acid extraction protocols were evaluated for use with GLRaV-3 specific RT-LAMP. In each protocol a sample with a high viral titre and low viral titre were tested to ensure the sensitivity of the protocol. No amplification was observed when using the print capture method (1) (La Notte, 1997) or RNA extraction protocols (2), (4), (5). Further, the addition of extraction buffers (2) and (4) caused the HNB indicator to change to sky blue before incubation without any amplification of nucleic acid occurring. Protocol (3) amplified sample 01-2857, however was not able to amplify sample 01-2639. Using the ELISA extraction buffer (protocol (7)), RT-LAMP detected both the strong and the weak positive when RT-LAMP was evaluated using turbidity. However, the PVPP in the buffer caused the HNB to precipitate out of the reaction and therefore this buffer cannot be used where HNB is used as the means of evaluating RT-LAMP reaction. The optimal method was found to be where virus was immobilised on nitrocellulose membrane and then released using GES buffer was shown to be the most reliable protocol for detecting GLRaV-3, with no cross reaction with the HNB. This protocol (1) was used in all further testing of the samples.

2.3.6 Comparison of the relative sensitivity ELISA, nested PCR and LAMP

In order to ensure that RT-LAMP could detect GLRaV-3 as reliably as ELISA and nested PCR, petioles were collected from the same 10 grapevines infected with GLRaV-3 and were tested using ELISA, nested PCR and RT-LAMP. The samples represented the major phylogenetic groups of GLRaV-3 present in South Africa; Group 1, 2, 3 (Jooste et al., 2011) as well as Group 6. All 10 samples were detected using RT-LAMP, ELISA and nested PCR (Table 4).

Table 4: Comparison of ELISA, RT-PCR and RT-LAMP for the detection of *Grapevine leafroll associated virus* type 3 in infected grapevines

	ELISA		RT-LAMP	Nested PCR
Sample	OD±	SD		
623	0.239	0.038	+	+
GH 30	2.403	0.134	+	+
PL-20	1.790	0.080	+	+
74/2/56	1.269	0.122	+	+
37/71/84	1.113	0.044	+	+
74/02/02	2.308	0.187	+	+
621	0.960	0.092	+	+
93/0944	2.155	0.066	+	+
74/7/58	2.496	0.148	+	+
92/1027	0.485	0.043	+	+
Healthy Control (Black Spanish)	0.068	0.002	-	-

± - Average of two repetitions using three ELISA wells per assay; SD: Standard Deviation. A sample was considered positive if the OD (405 nm) measured after 1 h substrate incubation, was higher than 0.072 (Healthy Control O.D + 3*SD)

+ - Positive reaction (in both replicates) of two repetitions of RT-LAMP/ nested PCR

Once it had been established that RT-LAMP could be used to detect the same diversity of GLRaV-3 as ELISA and nested PCR the sensitivity of the three techniques relative to one another of the was compared. Three samples (623,

621 and PL-20) were selected and were pooled with healthy samples at 1:1 (1 infected plant: 1 Healthy Plant), 1:10, 1:20, 1:50, 1:60 and 1:100 ratio. The detection limit of each test was the point where the test could no longer detect all three samples. The detection limit of ELISA was found to be at a ratio of 1:30 infected plants (Table 5), the detection limit of nested PCR (Figure 13) and RT-LAMP (Figure 14) was found to be a ratio of 1: 50 infected plants. Based on these results, RT-LAMP has a comparably sensitivity to nested PCR and both molecular techniques are more sensitive than ELISA.

Table 5: Relative sensitivity of ELISA for detecting *Grapevine leafroll-associated virus* type 3 (PL-20, 623 and 621) infected vines diluted with healthy plant

Sample	1:10±SD	1:20±SD	1:30±SD	1:50±SD
PL-20	0.81± 0.03	0.54± 0.04	0.34± 0.04	0.10± 0.1
623	1.17±0.05	0.86± 0.0.2	0.47± 0.04	0.11± 0.00
621	0.62± 0.03	0.46± 0.03	0.24± 0.05	0.12± 0.01
Healthy Control (Black Spanish)	0.078± 0.01			

± - Average of two repetitions using three ELISA wells per assay; SD: Standard deviation. A sample was considered positive if the OD (405 nm) measured after 1 h substrate incubation, was higher than 0.12 (Healthy Control O.D + 3*SD)

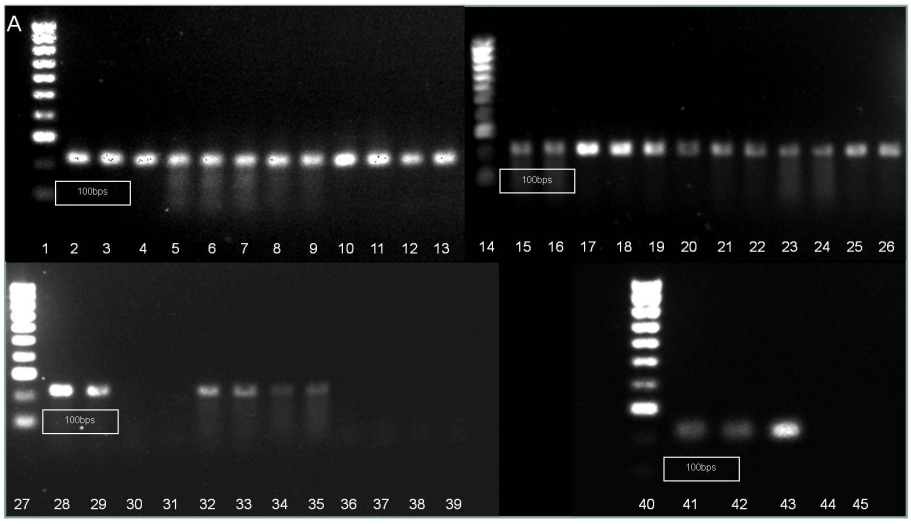

Figure 13: The relative sensitivity of nested RT-PCR for detecting *Grapevine leafroll associated* virus type 3 in infected grapevines . Lane 1, 14, 27, 40: Molecular Marker Ladder (100bps); Lane 2-3: PL-20 1:10; Lane 4-5: 623 1:10; Lane 6-7: 621 1:10; Lane 8-9: PL-20 1:20; Lane 10-11: 623 1:20; Lane 12-13: 621 1:20; Lane 15-16: PL-20 1:30; Lane 17-18: 623 1:30; Lane 19-20: 621 1:30; Lane 21-22: PL-20 1:50; Lane 23-24: 623 1:50;

Lane 25-26: 621 1:50; Lane 28-29: PL-20 1:60; Lane 30-31: 623 1:60; Lane 32-33: 621 1:60; Lane 34-35: PL-20 1:100; Lane 36-37: 623 1:100;Lane 38-39: 621 1:100; Lane 41: PL-20 (positive control); Lane 42: 623 (positive control); Lane 43: 621 (positive control); Lane 44: Healthy Control (Black Spanish); Lane 45: Negative Control (water).

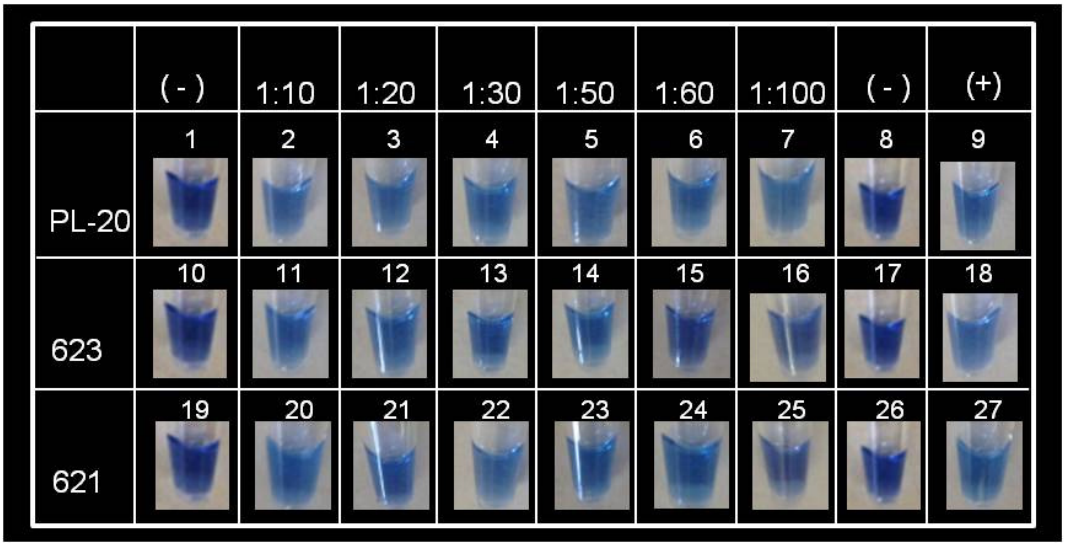

Figure 14: : The relatively sensitivity of RT-LAMP for detecting *Grapevine leafroll associated virus* type in infected grapevines. (for easy of interpretation, a copy of the Black Spanish negative control has been shown included at the beginning of each dilution series and a water negative control at the end) Tube 1: Negative control (Black Spanish); Tube 2: PL-20 1:10; Tube 3: PL-20 1:20; Tube 4: PL-20 1:30; Tube 5 PL-20 1:50; Tube 6 PL-20 1:60; Tube 7: PL-20 1:100; Tube 8: Negative Control (Water); Tube 9: Positive Control (PL-20); Tube 10: Negative control (Black Spanish); Tube 11: 623 1:10; Tube 12: 623 1:20; Tube 13: 623 1:30; Tube 14: 623 1:50; Tube 15: 623 1:100; Tube 16: 623 1:100; Tube 17: Negative Control (Water); Tube 18: Positive Control (623); Tube 19: Negative control (Black Spanish); Tube 20: 621 1:10; Tube 21: 621 1:20; Tube 22: 621 1:30; Tube 23: 621 1:50; Tube 24: 1:60; Tube 25: 1:100; Tube 26: Negative Control (water);Tube 27: Positive Control (621) *Only one replicate of RT-LAMP is shown

2.4 Discussion

The GLRaV-3 RT-LAMP developed here uses primers designed to detect a conserved region of the GLRaV-3 genome, to detect RNA targets extracted from GLRaV-3 infected Grapevine samples in 1 hour using only a heating block and HNB, a simple colourimetric indicator, to monitor the reaction. Additionally, a simple RNA extraction method was developed which

decreases the complexity and cost of template preparation and allows for RT-LAMP to be performed in even the most basic laboratories.

Detecting GLRaV-3 has several hurdles, firstly GLRaV-3 may have a low viral titre and erratically distribution in the plant tissue and as such, diagnostics techniques do not always detect the virus satisfactorily due to low sensitivity (La Notte, 1997). Several papers have discussed the sensitivity of LAMP and advantages associated with using LAMP in situation where low titres of the target are present (Parida et al., 2008; Thai et al., 2004). The sensitivity, specificity and efficiency of LAMP are affected by primer concentration, the ratio of inner: outer primer, Mg^{2+} concentration and temperature (Nie, 2005; Notomi et al., 2000) and as such the optimisation of these reagents is vital for the detection of GLRaV-3. Sensitivity in particular was linked to the ratio of inner: outer primers with sensitivity increasing 2.5-fold where the ratio of inner: outer primer was greater than 8:1. Specificity and efficiency was found be highly dependent on Mg^{2+} concentration. Mg^{2+} is known to affect primer annealing (Nie 2005; Gunimaladevi et al. 2005; Teng et al. 2007; Liu et al. 2010) and it is possible that the LAMP conditions make those conditions too stringent for primers to anneal to the slight variations in sequence. RT-LAMP ideally should be able to detect all strains of the GLRaV-3 and as such it is important that the conditions not be too stringent.

The sensitivity of a detection system can be highly dependent on the manner in which the target is extracted (Osman et al. 2012). Extraction of GLRaV-3 RNA from grapevine samples is complicated by the presence of inhibitory substances in the plant which decrease detection sensitivity as well as inhibit enzymes used in RT-PCR (Nakaune and Nakano, 2006). RNA extraction methods are usually either expensive, time consuming or require equipment which makes them unsuitable for use in the context of large scale testing. LAMP is a highly robust technique (Francois et al. 2011) and less likely to be affected by compounds present in grapevines. Based on this, several crude RNA extraction techniques and extraction buffers were tested for use with LAMP, with varying degrees of success. The majority of the systems tested yielded no results, while others could only detect the virus in plants where

there was a high viral titre. Only one system, which combined viral immobilization on a nitrocellulose membrane with viral release using GES buffer could reliably detect the virus in all samples tested. This system is simple to use and does not require sophisticated equipment which lends it to the basic labs found on wine farms. The extraction procedure is simple and once the membranes have been dried there is little risk of contamination as the nucleic acid is bound to the membrane until released. While basic laboratory practices, such as separation of nucleic acid extraction and LAMP amplification, are necessary these practices are already followed as part of the ELISA procedure in the basic laboratories, which limits the amount of training necessary.

Pooling samples allows for a larger number of samples to be tested quickly and more cheaply (Muñoz-Zanzi et al., 2000; Wells et al.,2003; Maherchandani et al., 2004; Brinkhof et al., 2007) .However one of the greatest disadvantages of pooling samples is that it can result in a loss sensitivity of the diagnostic tests (Brinkhof et al., 2007). In the case of ELISA, the pooling of samples can also result in unacceptably high OD of samples which limits the amount of samples which can be tested together (Brinkhof et al., 2007). In the case of the ELISA currently used in the detection of GLRaV-3 in white cultivars (Pietersen and Walsh, 2012), this means that very few samples can be pooled together which increases both the cost and time needed to test a vineyard. In order for RT-LAMP to be useful in testing of grapevines for GLRaV-3 infection, its ability to detect GLRaV-3 should be comparable to molecular techniques such as nested PCR and it should be able to be used on pooled sample.

In order to compare the relative sensitivity of RT-LAMP, ELISA and nested PCR to detect GLRaV-3 in collected GLRaV-3 samples, samples processed according to the various methods, were pooled and the end point at which a single infected individual could be detected amongst healthy individuals was determined. Results showed that nested PCR and RT-LAMP could detect a single infected plant amongst 50 healthy plants while ELISA could only detect 1 amongst 30. The sensitivity of RT-LAMP compared to ELISA combined with

the fact that the system does not require expensive equipment may contribute to the expedient screening large amounts of grapevines for GLRaV-3.

LAMP is a sensitive, reliable, robust system (Francois et al., 2011). However, in order for the system to be used effectively, there are several aspects of the test must be considered which affect the accuracy of the system. Firstly, the robustness of the system can be dependent on the indicator used. HNB was chosen as the indicator for the GLRaV-3 RT-LAMP as positive reactions are easily discernible and HNB has none of the inhibitory effects associated with other indicators (Tomlinson et al., 2010). However, several of the buffers tested for crude RNA extract protocols interfered with HNB indicator, causing false positives or inhibiting the reaction. This affects the overall robustness of LAMP and should be considered when using LAMP. Secondly, great care must be taken to separate sample preparation and the preparation of LAMP reagents as the system is highly prone to contamination and false positive reactions are difficult to ascertain.

Despite this, the relative simplicity of the reaction setup and product detection combined with its comparative sensitivity give RT-LAMP inherent advantages over ELISA and RT-PCR. The sensitivity of RT-LAMP is comparable to nested RT-PCR and greater than ELISA. Easy reaction setup and incubation make it simpler than nested PCR and the need for only basic equipment (a water bath or heating block) means it is a viable option for laboratories which do not have access to thermocyclers.

2.5 Conclusion

LAMP is a simple diagnostic technique which provides a simple way to detect GLRaV-3. With relative sensitivity comparable to nested RT-PCR assay, the one-step RT- LAMP HNB analysis exhibits potential for the detection of GLRaV-3 in both research and diagnostic labs, and offers a sensitive and efficient alternative for diagnosis of the virus that may help contribute to the eventual control of GLD, especially in symptomless white cultivars (Pietersen and Walsh, 2012).

CHAPTER 3: Using a Grapevine leafroll-associated virus type 3 (GLRaV-3) specific Reverse transcriptase loop-mediated amplification (RT-LAMP) to test for the presence of GLRaV-3 in grapevine Rootstocks

3.1 Introduction

Grapevine leafroll diseases is the most important disease of grapevines in South Africa (Pietersen, 2004) and is known to lower the quality and quantity of grapes as well as lowering the over-all vigour of the plants (Over de Linden & Chamberlain, 1970). Globally, the disease is associated with several viruses within the *Closteroviridae* family known as Grapevine leafroll associated viruses (GLRaV). Due to its wide spread occurrence; GLRaV-3 is considered the greatest threat to South African vineyards.

In a study at Vergelegen Wine Farm (Somerset West, South Africa), it was shown that GLD can be controlled using an integrated control strategy (Pietersen and Walsh, 2012; Pietersen et al., 2013) . This strategy involves the use of certified planting material to establish vineyards, controlling insect vectors using systemic insecticide and monitoring and removing infected individuals by roguing (Pietersen & Walsh, 2012).

This strategy is reliant on the ability to produce virus free planting material and reliably detect infected individuals. Virus free planting material is mainly supplied from mother blocks which are screened yearly; however virus free plants are still susceptible to virus infection in the field. Infection in rootstocks is more difficult to detect than in scion material and rootstocks could therefore act as symptomless carriers of GLRaV.

3.2 Rootstocks

The practice of grafting grapevines as a means of propagation has been around since the second century (Gu, 2003), however it only became common after the 1880's, when it was shown that native American *Vitis.* species (including *V. riparia*, *V. berlandeiri,* and *V. rupestris*) had natural

resistance to the root louse *Phylloxera vitifoliae* (Lider et al., 1993). Rootstocks have also displayed resistance to several other pests and diseases of grapevines (Cousins and Goolsby, 2010; Gao et al., 1993; Magoon and Magness, 1937) however no natural resistance to any GLRaV has ever been observed (Martelli and Boudon-Padieu, 2006). While no complete resistance to GLRaV has ever been observed, studies of the prevalence of viruses in vineyards have led to the suggestion that some rootstocks are less susceptible to GLRaV-3 (Ioannou et al., 1999) than others and that the rootstocks response to infection depends on the rootstock and virus type present (Golino et al., 1993; Kasdorf, 2006). These observations, in conjunction with the fact that GLRaV is found in very low concentrations in rootstock leaves makes detection of GLRaV in rootstock material problematic (Beuve et al., 2007), especially where virus free scion material is grafted onto an infected, symptomless rootstock.

3.3 The South African Plant Improvement Scheme

The South African Plant Improvement Scheme is responsible for the production of virus free material. In this system virus free nuclear material, which is produced by eliminating viruses using heat therapy or meristem tip culturing, is used to establish foundation blocks and mother blocks (Jooste et al., 2010). These blocks in turn provide the material used for the establishment of virus-free vineyards. Mother blocks are tested for the presence of viruses each year and where infection is above 3% the vineyard loses its mother block status.

The plant material used in the Scheme is tested using ELISA, immunosorbent electron microscopy (ISEM) and PCR which supplement traditional indexing (Kasdorf, 2006). This makes it possible to test a large amount of samples quickly and, in the case of scion material, reliably. In the case of rootstocks, several studies have shown that GLRaV's are not readily detected in the field, especially where American rootstocks lacking *V. vinifera* parent are used (Cid et al., 2003; Credi and Santucci, 1990; Kasdorf, 2006) .

While every care is taken to maintain the virus free status of these vines, they are still susceptible to GLD and although they are monitored for signs of infection and tested using ELISA and PCR, the lag time between infection and the time when viral titres become high enough to be detected by conventional detection techniques means that infected plants could still be used in the establishment of new vineyards (Pietersen, 2004). Globally, GLRaV-3 spread is thought to have been caused mainly by the use of infected planting material, with insect vectors contributing to spread in and between vineyards the vineyard (Cabaleiro and Segura, 1997b).

3.4 Detection of GLRaV-3 in rootstocks

In 2003 a project was launched in order to evaluate the sensitivity of the detection methods and the factors which would influence the sensitivity in the testing of rootstocks used in the South African grapevine industry. In this study 5 of the most important rootstocks in the South African grapevine industry were selected and were infected by several viruses including GLRaV-1, 2, and 3, Grapevine Virus A (GVA) and Grapevine Fleck virus (GFkV) virus-free rootstocks by grafting infected scion material onto the rootstocks. The rootstocks were then tested at various intervals over a three year period using ISEM, ELISA and PCR.

Fourteen months post grafting (mpg) the first positive GLRaV-3 samples were detected in the rootstocks using PCR, however subsequently it was found that PCR gave inconsistent results over the rest of the trial. At 33 mpg less than 28 % of samples tested positive for GLRaV-1, -2, 3 or GLRaV-3 alone with antigen-based detection methods. This is thought to be due to the low levels of virus found in the rootstocks (compared to the original scion material used to inoculate the rootstocks) as well as the erratic distribution of virus in the plant. Similar tests in other countries using other rootstocks yielded similar results (Credi and Santucci, 1990).

One of the largest hurdles for the testing of rootstocks is that the low viral titres and erratic distribution makes reliable detection difficult. Nested RT-PCR and real time reverse transcription PCR (qRT-PCR) have higher sensitivity

levels than antigen based testing methods and have been suggested as alternative methods for the reliable detection of GLRaV-3 (Ling et al., 2001; Malan, 2009) however, these methods require specific equipment (such as thermocyclers) and reagents (especially in the case of qPCR) and are not therefore viable alternative for most small basic field labs with little equipment where rootstocks are normally tested.

Here we report on the use of a GLRaV-3 specific RT- LAMP to detect GLRaV-3 in rootstocks. The RT-LAMP uses minimal equipment (heating block) and results can be monitored simply and quickly.

3.5 Materials and Methods

3.5.1 Plant Material

Rootstock material from the original 2006 trial was obtained from Kassie Kasdorf at the PPRI, Roodeplaat where it was maintained in grapevine potting mixture in insect free glasshouses The material consisted of replicates of 5 different rootstocks clones (101-14 Mgt (*V. riparia* x *V. rupestris*) (101-140), Salt creek/ Ramsey (*V. Champini*) (SC), Richter 99 (R99), Ruggeri 140 (Rug) and Richter 110 (R110) (*V. Berlandieri* x *V.* rupestris) which had been infected by means of clip- or cleft- grafting of *V. vinifera* Black Spanish canes which contained GLRaV-1, -2, -3, -4, -5 , Grapevine virus A (GVA) and Grapevine fleck Virus (GFkV).

3.5.2 RNA extraction

Six petioles from each rootstock were ground in liquid nitrogen and RNA was extracted from petioles using Total RNA extract kit (Bioline) according to manufacturer's instructions.

3.5.3 RT-LAMP

The final LAMP reaction mixture (25ul total volume in a 0.2ml tube) was made up as follows; 1.6µM FIP and BIP, 0.2µM F3 and B3, 8U Bst (Lucigen, Middleton, WI) and 1x Bst Buffer B (20mM Tris-HCl (pH 8.8), 10mM $(NH_4)_2SO_4$, 10mM KCl, 2mM $MgSO_4$ and 0.1% Triton X-100) (Lucigen), 1M Betaine (Sigma- Aldrich, St Louis, MO, USA), 1.4mM dNTPs (Promega, Madison, WI USA), 7mM $MgCl_2$ (Fermentas, Vilnuis, Lithuania), 120µM

Hydroxy naphthol blue (HNB) (Acros Organics, Geel, Belgium), 10U AMV reverse transcriptase (Roche, Palo Alto, CA USA) and 2µl of RNA. The mixture was then incubated at 60℃ using a heating block (Eppendorf Thermostat Plus 3130, Hamburg, Germany) for 1 hour followed by heating to 80℃ for 10 minutes to terminate the reaction. The reaction was monitored by the colour change from violet to sky blue using the naked eye.

3.5.4 Nested PCR

Nested PCR was carried out according to Ling et al. (2001) on petioles collected and macerated in liquid nitrogen, after which 0.1g of each sample was suspended in grapevine ELISA extraction buffer (Ling et al., 2001).

A PCR reaction mixture (to a total volume of 50ul in 0.2ml tubes) (2% Triton X-100, 1x NH_4 Biotaq buffer (Bioline, London England), 3.5mM dNTP (promega), 1µM each of primer 93-110 and 92-98 (IDT) (Ling et al., 2001) 10mM dithiothreitol (DTT) (Sigma-Aldrich), 1.5mM $MgCl_2$ (Fermentas), 18U Protector RNase inhibitor (Roche), 8U Avian myeloblastis virus (AMV) reverse transcriptase (Roche) and 0.5U Biotaq DNA polymerase (Bioline)) and 0.5µl of crude RNA extract was made up. A healthy plant control (Black spanish) and a water (no template control) were included for all PCR tests. The reaction temperature profile was of reverse transcription at 37ºC for 45min; denaturation at 94ºC for 2min; 35 cycles of 94ºC for 60sec, 50ºC for 1 min, 72ºC for 2min, and a final elongation step of 72ºC for 10min using a Biorad (Hercules, California) T100™ thermocycler.

The product of the first round RT- PCR (0.5µl) was added to 50 µl of PCR reaction mixture (1 x Biotaq NH_4 buffer (Bioline); 175µM dNTP ((Promega), 1µM primer 93-23, 1µM primer 93-40 (IDT), 1.5mM $MgCl_2$ (Fermentas, Vilnuis, Lithuania) and 0.5U Biotaq (Bioline)). The cycling profile was denaturation at 94ºC for 2min; 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 2min and a final elongation of 72ºC for 10min. Products of the reaction were analysed by electrophoresis as above.

3.6 Results

Results of RT-LAMP showed that of the 61 rootstock samples tested, 13 tested negative while the other samples tested positive (48 samples) (

Table 6).

Table 6: Results of RT-LAMP to detect *Grapevine Leafroll associated virus* type 3 in 5 different infected rootstocks (R99, 101-14, SC, R110, RUG) grafted onto Black Spanish scions

Replicate	BS/R99	BS/ 101-14	BS/ SC	BS/ R110	BS/RUG	Healthy Control
1	-	+	+	+	-	-
2	-	+	+	+	+	
3	+	+	+	+	-	
4	+	-	+	-	+	
5	+	+	+	+	+	
6	+	-	+	-	+	
7	+	+	+	-	+	
8	+	+	+	+	+	
9	+	-	-	+	N/A	
10	-	+	+	N/A	N/A	
11	+	+	+	N/A	N/A	
12	+	+	N/A	N/A	N/A	
13	+	-	N/A	N/A	N/A	
14	+	+	N/A	N/A	N/A	
15	+	+	N/A	N/A	N/A	
16	+	N/A	N/A	N/A	N/A	
17	+	N/A	N/A	N/A	N/A	
18	+	N/A	N/A	N/A	N/A	
19	+	N/A	N/A	N/A	N/A	
Total replicates	19	15	11	9	8	1[†]

* BS- Black spanish
N/A- Not appicable

+ - Positive
- - Negative

To confirm the negative results obtained using RT-LAMP, negative samples were then tested using nested PCR to confirm the negative results (Figure 15) .None of the samples which tested negative using RT-LAMP were amplified using nested PCR.

Further it was noticed that there was a large variation between the numbers of positive samples which were detected in different rootstocks (Table 7). Richter 110 had the least infected individuals, with a 67% of individuals while the Salt creek/ Ramsey rootstock had the most infected individuals (91%).

Table 7: Variation in number of positive samples detected in 5 different grapevine rootstocks (R110, 101-14, RUG, R99 and SC) by a *Grapevine leafroll-associated virus* type 3 specific RT-LAMP

Cultivar	Total	Positive	Negative	Percentage infection
R 110	9	6	3	67
101-14	14	10	4	71
RUG	8	6	2	75
R 99	19	16	3	84
SC	11	10	1	91
Total	61	48	13	78

*** - Percentage infection determined as (number of infected samples/ total number of samples)**

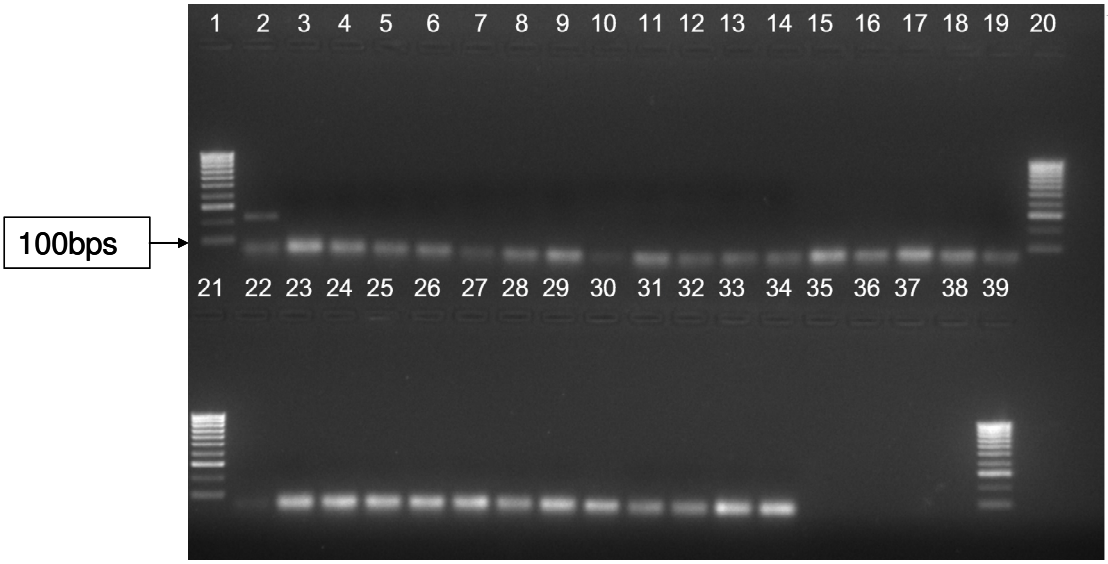

Figure 15: GLRaV-3 specific nested RT-PCR on 5 different grapevine Rootstocks (R99, R110, 101-14, RUG, SC). Lane 1: Ladder (100bps); Lane 2: Positive control (11-3014); Lane 3: Negative Control (Healthy plant (BS)); Lane 4: Negative Control (Water); Lane 5-6: BS/R99 replicate 1; Lane 7-8: BS/R99 replicate 2; Lane 9-10: BS/R99 replicate 10; Lane 11-12: BS/R110 replicate 5; Lane 13-14: BS/R110 replicate 7; Lane 15-16: BS/R110 replicate 8; Lane 17-18: BS/101-14 replicate 4; Lane 19: BS/101-14 replicate 6; Lane 20: Ladder; Lane 22: BS/101-14 replicate 6; Lane 23-24: BS/101-14 replicate 9; Lane 25-26: BS/101-14 replicate 13; Lane 27-28: BS/ RUG replicate 1; Lane 29-30: BS/RUG replicate 3; Lane 30-32: BS/SC replicate 1 Lane 33-34: Healthy rootstock

3.7 Discussion

GLRaV are not easily detected in rootstocks, especially in American rootstocks were infected individuals do not produce visual symptoms and viral titres tend to be low (Cid et al., 2003; Credi and Santucci, 1990; Kasdorf, 2006) . Established method of detecting GLRaV including ISEM, ELISA and PCR have shown to have very low detection rates or have been shown to give unreliable results (Kasdorf, 2006) Both nested PCR and qPCRs, which have sensitivity several orders higher than conventional PCR and ELISA, have been developed (Ling et al., 2001; Malan, 2009) and could be used in the detection of GLRaV-3 in rootstocks. However, both of these techniques have the inherent disadvantage that they require expensive thermocyclers and (especially in the case of qPCR) expensive reagents. This makes their use in testing large quantities of samples less feasible as most basic diagnostics

labs cannot afford basic or real-time thermocyclers. Here we reported on the ability of a GLRaV-3 specific RT-LAMP to detect GLRaV-3 in rootstocks (R110, R99, 101-14, SC and RUG) which had been infected with various viruses including GLRaV-1, -2, -3, GVA and GVB.

These rootstocks had been used in a previous study which aimed to test the efficiency of ISEM, ELISA and PCR as methods of testing for the presence of various viruses in grapevine rootstocks. In this study Kasdorf (2006) found that after 33 mpg 28% of samples tested positive for GLRaV-1,-2,-3 or -3 alone. Kasdorf hypothesised that this low detection rate was due to the low levels of virus in the samples. Using the GLRaV-3 specific RT-LAMP, 78 % of these same rootstocks tested were infected with GLRaV-3. LAMP is a highly sensitive technique and has the advantage over other diagnostic techniques that , because of the amount product that is produced during incubation, it is not affected by the amount of starting material (Tomlinson et al., 2010), which allows it to detect even small amount of virus present in rootstocks.

Cid et al. (2003) studied GLRaV-3 movement in Cabernet franc (*V. vinifera*) and found that GLRaV-3 particles organized themselves into bundles and that this did not occur in any rootstock variety. This suggests that rootstocks have a biochemical or physical mechanism which results in resistance against GLRaV-3. Although overall 78% of samples tested positive for GLRaV-3, the percentage of positive samples of different cultivars varied significantly. Richter 110 had the lowest percentage infection (67%) and the Salt creek/ Ramsey had the highest percentage infection (91 %). These results are similar to those found in Kasdorf (2006). It was hypothesised that this variation amongst rootstocks was due to a difference in susceptibility of different cultivars to virus transmission. Studies have shown that there is a link between the rootstock, the virus present and the rootstocks response to infection (Golino et al., 1993) and it may be possible that the variation in detection between cultivars is due to a variation in viral titres in the various cultivars, caused by a natural resistance to GLRaV-3.

For the Plant improvement scheme this information may be useful in two ways; firstly by identifying which rootstock are more likely to have lower viral titres it may give some indication as to which rootstocks should be tested using multiple methods (for example using ISEM and RT-LAMP to confirm results) or which should be sampled in duplicate to confirm virus status before it is used for grafting. Secondly, (although further studies need to be done to confirm this hypothesis) if in fact some rootstocks are more resistant to viral transmission, grafting of Scion onto this material may lower the incident of GLRaV-3 in the field by lowering the amount of infected material introduced into the field. It may also be possible to study the exact mechanism of resistance in the rootstocks and use this to create scion material which has a higher resistance to GLRaV-3.

LAMP sensitivity is comparable to PCR and qPCR, however it could still not detect GLRaV-3 in all the rootstock samples. One of the possible reasons for this could be the erratic distribution of viruses in rootstocks, which makes consistent detection of viruses problematic. In this study, as well as in Kasdorf (2006) only petioles and bark scrapings were used, however Credi and Santucci (1990) found that root material contained much higher concentrations of the virus. Further it has been shown (Tobie Oosthuizen, unpublished results.) that some areas of rootstocks have higher concentrations of virus than others. Viral titers have also been shown to vary in vines at specific times of the year. In order to better detect GLRaV-3 in rootstock efficiently it is important to understand where the greatest concentration of the virus is likely to be found in the plant and whether time of year will influence detection of the virus.

This study served as a preliminary test to establish whether RT-LAMP was able to detect GLRaV-3 in rootstocks, GLRaV-3 is notoriously difficult to detect. RT-LAMP has been shown to be sensitive and capable of detecting GLRaV-3, however there are still further studies that need to be done on including confirming the presence of GLRaV-3 in the rootstocks using (possibly using) biological indexing evaluating whether RT-LAMP results will be affected by the time of year when sampling occurs or the type of material

which is tested (petiole, bark scraping or root sampled). These results will affect whether RT-LAMP can be used exclusively in the field as a means of testing rootstocks. At this point the utility of RT-LAMP lies in the fact that it requires less expensive equipment and requires less time than other comparatively sensitive techniques (nested PCR and qPCR).

3.8 Conclusion

GLRaV-3 specific RT-LAMP can be used in the detection of GLRaV-3 in rootstocks, where its sensitivity is comparable to nested PCR. RT-LAMP has the advantage over other sensitive techniques that it does not require expensive equipment and faster results can be obtained using this technique. However, due to the (assumed) erratic distribution of GLRaV-3 in the plant, RT-LAMP could not detect GLRaV-3 in all samples and more studies need to done to optimise sampling of rootstocks in order to develop an optimal system of detection of GLRaV-3 in rootstocks.

Chapter 4: Conclusions

GLD, most commonly associated with GLRaV-3, is one of the most important diseases of the South African grapevine industry. GLD, which results in a reduction the quality and quantity of grapevine crops, can be controlled using an integrated strategy which includes the use of virus-free planting material to establish vineyards; controlling insect vectors using systemic insects and removing infected vines through roguing.

Before vines can be rogued each autumn infected vines are identified using either symptom display (red cultivars which display characteristic symptoms) or ELISA (asymptomatic white cultivars). While other methods for the detection of GLRaV-3, including PCR and Q-PCR, have been developed the vine testing is usually carried out in very basic laboratories on wine farms and the equipment needed for these techniques is too specialised and usually too costly to make these techniques feasible alternatives. Alternatively, ELISA uses very little specialised equipment and is robust and simple and lends itself to being used in very basic laboratories. However the greatest disadvantage of ELISA is that it is laborious and time consuming and does not have high sensitivity and a more rapid, sensitive technique is needed.

The aim of this study was to develop a rapid, sensitive RT-LAMP which could be used to test grapevine samples for GLRaV-3 infection in very basic laboratory settings and ultimately contribute towards the control of GLD. This technique should be simple, should not use specialised equipment and the results should be easy to interpret.

A GLRaV-3 specific RT-LAMP combined with a crude RNA extraction protocol was developed which was able to reliably detect GLRaV-3 from grapevine leaf petioles in two hours. The inclusion of HNB ,a colourimetric indicator, which changes from violet to sky blue only were amplification has occurred, allowed for positive results to be easily interpreted. RT-LAMP was compared to ELISA and nested RT-PCR in order to determine its level of reliability and relative

sensitivity. The results of the comparison showed that RT-LAMP is as reliable as ELISA and nested RT-PCR and can detect the same GLRaV-3 variants as nested RT-PCR. Further sensitivity testing showed that the relative sensitivity of RT-LAMP is comparable to nested RT-PCR and higher than ELISA and that using this method up to 50 samples can be pooled together with a single infected plant still being detected.

Several articles which evaluate the use of LAMP systems in detection of pathogens have discussed the possibility of it being used as an alternative where the current methods are not viable due to a lack of specialised equipment or technical training. The majority of the systems which have been evaluated and found to have promise have been designed to detect DNA pathogens and involve very basic total nucleic acid extractions with basic LAMP reagents. One of the major hurdles found in the RT-LAMP system developed here was the RNA; firstly because a reliable RNA extraction method which did not involve many complicated steps or reagents was difficult to establish and secondly; RNA necessitates the inclusion of AMV which both reduces the robustness of the LAMP mixture and increases the overall price per reaction.

The final method used here involves the immobilisation of nucleic acid on a membrane and then the release of nucleic acid from the membrane. This process is simple and requires only very basic equipment. Further, as long as good laboratory practices, such as ensuring that surfaces are clean and gloves are worn when handling samples, contamination can be limited. The RT-LAMP can also be incubated in a simple water bath and results and easily interpreted with the naked eye which makes it an option in very basic environment. It terms of reagents availability, while RT- LAMP kits are available, most kits tend to include intercalating dyes such as SYBR green, which require a real-time monitoring system, or are expensive or difficult to acquire in South Africa which means that reagents need to be ordered and reaction mixture must be made up. This group did try to make up a pre-made mixture and transport it to lab facilities however the mixture lacks robustness and at this point this seem to be the biggest limitation to the method and

further studies will have to be done to develop a reliable way of transporting the RT-LAMP mixture to the field.

The other limitation to using RT-LAMP is that it requires the use of AMV, which increases the price per reaction and for a single sample, does not compare with ELISA. However, the greater sensitivity of LAMP means that, in terms of testing entire vineyards, samples can be pooled into much larger groups than can ELISA which decreases the overall price of using this system. Also, because it does not require specialised equipment it is a feasible solution for labs where equipment such as Q-PCR is not available.

In light of the high sensitivity and simplicity of the RT- LAMP system, another area where it might be used is in the testing of grapevine rootstocks. GLRaV-3 is (presumed to be) erratically distributed and has low viral titres in rootstocks which make reliable detection of the virus difficult. ELISA is too insensitive to reliably detect the virus and therefore molecular methods such as nested RT-PCR and qRT–PCR are used. These methods have greater sensitivity than ELISA but they require specialised laboratory equipment such as thermocyclers and trained technicians are therefore not suitable for large scale testing in small basic laboratories. RT-LAMP, shown to be as sensitive as nested RT-PCR for the testing of scion material and does not require specialised equipment, was tested as an alternative for nested RT-LAMP.

Petioles collected from various Rootstocks, grafted onto Black Spanish scion material were collected and tested for the presence of GLRaV-3. Samples that were found to be negative using RT-LAMP was then retested using RT-PCR in order to compare the two techniques. RT-LAMP could detect GLRaV-3 in 78% of the samples and no sample which was found to be negative using RT-LAMP was amplified using nested RT-PCR showing that RT-LAMP is at least as sensitive as nested RT-PCR. RT-LAMP amplified samples in a much smaller amount of time than is required by nested RT-PCR and the HNB indicator reduces the possibility of contamination as tubes remained sealed once the test has been performed. Therefore, although not 100% reliable, RT-LAMP may be an alternative for laboratories which could not use nested RT-

PCR. However, this testing is still in its infancy and many further tests need to be carried to confirm this preliminary testing.

RT-LAMP is highly sensitive, reproducible, and reliable and is efficient means of detecting nucleic acid, making it a powerful tool in the control of GLD in South Africa. The system is simple and easy to learn which increases its versatility for use in the field and it is may be a viable alternative for the testing of GLRaV-3 in grapevines.

Bibliography

Al-Musa, A., Anfoka, G., Misbeh, S., Abhary, M., Ahmad, F.H., 2008. Detection and Molecular Characterization of *Squash leaf curl viru*s (SLCV) in Jordan. Journal of Phytopathology 156, 311–316.

Arora, S., Agarwal, R.K., Bist, B., 2006. Comparison of ELISA and PCR vis-a ` -vis cultural methods for detecting *Aeromonas* spp . in foods of animal origin. International Journal of Food Microbiology 106, 177–183.

Bertamini, M., Muthuchelian, K., Nedunchezhian, N., 2004. Effect of Grapevine Leafroll on the Photosynthesis of Field Grown Grapevine Plants (*Vitis vinifera* L. cv. Lagrein). Journal of Phytopathology 152, 145–152.

Bertolini, E., Olmos, A., Martínez, M.C., Gorris, M.T., Cambra, M., 2001. Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. Journal of Virological Methods 96, 33–41.

Beuve, M., Sempé, L., Lemaire, O., 2007. A sensitive one-step real-time RT-PCR method for detecting *Grapevine leafroll-associated virus* 2 variants in grapevine. Journal of Virological Methods 141, 117–24.

Boldbaatar, B., Inoue, S., Sugiura, N., Noguchi, A., Orbina, J.R.C., Demetria, C., Miranda, M.E., Yamada, A., 2009. Rapid detection of rabies virus by reverse transcription loop-mediated isothermal amplification. Japanese Journal of Infectious Diseases 62, 187–91.

Boyko, V.P., Karasev, a V, Agranovsky, a a, Koonin, E. V, Dolja, V. V, 1992. Coat protein gene duplication in a filamentous RNA virus of plants. Proceedings of the National Academy of Sciences of the United States of America 89, 9156–60.

Brinkhof, J., Houwers, D., Van Maanen, C., 2007. Development of a sample pooling strategy for the serodiagnosis of small ruminant lentiviral infections using the ELITEST-MVV ELISA. Small Ruminant Research 70, 194–199.

Bush, C.E., Donovan, R.M., Peterson, W.R., Jennings, M.B., Bolton, V., Sherman, D.G., Brink, K.M. Vanden, Beninsig, L.A., Godsey, J.H., 1992. Detection of *Human Immunodeficiency Virus* Type 1 RNA in Plasma Samples from High-Risk Pediatric Patients by Using the Self-Sustained Sequence Replication Reaction. Journal of Clinical Microbiology 30, 281–286.

Cabaleiro, C., Couceiro, C., Pereira, S., Cid, M., Barrasa, M., Segura, A., 2007. Spatial analysis of epidemics of *Grapevine leafroll associated virus*-3. European Journal of Plant Pathology 121, 121–130.

Cabaleiro, C., Segura, A., 1997a. Field transmission of *Grapevine leafroll-associated virus* 3 (GLRaV-3) by the mealybug *Planococcus citri*. Plant Disease 81, 283–287.

Cabaleiro, C., Segura, A., 1997b. Some characteristics of the transmission of *Grapevine leafroll-associated virus* 3 by *Planococcus citri* Risso. European Journal of Plant Pathology 103, 373–378.

Cabaleiro, C., Segura, A., 2006. Temporal Analysis of *Grapevine leafroll associated virus* 3 Epidemics. European Journal of Plant Pathology 114, 441–446.

Cardoso, T.C., Ferrari, H.F., Bregano, L.C., Silva-Frade, C., Rosa, A.C.G., Andrade, A.L., 2010. Visual detection of *turkey coronavirus* RNA in tissues and feces by reverse-transcription loop-mediated isothermal amplification (RT-LAMP) with hydroxynaphthol blue dye. Molecular and Cellular Probes 24, 415–7.

Charles, J.G., Cohen, D., Walker, J., Forgie, S., Bell, V., Breen, K., 2006. A review of *Grapevine leafroll-associated virus* type 3 (GLRaV-3) for the New Zealand wine industry. Hort Research Client Report 18447.

Charles, J.G., Froud, K.J., van den Brink, R., Allan, D.J., 2009. Mealybugs and the spread of *Grapevine leafroll-associated virus* 3 (GLRaV-3) in a New Zealand vineyard. Australasian Plant Pathology 38, 576.

Chiba, M., Reed, J., Prokhnevsky, A., 2006. Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. Virology 346, 7–14.

Cid, M., Cabaleiro, C., Segura, A., 2003. Detection of *Grapevine leafroll-associated Virus* 3 in rootstocks, in: Proceedings of the 14th Meeting of the ICVG Congress, Locorotondo, Italy, 12-17 September. p. 41.

Cousins, P., Goolsby, J., 2010. Grape rootstock variety influence on Pierce's Disease symptoms in chardonnay. CDFA Pierce's Disease Control Program Research Symposium. 99–100.

Credi, R., Santucci, A., 1990. Serological detection of grapevine leafroll-associated closteroviruslike particles: Apparent absence of viral antigens in leaves of graft-inoculated American rootstocks, in: Proceedings of the 10th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine (ICVG). Volos, Greece. pp. 71–80.

Dolja, V. V, Kreuze, J.F., Valkonen, J.P.T., 2006. Comparative and functional genomics of *closteroviruses*. Virus Research 117, 38–51.

Dolja, V.V., Karasev, A.V., Koonin, E.V., 1994. Molecular biology and evolution of *closteroviruses*: Sophisticated build-up of large RNA genomes. Annual Review of Phytopathology 32, 261–285.

Douglas, N., Krüger, K., 2008. Transmission efficiency of *Grapevine leafroll-associated virus 3* (GLRaV-3) by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: *Pseudococcidae*). European Journal of Plant Pathology 122, 207–212.

Engel, E., Girardi, C., Escobar, P.F., Arredondo, V., Domínguez, C., Pérez-Acle, T., Valenzuela, P.D.T., 2008. Genome analysis and detection of a Chilean isolate of *Grapevine leafroll associated virus-3*. Virus Genes 37, 110–8.

Engelbrecht, D.J., Kasdorf, G.G.F., 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus*. Phytophylactica 22, 341–346.

Enosawa, M., Kageyama, S., Sawai, K., Watanabe, K., Notomi, T., Onoe, S., Mori, Y., Yokomizo, Y., 2003. Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. paratuberculosis. Journal of Clinical Microbiology 41, 4359.

Fahy, E., Kwoh, D.Y., Gingeras, T.R., 1991. Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. Genome Research 1, 25–33.

Frackman, B.S., Kobs, G., Simpson, D., Storts, D., Corporation, P., 1998. Betaine and DMSO□: Enhancing Agents for PCR 9–12.

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E.-J., Boehme, C.C., Notomi, T., Perkins, M.D., Schrenzel, J., 2011. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. FEMS Immunology and Medical Microbiology 62, 41–8.

Freeborough, M., 2008. Leafroll: Economic implications [WWW Document]. Wynboer: A Technical Guide for Wine. URL http://www.wineland.co.za/index.php?option=com_zine&view=article&id=137:leafroll-economic-implications&Itemid=10 (accessed 5.4.12).

Fuchs, M., Marsella-Herrick, P., Loeb, G.M., Martinson, T.E., Hoch, H.C., 2009a. Diversity of *ampeloviruses* in mealybug and soft scale vectors and in grapevine hosts from leafroll-affected vineyards. Phytopathology 99, 1177–84.

Fuchs, M., Martinson, T., Loeb, G., 2009b. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. Plant Disease 93, 395– 401.

Gao, X.P., Guo, X.W., Wang, K., Fu., W.H., 1993. The resistance of grape rootstocks to cold and crown gall. Acta Hort. Sinica 20, 313–318.

Gill, P., Ghaemi, A., 2008. Nucleic Acid Isothermal Amplification Technologies — A Review. Nucleosides , Nucleotides and Nucleic Acids 27, 224– 243.

Golino, D.A., Sim, S., Gill, R., Rowhani, A., 2002. California mealybugs can spread grapevine leafroll disease. California Agriculture 56.

Golino, D.A., Sim, S., Rowhani, A., 1993. Growth and Survival of Grapevine Rootstocks Infected with Grapevine Leafroll and Vitiviruses [WWW Document]. iv.ucdavis.edu. URL http://iv.ucdavis.edu/files/108796.pdf (accessed 3.13.13).

Golino, D.A., Weber, E., Sim, S., 2008a. Leafroll disease is spreading rapidly in a Napa Valley vineyard. California Agriculture 62, 156– 160.

Golino, D.A., Wolpert, J., Sim, S., 2008b. Virus effects on vine growth and fruit components of Cabernet Sauvignon on six rootstocks, in: Proceedings of the 2nd Annual National Viticultue Research Conference. pp. 28–29.

Goszczynski, D.E., Kasdorf, G.G.F., Pietersen, G., 1996. Western blots reveal that isolated *grapevine virus* B is serologically related to grapevine virus A. Journal of Phytopathology 144, 15–26.

Goszczynski, D.E., Kasdorf, G.G.F., Pietersen, G., 1997. Chapter 5: Production of antisera to grapevine leafroll-associated viruses using electrophoretically resolved antigens., in: Monette, P.L. (Ed) Filamentous Viruses of Woody Plants. pp. 49 – 58.

Goszczyński, D.E., Kasdorf, G.G.F., Pietersen, G., 1995. Production and use of antisera specific to grapevine leafroll-associated virusses following electrophoretic separation of their proteins and transfer to nitrocellulose. African Plant Protection 1, 1–8.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K.-I., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. BioTechniques 46, 167–72.

Gouveia, P., Santos, M.T., Eiras-Dias, J.E., Nolasco, G., 2011. Five phylogenetic groups identified in the coat protein gene of *Grapevine leafroll-associated virus*3 obtained from Portuguese grapevine varieties. Archives of Virology 156, 413–20.

Grasswitz, T.R., James, D.G., 2008. Movement of grape mealybug, *Pseudococcus maritimus* , on and between host plants. Entomologia Experimentalis et Applicata 129, 268–275.

Gu, H., Qi, X., Li, X., Jiang, H., Wang, Y., Liu, F., Lu, S., Yang, Y., 2010. Rapid and specific detection of *H3 swine influenza* virus using reverse

transcription loop-mediated isothermal amplification method. Journal of Applied Microbiology 108, 1145–54.

Gu, S., 2003. Effect of Rootstocks on Grapevines [WWW Document]. URL http://www.pawpaw.kysu.edu/viticulture/Information/Rootstock review.pdf (accessed 7.25.13).

Guatelli, J.C., Whitfield, K.M., Kwoh, D.Y., Barringer, K.J., Richman, D.D., 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 87, 1874.

Gunimaladevi, I., Kono, T., Lapatra, S.E., Sakai, M., 2005. A loop mediated isothermal amplification (LAMP) method for detection of infectious *hematopoietic necrosis virus* (IHNV) in rainbow trout (*Oncorhynchus mykiss*). Archives of Virology 150, 899–909.

Habili, N., Fazeli, C.F., Rezaian, M.A., 1997. Identification of a cDNA clone specific to *Grapevine leafroll-associated virus*1, and occurrence of the virus in Australia. Plant Pathology 46, 516–522.

Harper, S., Ward, L., Clover, G., 2010. Development of LAMP and Real-Time PCR Methods for the Rapid Detection of *Xylella fastidiosa* for Quarantine and Field Applications. Phytopathology 100, 1282–1288.

Higashimoto, Y., Ihira, M., Ohta, A., Inoue, S., Usui, C., Asano, Y., Yoshikawa, T., 2008. Discriminating between *varicella-zoster virus* vaccine and wild-type strains by loop-mediated isothermal amplification. Journal of Clinical Microbiology 46, 2665–70.

Hu, J.S., Gonsalves, D., Boscia, D., Namba, S., 1990. Use of monoclonal antibodies to characterize grapevine leafroll associated closteroviruses. Phytopathology 80, 920–925.

Inami, H., Tsuge, K., Matsuzawa, M., Sasaki, Y., Togashi, S., Komano, A., Seto, Y., 2009. Semi-automated bacterial spore detection system with micro-fluidic chips for aerosol collection, spore treatment and ICAN DNA detection. Biosensors & Bioelectronics 24, 3299–305.

Ioannou, N., Hadjinicolis, A., Hadjinicoli, A., 1999. Mealybug transmission and natural spread of grapevine *closterovirus* in Cyprus. Options Méditerranéennes, Série B / n 29 - Proceedings of the Mediterranean Network on Grapevine Closteroviruses 93–101.

Isogai, E., Makungu, C., Yabe, J., Sinkala, P., Nambota, A., Isogai, H., Fukushi, H., Silungwe, M., Mubita, C., Syakalima, M., Hang'ombe, B.M., Kozaki, S., Yasuda, J., 2005. Detection of *Salmonella* invA by isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) in Zambia. Comparative Immunology, Microbiology and Infectious Diseases 28, 363–70.

Iwamoto, T., Sonobe, T., 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, 2616.

Jarugula, S., Gowda, S., Dawson, W., 2010. 3'-coterminal subgenomic RNAs and putative cis-acting elements of *Grapevine leafroll-associated virus 3* reveals "unique"features of gene expression strategy. Virol. J.

Jooste, A.E.C., Maree, H.J., Bellstedt, D.U., Goszczynski, D.E., Pietersen, G., Burger, J.. T., 2010. Three genetic *Grapevine leafroll-associated virus*3 variants identified from South African vineyards show high variability in their 5'UTR. Archives of Virology 155, 1997–2006.

Jooste, A.E.C., Pietersen, G., Burger, J.T., 2011. Distribution of *Grapevine leafroll associated virus-3* variants in South African vineyards. European Journal of Plant Pathology 131, 371– 381.

Kaneko, H., Kawana, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. Journal of Biochemical and Biophysical methods 70, 499–501.

Karasev, A.V., 2000. Genetic diversity and evolution of *closteroviruses*. Annual Review of Phytopathology 38, 293–324.

Karasev, A.V., Boyko, V., Gowda, S., Nikolaeva, O.V., Hilf, M.., Koonn, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., Lee, R.F., Garnsey, S.M., Lewandowski, D.., Dawson, W.O., 1995. Complete sequence of the *citrus tristeza virus* RNA genome. Virology 208, 511–520.

Kasdorf, G.G.F., 2006. Final report: Detection of grapevine viruses in grapevine rootstocks [WWW Document]. sawis library. URL http://www.sawislibrary.co.za/dbtextimages/FinalReport118.pdf (accessed 3.18.13).

Krake, L., Scott, N., Rezaian, M., Taylor, R., 1999. Graft-transmitted diseases of grapevines, illustrate. ed. CSIRO PUBLISHING.

La Notte, P., 1997. A spot-PCR technique for the detection of phloem-limited grapevine viruses. Journal of Virological Methods 66, 103–108.

La Notte, P., Minafra, A., 1997. A spot-PCR technique for the detection grapevine viruses of phloem-limited. Virological Methods 66, 103–108.

Le Roux, C. a, Kubo, T., Grobbelaar, a a, van Vuren, P.J., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K., Paweska, J.T., 2009. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of *Rift Valley fever* virus in clinical specimens. Journal of clinical microbiology 47, 645–51.

Lee, J., Keller, K.E., Rennaker, C., Martin, R.R., 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir: Free amino acids, sugars, and organic acids. Food Chemistry 117, 99–105.

Lee, M.-S., Yang, M.-J., Hseu, Y.-C., Lai, G.-H., Chang, W.-T., Hsu, Y.-H., 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, 43–8.

Li, Q., Xue, C., Qin, J., Zhou, Q., Chen, F., Bi, Y., Cao, Y., 2009. An improved reverse transcription loop-mediated isothermal amplification assay for sensitive and specific detection of *Newcastle disease virus*. Archives of Virology 154, 1433–40.

Lider, L., Walker, M.A., Wolpert, J., 1993. Grape rootstocks in California vineyards: The changing picture. International Symposium on Viticulture and Enology 388, 13–18.

Lin, Y., Brlansky, R., 2002. Inefficient transmission of *Citrus tristeza virus* from grapefruit by single brown citrus aphids. HortScience 37, 936–939.

Ling, K.S., Zhu, H., Drong, R., Slightom, J., McFerson, J., Gonsalves, D., 1998. Nucleotide sequence of the 3'-terminal two-thirds of the *Grapevine leafroll-associated virus-3* genome reveals a typical monopartite closterovirus. Journal of General Virology 79, 1299.

Ling, K.S., Zhu, H., Gonsalves, D., 2004. Complete nucleotide sequence and genome organization of *Grapevine leafroll-associated virus 3* , type member of the genus *Ampelovirus*. Journal of General Virology 2099–2102.

Ling, K.S., Zhu, H., Jiang, Z., Gonsalves, D., 2000. Effective application of DAS-ELISA for detection of grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein. European Journal of Plant Pathology 106, 301–309.

Ling, K.S., Zhu, H.-Y., Petrovic, N., Gonsalves, D., 2001. Comparative Effectiveness of ELISA and RT-PCR for Detecting Grapevine Leafroll-Associated Closterovirus-3 in Field Samples. American Journal of Enology and Viticulture 52, 21–27.

Little, M., Andrews, J., Moore, R., 1999. Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clinical Chemistry 45, 777–784.

Liu, Y., Wang, Z., Qian, Y., Mu, J., Shen, L., Wang, F., Yang, J., 2010. Rapid detection of *tobacco mosaic virus* using the reverse transcription loop-

mediated isothermal amplification method. Archives of Virology 155, 1681–5.

Lu, R., 2003. Virus-induced gene silencing in plants. Methods 30, 296–303.

Maeda, H., Kokeguchi, S., Fujimoto, C., Tanimoto, I., Yoshizumi, W., Nishimura, F., Takashiba, S., 2005. Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method. FEMS Immunology and Medical Microbiology 43, 233–9.

Magoon, C.., Magness, J.., 1937. Investigation on the adapability of grape rootstock to Gulf Coast conditions. Proc. Amer. Soc. Hort. Sci 35, 466 – 470.

Maherchandani, S., Muñoz-Zanzi, C.A., Patnayak, D.P., Malik, Y.S., Goyal, S.M., 2004. The Effect of Pooling Sera on the Detection of Avian *Pneumovirus* Antibodies using an Enzyme-Linked Immunosorbent Assay Test. Journal of Veterinary Diagnostic Investigation 16, 497.

Mahfoudhi, N., Digiaro, M., Dhouibi, M., 2009. Transmission of grapevine leafroll viruses by *Planococcus ficus* (Hemiptera: *Pseudococcidae*) and *Ceroplastes rusci* (Hemiptera: *Coccidae*). Plant disease 999–1002.

Malan, S., 2009. Real time PCR as a versatile tool for virus detection and transgenic plant analysis. University of Stellenbosch.

Maree, H.J., Almeida, R.P.P., Bester, R., Chooi, K.M., Cohen, D., Dolja, V. V, Fuchs, M.F., Golino, D.A., Jooste, A.E.C., Martelli, G.P., Naidu, R. a, Rowhani, A., Saldarelli, P., Burger, J.T., 2013. *Grapevine leafroll-associated virus* 3. Frontiers in Microbiology 4, 82.

Maree, H.J., Freeborough, M.J., Burger, J.., 2008. Complete nucleotide sequence of a South African isolate of *Grapevine leafroll-associated virus* 3 reveals a 5'UTR of 737 nucleotides. Archives of Virology 153, 755–7.

Martelli, G.P., 1993. Graft-transmissible diseases of grapevines: handbook for detection and diagnosis. Food & Agriculture Org., Rome, Italy.

Martelli, G.P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A.A., Al Rwahnih, M., Dolja, V.V., Dovas, C.I., Fuchs, M., Gugerli, P., Hu, J.S., Jelkmann, W., Katis, N.I., Maliogka, V.I., Melzer, M.J., Menzel, W., Minafra, A., Rott, M.E., Rowhani, A., Sabanadzovic, S., Saldarelli, P., 2012. Taxonomic revision of the family *Closteroviridae* with special reference to the grapevine leafroll-associated members of the genus *Ampelovirus* and the putative species unassigned to the family. Journal of Plant Pathology 94, 7–19.

Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R., Dolja, V., Falk, B., Gonsalves, D., Jelkmann, W., Karasev, A.,

Minafra, A., Namba, S., Vetten, H.., Wisler, G.., Yoshikawa, N., 2002. The family *Closteroviridae* revised. Archives of Virology 147, 2039–2044.

Martelli, G.P., Boudon-Padieu, E., 2006. Directory of infectious diseases of grapevines and viroses and virus-like diseases of the grapevine: bibliographic report, in: Options Méditerranéennes, Série B n 55: CIEHEM-IAMB.

Mirelman, D., Nuchamowitz, Y., Stolarsky, T., 1997. Comparison of use of enzyme-linked immunosorbent assay-based kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E.dispar*. Journal of Clinical Microbiology 35, 2405–2407.

Monis, J., Bestwick, R., 1996. Detection and localization of grapevine leafroll associated closteroviruses in greenhouse and tissue culture grown plants. American Journal of Enology and Viticulture 47, 199– 205.

Morgan, B.P., 2000. Complement Methods and Protocols. Humana Press, p. 280.

Mori, Y., Kitao, M., Tomita, N., Notomi, T., 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. Journal of Biochemical and Biophysical Methods 59, 145–57.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical and Biophysical Research Communications 289, 150–4.

Mori, Y., Notomi, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. Journal of Infection and Chemotherapy□: official journal of the Japan Society of Chemotherapy 15, 62–9.

Mukai, H., Takeda, O., Usui, K., Asada, K., Kato, I., 2008. SNP typing of aldehyde dehydrogenase2 gene with Cycleave ICAN. Molecular and Cellular probes 22, 333–7.

Mukai, H., Uemori, T., Takeda, O., Kobayashi, E., Yamamoto, J., Nishiwaki, K., Enoki, T., Sagawa, H., Asada, K., Kato, I., 2007. Highly efficient isothermal DNA amplification system using three elements of 5'-DNA-RNA-3' chimeric primers, RNaseH and strand-displacing DNA polymerase. Journal of Biochemistry 142, 273–81.

Muñoz-Zanzi, C.A., Johnson, W.O., Thurmond, M.C., Hietala, S.K., 2000. Pooled-sample testing as a herd-screening tool for detection of *bovine viral diarrhea virus* persistently infected cattle. Journal of Veterinary Diagnostic Investigation 12, 195.

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

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., Notomi, T., 2001. Loop-mediated isothermal amplification reaction using a nondenatured template. Clinical chemistry 47, 1742.

Nakaune, R., Nakano, M., 2006. Efficient methods for sample processing and cDNA synthesis by RT-PCR for the detection of grapevine viruses and viroids. Journal of Virological Methods 134, 244–9.

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

Nolasco, G., 2003. Diagnosis: recent developments and routine implementation., in: Extended Abstracts 14th Meeting of the ICVG, Locorotondo, Italy. pp. 184–187.

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

Osman, F., Leutenegger, C., Golino, D.A., Rowhani, A., 2007. Real-time RT-PCR (TaqMan ®) assays for the detection of Grapevine Leafroll associated viruses 1 – 5 and 9. Journal of Virological Methods 141, 22–29.

Osman, F., Olineka, T., Hodzic, E., Golino, D.A., Rowhani, A., 2012. Comparative procedures for sample processing and quantitative PCR detection of grapevine viruses. Journal of Virological Methods 179, 303–10.

Osman, F., Rowhani, A., 2006. Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). Journal of virological methods 133, 130–6.

Over de Linden, A., Chamberlain, E., 1970. Effect of grapevine leafroll virus on vine growth and fruit yield and quality. New Zealand Journal of Agricultural Research 37–41.

Pacifico, D., Caciagli, P., Palmano, S., Mannini, F., Marzachì, C., 2011. Quantitation of *Grapevine leafroll associated virus-1* and -3, *Grapevine virus A*, *Grapevine fanleaf virus* and *Grapevine fleck virus* in field-collected *Vitis vinifera* L. “Nebbiolo” by real-time reverse transcription-PCR. Journal of Virological Methods 172, 1–7.

Pandey, B.D., Poudel, A., Yoda, T., Tamaru, A., Oda, N., Fukushima, Y., Lekhak, B., Risal, B., Acharya, B., Sapkota, B., Nakajima, C., Taniguchi, T., Phetsuksiri, B., Suzuki, Y., 2008. Development of an in-house loop-

mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients. Journal of Medical Microbiology 57, 439–43.

Parida, M., Horioke, K., Ishida, H., Dash, P.K., Saxena, P., Jana, A.M., Islam, M.A., Inoue, S., Hosaka, N., Morita, K., 2005. Rapid detection and differentiation of *dengue virus* serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. Journal of Clinical Microbiology 43, 2895.

Parida, M., Posadas, G., Inoue, S., Hasebe, F., Morita, K., 2004. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of *West Nile virus*. Journal of Clinical Microbiology 42, 257.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L., 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, 407–421.

Paris, D.H., Imwong, M., Faiz, A.M., Hasan, M., Yunus, E. Bin, Silamut, K., Lee, S.J., Day, N.P.J., Dondorp, A.M., 2007. Loop-mediated isothermal PCR (LAMP) for the diagnosis of *falciparum malaria*. The American journal of tropical medicine and hygiene 77, 972–6.

Petersen, C., 1997. Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinu*s and *P. calceolariae*. Plant Pathology 509–515.

Pietersen, G., 2004. Spread of grapevine leafroll disease in South Africa-a difficult but not insurmountable problem [WWW Document]. Technical Yearbook 2004/5. URL http://www.wynboer.co.za/recentarticles/0406leaf.php3 (accessed 1.18.13).

Pietersen, G., Spreeth, N., Oosthuizen, T., Rensburg, A. van, Rensburg, M. van, Lottering, D., Rossouw, N., Tooth, D., 2013. Control of Grapevine Leafroll Disease Spread at a Commercial Wine Estate in South Africa: A Case Study. American Journal of Enology and Viticulture February.

Pietersen, G., Spreeth, N., Oothuizen, T., Van Rensburg, A., Lottering, D., Tooth, D., Rossouw, N., 2009. A case study of control of grapevine leafroll disease spread on Vergelegen Wine Estate, South Africa, 2002-2008, in: Extended Abstracts, 16th Meeting of ICGV, Dijion, France, 31 August – 4 September. pp. 230–231.

Pietersen, G., Walsh, H.A., 2012. Development of a LAMP Technique for Control of *Grapevine Leafroll Associated Virus Type 3* (GLRaV-3) in Infected White Cultivar Vines by Roguing, in: Extended Abstracts of 17th Congress of the International Council for the Study of Virus and Virus-like

Diseases of the Grapevine (ICVG), Davis, California, USA, October 7-14th. pp. 50–51.

Rayapati, A., O'Neil, S., Walsh, D., 2008. Grapevine leafroll disease [WWW Document]. WSU Extension Bulletin …. URL http://cru.cahe.wsu.edu/CEPublications/eb2027e/eb2027e.pdf (accessed 11.13.12).

Reed, J., Kasschau, K., Prokhnevsky, A., 2003. Suppressor of RNA silencing encoded by *Beet yellows virus*. Virology 306, 203–209.

Rosciglione, B., Martelli, G.P., 1983. Mealybug transmission ot *grapevine Virus A*. Vitis 22, 331–347.

Rowhani, A., Chay, C., Golino, D.A., Falk, B., 1993. Development of a polymerase technique for the detection of *grapevine fanleaf virus* in grapevine tissue. Phytopathology 83, 749–753.

Rowhani, A., Uyemoto, J., Golino, D.A., 1997. A comparison between serological and biological assays in detecting grapevine leafroll associated viruses. Plant disease 799–801.

Saitou, Y., Kobayashi, Y., Hirano, S., Mochizuki, N., Itou, T., Ito, F.H., 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, 13–7.

Sampol, B., Bota, J., Riera, D., Medrano, H., Flexas, J., 2003. Analysis of the virus-induced inhibition of photosynthesis in malmsey grapevines. New Phytologist 160, 403–412.

Sforza, R., Boudon-Padieu, E., Greif, C., 2003. New Mealybug Species Vectoring Grapevine Leafroll-Associated Viruses-1 and -3 (GLRaV-1 and -3). European Journal of Plant Pathology 109, 975–981.

Shi, W., Li, K., Ji, Y., Jiang, Q., Shi, M., Mi, Z., 2011. Development and evaluation of reverse transcription-loop-mediated isothermal amplification assay for rapid detection of *enterovirus* 71. BMC Infectious Diseases 11, 197.

Singh, R.P., Dilworth, A.D., Singh, M., Babcock, K.M., 2006. An alkaline solution simplifies nucleic acid preparation for RT-PCR and infectivity assays of viroids from crude sap and spotted membrane. Journal of Virological Methods 132, 204–11.

Soliman, H., El-Matbouli, M., 2006. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid detection of viral *hemorrhagic septicaemia virus* (VHS). Veterinary Microbiology 114, 205–213.

Teng, P.H., Chen, C.L., Sung, P.F., Lee, F.C., Ou, B.R., Lee, P.Y., 2007. Specific detection of reverse transcription-loop-mediated isothermal amplification amplicons for *Taura syndrome virus* by colorimetric dot-blot hybridization. Journal of Virological Methods 146, 317–326.

Thai, H.T.C., Le, M.Q., Vuong, C.D., Parida, M., Minekawa, H., Notomi, T., Hasebe, F., Morita, K., 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of *severe acute respiratory syndrome coronavirus*. Journal of Clinical Microbiology 42, 1956.

Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nature Protocols 3, 877–882.

Tomlinson, J. a., Boonham, N., Dickinson, M., 2010. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. Plant Pathology 59, 465–471.

Tsai, C.W., Daugherty, M.P., Almeida, R.P.P., 2011. Seasonal dynamics and virus translocation of *Grapevine leafroll-associated virus 3* in grapevine cultivars. Plant Pathology 61, 977– 985.

Turturo, C., Minafra, A., Ni, H., Wang, G., Di Terlizzi, B., Savino, V., 1998. Occurrence of *peach latent mosaic viroid* in China and development of an improved detection method. Journal of Plant Pathology 80, 165–169.

Turturo, C., Saldarelli, P., Yafeng, D., Digiaro, M., Minafra, A., Savino, V., Martelli, G.P., 2005. Genetic variability and population structure of *Grapevine leafroll-associated virus* 3 isolates. Journal of General Virology 86, 217 – 224.

Uemori, T., Mukai, H., Takeda, O., Moriyama, M., Sato, Y., Hokazono, S., Takatsu, N., Asada, K., Kato, I., 2007. Investigation of the molecular mechanism of ICAN, a novel gene amplification method. Journal of Biochemistry 142, 283–92.

Uemura, N., Makimura, K., Onozaki, M., Otsuka, Y., Shibuya, Y., Yazaki, H., Kikuchi, Y., Abe, S., Kudoh, S., 2008. Development of a loop-mediated isothermal amplification method for diagnosing *Pneumocystis pneumonia*. Journal of Medical Microbiology 57, 50–7.

Urasaki, N., 2008. Detection of “Candidatus Liberibacter asiaticus” by cycleave isothermal and chimeric primer-initiated amplification of nucleic acids (Cycleave ICAN). plantmanagementnetwork.org 2007–2009.

Urasaki, N., Kawano, S., Mukai, H., Uemori, T., Takeda, O., Sano, T., 2008. Rapid and sensitive detection of “*Candidatus Liberibacter asiaticus*” by

cycleave isothermal and chimeric primer-initiated amplification of nucleic acids. Journal of General Plant Pathology 74, 151–155.

Walker, G., Fraiser, M., 1992. Strand displacement amplification—an isothermal, in vitro DNA amplification technique. Nucleic Acids Research 20, 1691–1696.

Walton, V., 2004. Vine mealybug, *Planococcus ficus* (Signoret)(Hemiptera: Pseudococcidae), a key pest in South African vineyards. A review. South African Journal of Enology and Viriculture 25, 54–62.

Wang, J., Sharma, A.M., Duffy, S., Almeida, R.P.P., 2011. Genetic Diversity in the 3'Terminal 4.7-kb Region of *Grapevine leafroll-associated virus* 3. Phytopathology 101, 445–450.

Wastling, S.L., Picozzi, K., Kakembo, A.S.L., Welburn, S.C., 2010. LAMP for human African trypanosomiasis: a comparative study of detection formats. PLoS Neglected Tropical Diseases 4, e865.

Weber, E., Golino, D.A., Rowhani, A., 2002. Laboratory testing for grapevine diseases. Practical Winery and Vineyard. 22, 21–24.

Wells, S., Godden, S., Lindeman, C., Collins, J.E., 2003. Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. Journal of the American Veterinary Medical Association 223, 1022–1025.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I., Tsukamoto, T., 2007. Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. Journal of Medical Virology 79, 326–334.

Zorloni, A., Prati, S., Bianco, P., 2006. Transmission of *Grapevine virus A* and *Grapevine leafroll-associated virus 3* by *Heliococcus bohemicus*. Journal of Plant Pathology 88, 325–328.

1