Expertise
and insight
for the future

Irene Tukonen Pala

A Real-Time PCR Method for the Detection of Greedy Scale (*Hemiberlesia rapax*)

Metropolia University of Applied Sciences
Bachelor of Laboratory Services
The Degree Programme in Laboratory Sciences
Bachelor's Thesis
13 November 2019

Author Title Number of Pages Date	Irene Tukonen Pala A Real-Time PCR Method for the Detection of Greedy Scale (*Hemiberlesia rapax*) 38 pages + 2 appendices 13 November 2019
Degree	Bachelor of Laboratory Services
Degree Programme	Laboratory Sciences
Instructors	Alistair Mowat, Technical manager (Eurofins Bay of Plenty) Sean Bulley, Scientist (Plant and Food Research) Tiina Soininen, Lecturer

In New Zealand, kiwifruit and avocado orchard pest monitoring is based on the manual counting of insects found on leaf samples. This work is time-consuming and the results can be variable due to the mobility of insects. Traces of cellular DNA that insects excrete into the environment provide an opportunity to disrupt the current manual inspection methods for pest monitoring. In this study, the goal was to develop a real-time PCR method for the detection of greedy scale (*Hemiberlesia rapax*) insect, that is a major pest found in commercial crops in New Zealand and globally.

Firstly, in this study, a target gene, internal transcribed spacer (ITS 2), was selected. Then species-specific real-time PCR primers and primers for sequencing purposes were designed, and finally the optimal PCR conditions were found using Sybr Green chemistry. This project was led and funded by Eurofins Bay of Plenty and the laboratory work was carried out in Plant & Food Research facilities in New Zealand.

Designing the novel real-time PCR method for the detection of greedy scale was successful. Results were analyzed by observing Cq and amplification values of replicate samples. Amplification values less than 1.4 (optimal 2) and sample values similar to non-template control samples were treated as outliers. Melting curve analysis was conducted after each run to assure that the target gene region was only amplified. The target ITS2 region has a melting temperature of 85.7℃. Assay specificity was evaluated by testing the method against other insect pests and with different DNA matrix. The method resulted to be highly specific and sensitive for greedy scale, meeting the validation requirements.

This proof of concept study will be used as a platform for further studies of improving the pest monitoring method. The future studies would include designing a method to identify many pests found from orchards. Also, one aim is to study how the method would be applied in practice. The final goal would be to commercialize the method so that growers, for instance, could use it in packhouses as the new pest monitoring method.

Keywords	Greedy scale, primers, real-time PCR method, specificity, pest monitoring

Tekijä Otsikko Sivumäärä Aika	Irene Tukonen Pala Reaaliaikaisen PCR-menetelmän kehitys *greedy scale* (*Hemiberlesia rapax*) -hyönteislajille 38 sivua + 2 liitettä 05.11.2019
Tutkinto	Laboratorioanalyytikko (AMK)
Tutkinto-ohjelma	Laboratorioanalytiikka
Ohjaajat	Tekninen päällikkö Alistair Mowat (Eurofins Bay of Plenty) Tutkija Sean Bulley (Plant and Food Research) Lehtori Tiina Soininen

Uudessa-Seelannissa tuholaisseurantaa toteutetaan manuaalisesti tarkkaillen hyönteisten populaatiokokoja hedelmätarhoissa. Tämä tapa vie aikaa, eivätkä tulokset välttämättä ole valideja, sillä suurin osa hyönteisistä on liikkuvia. DNA-jäljet, joita hyönteiset erittävät ympäristöön solujen ja kudosten muodossa, luovat mahdollisen vaihtoehtoisen menetelmän manuaaliselle seurannalle. Tämän tutkimuksen tavoitteena oli kehittää reaaliaikainen PCR-metodi *greedy scale* -hyönteislajille, joka on suurtuholainen ja jota esiintyy kaupallisissa hedelmätarhoissa Uudessa-Seelannissa ja globaalisestikin.

Tutkimukseen kuului kohdegeenin, *internal transcribed spacer* (ITS2), valitseminen, lajispesifisten reaaliaikaisten PRC-alukkeiden ja sekvensointia varten olevien alukkeiden suunnittelu sekä optimaalisten PCR-olosuhteiden löytäminen Sybr Green -kemialla. Tämä projekti johdettiin ja rahoitettiin Eurofins Bay of Plenty -yrityksen puolesta ja työ suoritettiin Plant and Food Research -yrityksen tiloissa Uudessa-Seelannissa.

Reaaliaikaisen PCR-menetelmän kehitys *greedy scale* -hyönteislajin tunnistukseen onnistui. Tulokset analysoitiin tarkkailemalla Cq- ja monistusarvoja. Monistusarvoja, jotka olivat alle 1,4 (optimaali on 2), ja samankaltaisia arvoja näytteiden ja nollakontrollien välillä ei otettu huomioon tulosten arvioinnissa. Sulamiskäyräanalyysi suoritettiin jokaisen PCR-ajon jälkeen. Tällä varmistettiin, että vain kohdegeenin osuus monistui. ITS2-geenin sulamispiste on 85,7 ℃. Metodin spesifisyyttä arvioitiin kokeilemalla metodin toimivuutta muilla hyönteislajeilla ja testaamalla myös toisella DNA-matriisilla. Metodi on erittäin spesifinen ja herkkä *greedy scale* -hyönteislajille sekä täyttää validointivaatimukset.

Tämä tutkimus oli soveltuvuusselvitys, jota tullaan käyttämään lähtökohtana tuholaisseurannan kehittämisen jatkotutkimuksille. Seuraava tavoite projektissa on suunnitella metodi, jolla voidaan tunnistaa useita tuholaisia hedelmätarhoista samanaikaisesti. Tämän lisäksi yhtenä tavoitteena on tutkia menetelmän soveltamista käytäntöön. Lopullinen päämäärä on menetelmän kaupallistaminen, jotta esimerkiksi viljelijät voisivat käyttää sitä pakkaamoissa uutena tuholaistorjuntamenetelmänä.

Avainsanat	*Greedy scale* -hyönteislaji, alukkeiden suunnittelu, Reaaliaikainen PCR menetelmä, spesifisyys, tuholaisseuranta

Contents

List of Abbreviations

1 Introduction 1

2 Theoretical Background 2

2.1 Armoured Scales 2

Scale Monitoring in Avocado and Kiwifruit Orchards 4

2.2 Internal Transcribed Spacer 5

2.3 PCR Primer Design 6

2.4 Real-Time PCR 9

2.4.1 Melting Curve Analysis 11

2.4.2 Assay Design 11

3 Methods 12

3.1 Sample Collection and Pre-Treatment 12

3.2 DNA Extraction and Quality 14

3.3 Validating Primer Design 15

DNA sequencing 17

3.4 Gradient PCR 18

3.5 Real-time PCR Assay Design 18

3.6 Assay Specificity 19

4 Results 20

4.1 DNA Concentration 20

4.2 Primer Properties 21

Primer-Dimer Probabilities 22

4.3 qPCR Primer Verification 23

4.4 Optimal Annealing Temperature 24

4.5 The Optimal qPCR Method 25

Specificity Tests 28

5 Discussion & Conclusion 32

References 34

Appendices
Appendix 1. The Other qPCR Primer Pair
Appendix 2. Specificity Test qPCR Result

List of Abbreviations

Cq	Quantification cycle
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
ITS	Internal transcribed spacer
NTC	Non template control
PCR	Polymerase chain reaction
qPCR	Real-time PCR
rRNA	Ribosomal RNA
Ta	Annealing temperature
Tm	Melting temperature

1 Introduction

Some insects are considered as agricultural pests and disease vectors with agricultural and horticultural production systems. However, they also provide ecosystem services, for instance in form the of pollination or as predators providing natural biological control [1, 3]. Accurate detection, potential monitoring and control of insect taxa can have significant financial, economic and environmental benefits.

Currently, pest monitoring in kiwifruit and avocado orchards in New Zealand is done manually by counting insects found on leaves or fruits. This work is time-consuming and the results can be variable as most insects are mobile or can move with wind currents around the orchards. There is now interest towards new and faster pest monitoring methods.

Deoxyribonuclease, DNA, is chemically and physically a very stable molecule. Higher organisms excrete DNA in the form of cells and tissue to the surrounding environment. Besides intracellular DNA originating from small cells and mitochondria [2], extracellular DNA is also present in the environment due to membrane degradation over time [3]. This enables the detection of species even though the life stage or gender are not able to be determined [4]. This complex mixture of genomic DNA that is collected from the environment is called environmental DNA (eDNA).

Traces of DNA found in orchards provide an opportunity for an alternative pest monitoring method and biosecurity management tool. Ideally, this could give more reliable and statistically robust results of the insect populations found in orchards.

This thesis presents novel primers and a real-time PCR assay for the detection of greedy scale (*Hemiberlesia rapax*), which is a major pest found on commercial orchards in New Zealand. This was a proof of concept study that was part of research study on, the feasibility of using real-time PCR or similar genetic approach for pest monitoring. The end goal of the whole research is to study, whether a commercialized method using DNA diagnostic tools for pest identification can be developed. This study was funded and led by Eurofins Bay of Plenty, Katikati. The laboratory work was done in collaboration with Plant and Food Research, Te Puke in New Zealand.

2 Theoretical Background

2.1 Armoured Scales

Scales are Hemipterous insects that include all members of the superfamily Coccoidea (order: Hemiptera, Phylum: Arthropoda) [5, 4]. Two globally distributed scale species, greedy scale (*H. rapax*) and latania scale (*Hemiberlesia lataniae*), are major pest species found in New Zealand avocado and kiwifruit orchards [6, 6]. The third scale species, oleander scale (*Asipidiotus nerii*), has been found mainly from kiwifruit orchards [6, 6], which is why the focus in this study is on the more common greedy and latania scale species. Scales infest a variety of ornamental and food-bearing plants [7]. Latania scale has a wide variety of host plants, including banana, mango, orchids and rose [8]. Greedy scale is found in olive, apple, citrus, rose and many other plants [7]. In New Zealand, they can be found in many commercial horticultural crops [6, 7].

Scales are usually less than 5 mm in length. Taxonomically, scales are identified from the morphological characteristics of the cuticle of the adult female. [5, 4.] Adult females mature sexually in a juvenile form [5, 5] and they are soft-bodied and concealed under "armour" cap made of secretions from the scale. As the scale continues to grow, the cap is expanded at the same rate to maintain cover. [4, 1.] Latania and greedy scale are closely related species. They differ morphologically since latania scale has a yellow exuvial, whereas greedy scale has a browner exuvial forming a dark spot on the shell [7]. This difference between the shells of the species can be seen from figure 1 A and B. However, if the wax shell is removed both scale insects itself are yellow [7]. When the scale dies, the body dries out turning brown [6, 6].

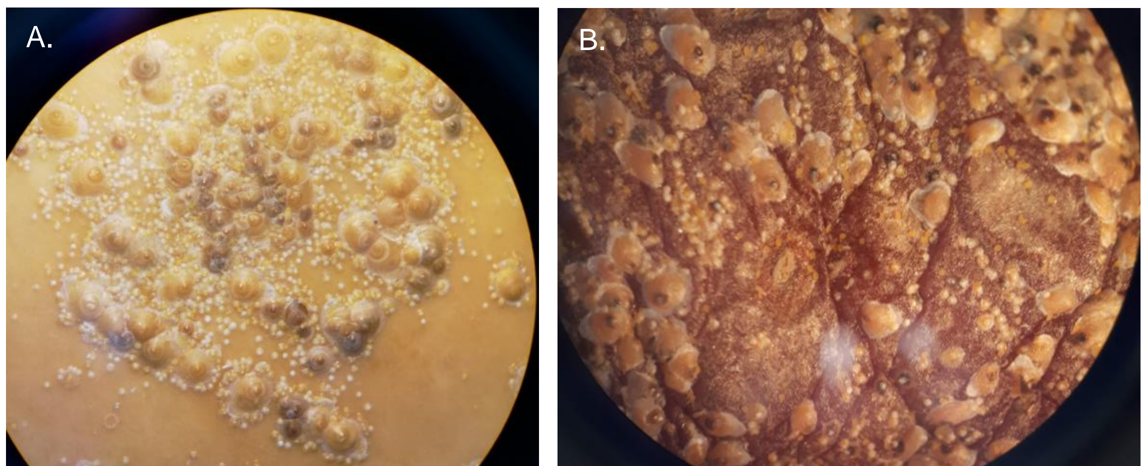

Figure 1. Microscopic magnification photos of A. Latania scale on a butternut squash (Plant & Food Research, Te Puke) and B. Greedy scale on a potato (Plant & Food Research, Auckland)

Reproduction of Armoured Scales can be sexual or parthenogenetic. One to three eggs a day are produced over a period lasting up to two months. Each adult produces a total of 30-120 eggs during this period. [9, 1.] Eggs hatch soon after being laid into mobile crawlers, which search for new plants to feed [10]. Besides dispersing by crawling, scales can also be blown away by wind currents. Female crawlers attach for good to the host plant and begin to secrete a waxy scale cap. [6, 6.] Male greedy and latania scale are not known in New Zealand and are not common in oleander scale [9, 2].

Scales can weaken or kill plants by feeding on their sap, injecting toxins or transmitting viral diseases. Also, scales excrete honeydew which can, in right conditions, serve as a growth medium for sooty mould. [5, 5.] Scales can cause a cosmetic defect to the fruit, such as being associated with a small “dimple” on the surface of the fruit as seen in figure 2. This fruit damage can result in market restrictions, thus limiting the fruit availability for specific markets. Because of this, removing fruit with scale infestation, as well as monitoring and control is important.

Figure 2. Cosmetic defect on avocado fruit caused by scale insects [6, 6].

Information on the natural enemies of the scales is limited. However, there are two small parasitic wasps (*Encarsia citrina* and *Signiphora merceti*) that have been recorded attacking both Greedy and Latania scales. [6, 7]

Scale Monitoring in Avocado and Kiwifruit Orchards

Monitoring is important, as armoured scales have become a major pest in the avocado industry due to changes in importing country quarantine status and the increased diversity of markets [11, 23]. In general, there are two main periods of scale multiplication when monitoring is essential. The first is from November to January and the second from March to May. During these periods, the scale crawlers have emerged and settled to form adult scales that can be identified more reliably. However, monitoring the presence of all pests at all times is best practice. Control of scales is most effective by using spray applications after harvest as well as during either of these scale multiplication periods. [11, 17.]

The most valuable feedback a grower can get from the presence of scales is analysing market pack-out assessments or harvest data in packhouses. In the orchard, monitoring can be applied to fruit and leaves by using at least an x10 magnifying glass [11, 17]. In practice, monitoring scales from leaves and fruit is done by examining 10 mature fruit or leaves per tree. Both the upper and lower surface of the leaf needs to be examined for the presence of scales, using a hand lens. If a scale is found, the scale cap needs to be carefully lifted to determine whether the scale is alive or dead. [11, 23.]

Going through 10 leaves per tree from an orchard is time-consuming work and also the results might change during monitoring since scale crawlers are mobile and can move with wind currents too. Thus, there is interest for new, faster and more robust pest monitoring methods that allow better assessment of the presence of insect pests like scales within orchards.

2.2 Internal Transcribed Spacer

Different gene regions can be potentially used for the identification of pests from eDNA found from orchards. One of these genes is internal transcribed spacer, ITS, which is part of the ribosomal DNA (rDNA) that encodes rRNAs. Eukaryotic nuclear rDNA is formed of tandem repeat units, that consists of genes coding for the 5.8S rDNA, nuclear small (SSU) and large (LSU) subunit. These coding regions are separated from each other by spacers. [12.] ITS is divided into ITS1 and ITS2 regions, which are separated by the 5.8S rDNA coding region as illustrated in figure 3.

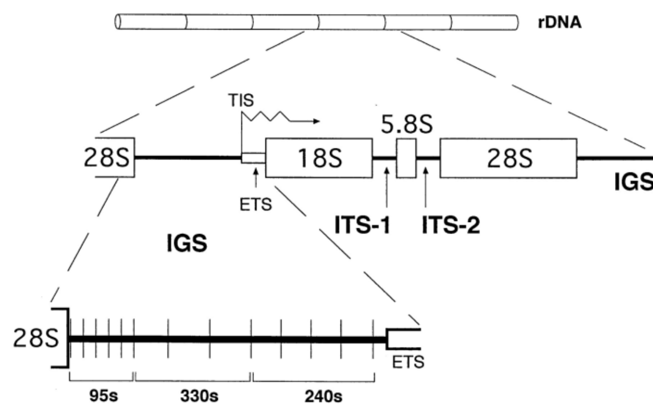

Figure 3. Structure of rDNA repeat units. 18S, 5.8S and 18S boxes indicate rRNA genes. The rest are; IGS, intergenic spacer region, TIS, transcription initiation site and ITS1 and ITS2, internal transcribed regions. [13.]

The rDNA spacer regions have evolved much faster than the coding regions, as mutations in spacer regions do not have lethal effects on the organisms [12]. This makes spacer regions variable between closely related species. Thus, ITS1 and ITS2 are frequently used nuclear markers for phylogenetic studies across many eukaryotic groups. [14.] Conserved regions of ITS2 indicate that it may function in pre-rDNA maturation, even though it has been assumed in the past that ITS has no function. [15] ITS is also a popular marker due to a high copy number of the gene clusters, which makes the amplification easy.

ITS has been mostly used in plant and fungi barcoding studies, where species are identified using a short segment of a gene. In animal barcoding, mitochondrial DNA markers, such as cytochrome c oxidase (COI and COII), are widely used. Nevertheless, ITS2 region was used in metabarcoding for the identification of Hexapoda species in the study by Toju H. and Baba G. Y [16]. Additionally, in the study by Yao H, *et al.* [17] 12,221 animal ITS2 sequences were downloaded from GenBank and analysed. Results showed that the success rate for identifying animals using ITS2 as a barcode was 91.7 %. In the study [17], it is suggested that the ITS2 region could be used as a complementary locus to COI for identifying animals.

These studies prove that ITS2 has sufficient variability to distinguish between closely related species [17] and to be a suitable barcoding gene for animals. Thus, for this study, the ITS2 was chosen as the target gene. Besides, the COI and COII sequences of scale insects are extremely A-T rich, approximately 75 %, which makes PCR primer and assay design complicated.

2.3 PCR Primer Design

The key part of a successful PCR reaction is the primer design. Correctly designed and validated primers are important when determining the specificity, efficiency, sensitivity and robustness of a PCR reaction. Specificity is defined as the frequency of mispriming events occurring during amplification. Primers with poor specificity tend to produce undesired amplicons. The efficiency of primers is determined as the number of amplified products in a PCR run compared to the theoretical two folded increase value of amplification. [18.]

Many factors influence the success of primer design. Primers must be homologous to the desired target sequence. [19.] Primer length of 18-24 base pairs tend to be target-specific at the optimal annealing temperature. A longer primer will be more inefficient producing a significant decrease in the amplified product since a smaller fraction of primed templates will be in the annealing step of the amplification. [20.]

Single nucleotide polymorphisms (SNPs) positioning should be considered when designing primers since SNPs can influence the melting temperature (Tm) of the primers, efficiency of polymerase extension and even target specificity. SNPs are the single base positions within a DNA region that differ in sequence among individuals or a population. [21.] In this study SNPs are in great importance, since the primers should be designed to target these polymorphisms areas of the target gene to be able to distinguish between latania and greedy scale species.

At the annealing stage of PCR, primers anneal from the primer 5'-end to the 3'- end of the DNA template as can be seen from figure 4. The DNA polymerase enzyme starts preparing the new DNA strand from where the primer is attached, which is why the 3'-end of the primer must have a low probability of forming primer dimers and has to be the most specific region of the primer. Primer dimers are formed when the oligos hybridize to one another [19]. Primer dimers can adversely affect primer and template binding [22] and cause poor amplification, especially in the last cycles when there are large amounts of primers available compared to the template.

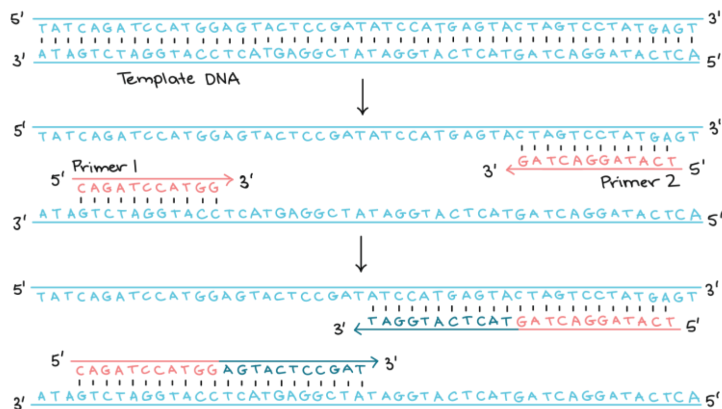

Figure 4. Annealing stage of PCR. Primers attach from the 5'-end to the 3'-end of the template and DNA polymerase starts producing the DNA strand copies from the primer's 3'-end. The forward primer attaches to the template DNA and the reverse primer to the complementary strand of DNA. [23.]

To ensure stable binding of primer to the template, primer pairs should have similar GC-content of 35-65 % [24]. Stability and specificity are aided by the presence of a GC clamp, where there are G and C bases at the 3'-end of the primer because of the stronger hydrogen bonds compared to A and T bases. However, more than three repeats of G and C bases in the sequence should be avoided in the first five bases from the 3'-end of the primer, as the probability for primer-dimer formations is higher. [25.]

Primer-dimer probabilities are evaluated with ΔG values, which is the Gibbs Free Energy G value and it measures the spontaneity of the reaction. There are two types of intermolecular primer-dimers: self-dimer is formed by primers hybridized with itself and cross dimer is formed by primer pairs hybridizing to each other. Generally, 3'-end dimer ΔG of -5 kcal/mol and internal ΔG of -6 kcal/mol values are tolerated in both primer-dimer cases. [22.] The two types of primer-dimers are presented in figure 5.

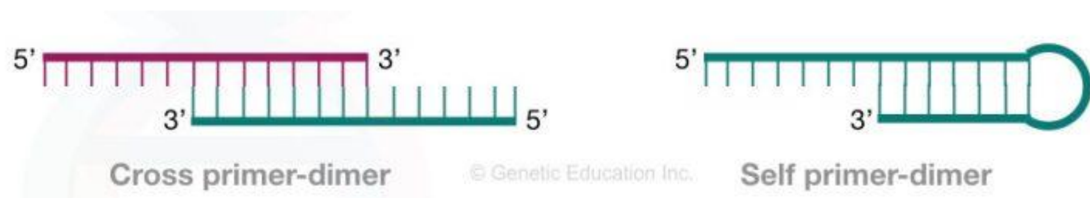

Figure 5. The two types of primer-dimer formations. Cross dimer is formed by primers hybridizing to each other and self-dimer to itself [26].

Primers melting temperature (Tm) is the temperature at which 50 % of the primer and its complement are hybridized. Primer Tm should be between 50-68 °C [27; 28], thus Tm of 54 °C or higher will provide the best result for specificity and efficiency [20]. Theoretically, for a qPCR assay, the optimal primer Tm is between 60-64 °C [24]. Ideally, primer pairs Tm should be within 5 °C of each other [26]. Primer pairs with similar Tm, differing less than 2 °C, theoretically promote simultaneous binding and efficient amplification of the product [24]. Poorly matched primer pairs can be less efficient and specific because the loss of specificity arises with a lower Tm and the primer with the higher Tm has a greater chance of mispriming [20].

Defining the annealing temperature (T_a) of the primers is a critical variability when designing qPCR assays. T_a defines the temperature, at which the maximum amount of primer is bound to the target sequence. The optimal primer T_a must be determined experimentally, since different buffers influence annealing temperature and not only the type of DNA polymerase used in a reaction. However, even at the optimal T_a, non-specific amplification can occur due to physical closeness of primer pairs at mismatched sites. [18.] Also, if the annealing temperature is too high, the primer with the lower Tm may not work at all [20].

Primer specificity is another important aspect when designing new primers. BLAST search is usually done to check the specificity of primers. Still, the fast algorithm of BLAST cannot be completely relied on. It may miss thermodynamically important hybridisation events, as it does not correctly score the gaps that generate duplex bulges which can be created in DNA during recombination between imperfectly homologous sequences. [18.] Bulges are formed when bases in one strand have no pairing partner in the opposite strand [29].

When designing or adopting primers from a previous publication or using commercially supplied assays, primers need to be validated [18]. A good primer pair has a low potential of forming primer-dimers, especially when using Sybr Green chemistry. Also, it is essential to check that preferably the oligos and amplicon should not adopt secondary structures, the primers are homologous to the target sequence and that the reverse complement primer is correct. After primer design is validated, the annealing temperature of the primers needs to be optimized, as this can affect the whole assay specificity and efficiency. [19.]

2.4 Real-Time PCR

In real-time PCR (qPCR), the amplification of DNA is monitored in real-time. qPCR is based on the ability to quantify exact amounts (relative or absolute) of amplified DNA in samples. The amplified DNA is fluorescently labelled with different dyes and the quantification occurs when a fluorescent signal is emitted during amplification. Thus, the amount of signal is relative to the amount of DNA amplified. [30.] The cycle in which detected fluorescence from amplification exceeds the background fluorescence, threshold line, is called the quantification cycle (Cq) value [31]. This means when the sample reaches the Cq value, it is first visible in the data.

In qPCR, there are two different types of dyes frequently-used for DNA quantification. 5' nuclease assays exploit the exonuclease activity of Taq DNA polymerase. Other dye groups are intercalating dyes, such as SYBR Green, EvaGreen and Cyto. This assay uses only primers and unbound dye which intercalates most efficiently into double-stranded DNA (dsDNA). Increase in fluorescence is detected whenever new double-

stranded DNA is formed. The action of SYBR Green dye is illustrated in figure 6. However, intercalating dyes will bind to any double-stranded product and can fluoresce non-specific products too. [31.]

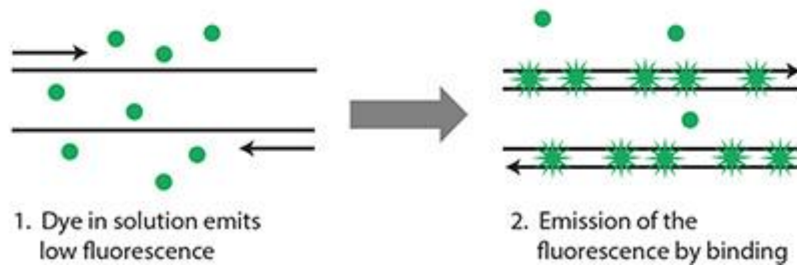

Figure 6. The action of SYBR Green I Dye. The dye binds to double-stranded DNA in annealing through extension steps of PCR. The fluorescence signal increases at the same rate as the number of amplicons increases. [32.]

Another important aspect of the PCR reaction is the usage of different reagents. A master mix that is produced commercially or prepared in a lab contains different components which in absence leads to a failed PCR reaction. Typically, the master mix includes a buffer to maintain pH and salt concentrations, Taq polymerase enzyme to synthesize the new DNA, magnesium chloride to stabilize double-stranded interactions and act as a cofactor for Taq polymerase, and dNTPs that are free nucleotides which are needed for building new DNA strands. [31.]

For visualization of PCR products, the number of product molecules must exceed the detection limit of the reaction. The sample's Cq value depends on the initial concentration of target DNA. A lower concentration of target DNA needs more cycles to reach the Cq value and vice versa. [33.] Lower Cq values, typically below 29 cycles is an indication of high amounts of target DNA. Higher Cq values of 35-40, depending on the machine's detection limits and the non-template control (NTC) and sample Cq value similarity, should be ignored as an outlier. Amplifications in NTC might be caused by primers binding to each other causing an increase in fluorescence signal. [34.] For example, if a sample is detected reliably at 35 cycles and an NTC is detected at 40 cycles, the NTC can be ignored and removed from the data [35, 7].

2.4.1 Melting Curve Analysis

When using intercalating dyes such as Sybr Green, the formation of the correct PCR product needs to be confirmed with melting curve analysis [31]. At the end of a qPCR run the temperature is increased gradually, usually starting above the primer Tm, measuring the amount of fluorescence. The fluorescence signal decreases as the temperature increases. Decrease of the signal is detected because double-stranded DNA (dsDNA) denatures becoming single-stranded and at the same time the dye dissociates. [36.] Every sequence has its signature melting temperature, which makes melting curve analysis also a powerful genotyping tool [37, 19].

G-C base pairs bind with three hydrogen bonds and A-T base pairs with two hydrogen bonds. This causes G-C rich DNA to be more stable and to have a higher melting temperature than A-T rich DNA. [37.] A single target amplicon peak should be observed in a melting curve analysis. However, some samples might produce shoulders which can be caused by DNA melting biphasic, meaning that the dye bound to A-T base pairs is released before the whole amplicon is melted [38]. In these cases, the PCR product should be observed in agarose gel for possible additional products.

2.4.2 Assay Design

The goal in qPCR assay design and optimization is that the specificity, efficiency and fidelity of the PCR reaction reach the validation requirements. A highly specific assay will generate only one specific product that is the target sequence. Efficient amplification generates more products with fewer cycles. PCR assay with high-fidelity contains a negligible amount of DNA polymerase errors in its product. Many factors influence these parameters, such as buffer conditions, PCR cycling temperature and duration of each step, as well as DNA polymerases. However, a highly specific assay might not produce as high a yield as a less specific assay. Optimizing the fidelity of an assay may also reduce the PCR efficiency. Thus, it is important to know what parameters are more significant for the intended application and mainly focus on optimizing those parameters. [39.]

PCR efficiency is influenced by the sample used. Practically all forms of DNA and RNA, such as genomic, plasmid, and phage DNA, cDNA, mRNA and previous amplicons, can be used as a template in PCR. Shearing of genomic DNA during DNA extraction should

not theoretically influence the efficiency of a PCR assay when the fragments are less than 2 kb. PCR efficiency is greater for a smaller size template, like plasmid and phage DNA than for high molecular DNA, such as eukaryotic genomic DNA. [39.]

The specificity of the assay can be assessed by observing the melting curve. A highly specific assay will have a single peak which represents the target sequence. Melting curve analysis can also serve as an identification tool since every sequence will have a unique melting point no matter what run conditions are used. This enables the identification of different species, even when using the same gene as the target if there are SNP regions in the sequences. As this assay uses Sybr Green chemistry, it is also crucial to find a PCR program that is specific to only the target sequence. If primer-dimers are formed, it will affect the specificity of the assay as the fluorescence signal is detected every time a new double-stranded DNA is produced.

3 Methods

3.1 Sample Collection and Pre-Treatment

The target insects, Latania and greedy scales were provided by an entomologist Kate Stannard from a colony growth of Plant & Food Research, Te Puke, New Zealand. The scale colony was formed from female scales that were collected in 2001 from kiwifruit block 20, an entomology spray-free block within the Te Puke Research orchard. Scales were identified by entomologists as latania or greedy scale morphologically and inserted to live on the surface of butternut squash fruit (*Cucurbita maxima*). Fifty individual live adults of both scale species were harvested from the squash one by one by spearing them with a flame-sterilized needle to microcentrifuge tubes under a microscope. This is illustrated in figure 7. Further, one individual from both species was collected for DNA extraction. Duplicate samples from both species were collected from the same squash. Samples were used for DNA extraction immediately after insect collection.

Figure 7. Latania scale infested butternut squash under a microscope ready for insect collection. Scales were collected to the Eppendorf tubes one by one with flame-sterilized needles.

Other pests that are normally found in avocado orchards were collected from colony growths of both Plant & Food Research, Auckland and Te Puke, New Zealand to validate the specificity of the assay. Greenhouse thrips were provided by Kate Stannard from a colony grown at Plant & Food Research, Te Puke. About 20 individual greenhouse thrips adults were swiped off a lemon fruit with a little brush into a microcentrifuge tube. Two duplicate samples were collected and used immediately for DNA extraction.

The rest of the insect species were provided from colonies grown at Plant & Food Research, Auckland. An entomologist, Jacqui Todd, provided live leafroller caterpillars in small tubes and one live adult bronze beetle. The leafrollers and the bronze beetle were removed from the tubes with flame-sterilized tweezers and killed in 80 % ethanol. A science team leader of invertebrate biology & rearing, David Logan, provided passion vine hopper nymphs and an entomologist Amanda Hawthorne supplied long-tailed mealybugs that were already deceased in 95 % ethanol. All the samples were stored in the freezer until DNA extraction. Table 1. lists all the collected species used in this study.

Table 1. The collected species for this study.

Common name	**Scientific name**	**Sample name**
Greedy scale	*Hemiberlesia rapax*	G.S
Latania scale	*Hemiberlesia Lataniae*	L.S
Greenhouse thrips	*Heliothrips haemorrhoidalis*	GHT
Passion vine hopper	*Scolypopa australis*	PVH
Long-tailed mealybug	*Pseudococcus longispinus*	LTM
Brown beetle	*Costelytra zealandica*	BB
Black-lyle leafroller	*Cnephasia jactatana*	Cnej
Light brown apple moth	*Epiphyas postvittana*	Epo
Brown headed leafrollers	*Ctenopseustis obliquana*	Cob
	Ctenopseustis herana	Cher
Green headed leafrollers	*Planotortrix excessana*	Pex
	Planotortrix octo	Poc

The greedy scale was the main target species of this study. Latania scale was used as a negative control in every experiment since they are closely related species. Other collected insect species were used in proving the assay's specificity.

3.2 DNA Extraction and Quality

The DNA extractions were undertaken using a DNeasy Blood and Tissue kit (Qiagen, Lot: 160036445). From that kit, the protocol ”purification of total DNA from insects” was followed. However, some of the samples were ground differently before the lysis step, elution time was extended and elution was done to 100 µl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9) for better DNA yield.

The passion vine hopper, long-tailed mealybug and bronze beetle samples were ground with an electric Bullet blender homogenizer using magnetic beads and the lysis proceeded with buffer ATL. Elution time for these samples was one hour instead of one minute. Duplicate DNA extractions containing 50 scale insects and one individual from both species were made. All the DNA extractions were made at both the earlier mentioned Plant & Food Research sites and the samples were stored in the freezer until further use.

All the DNA extractions were run on a 1.5 % agarose gel (data not shown) and concentrations were measured with Nanodrop 2000. The quality of the samples was estimated by the clarity of the bands and with A260/280 and A260/230 values. Generally, A260/280 ratio of ~1.8 is accepted as "pure" DNA and a ratio of ~2.0 is accepted as "pure" for RNA. However, the ratios vary depending on changes in sample acidity and the nucleotide mix of the sample. RNA values are usually higher because the uracil nucleotide ratio (4.00) is higher to that of thymine (1.47). [40.]

The expected A260/230 values are commonly in the range of 2.0-2.2. If the ratio is significantly lower than this, it might indicate that contaminants which absorb at 230 nm are present in the sample. [40.] However, in downstream applications, such as PCR, protein contaminations do not necessarily affect the reaction.

3.3 Validating Primer Design

All the primers were designed using Primer3 plugin in Geneious program [41;42]. Five ITS2 sequences of greedy and fifteen of latania scale from GenBank were used in primer design by aligning the sequences. The accession numbers of the sequences are listed in table 2.

Table 2. GenBank accession numbers of the ITS2 gene sequences used in primer design.

Greedy scale	Latania scale		
GQ284622	GQ284593	GQ284612	GQ284617
GQ284623	GQ284594	GQ284613	GQ284618
GQ284624	GQ284609	GQ284614	GQ284619
GQ284625	GQ284610	GQ284615	GQ284620
GQ284626	GQ284611	GQ284616	GQ284621

Primers were designed using the following criteria:

- template homologous
- length between 18-24 bp
- Tm ≥ 54 °C
- Primers Tm difference less than 5 °C
- GC% of 20-70 %
- product size
- low primer-dimer probability
- GC clamp

Primer3 properties for primer design were set as follows. Product size was set between for qPCR primers to 100 – 130 bp and for the sequencing primers to 500-700 bp. Melting temperature (Tm) range was chosen between 58-62℃ and GC% range from 20-70 %. Following concentration settings were used in Tm calculation (SantaLucia 1998): Monovalent 50 mM, divalent 1.5 mM, Oligo 200 nM, dNTPs: 0.6 mM.

Real time-PCR primer pairs targeting the ITS2 gene regions of greedy scale and one PCR universal primer pair targeting the whole ITS2 of both scale species for sequencing were designed. Primers were ordered desalted from Macrogen (lot number: OG190405-099; 10F, 254, Beotkkot-ro, Geumcheon-gu, Seoul, South Korea). Primer tubes were spun down prior to stock solution preparation. 100 µM stock solutions were prepared by adding 250 ul of AE buffer (DNeasy blood and tissue kit) for longer term preservation reasons.

The AE buffer contains 0.5 mM EDTA, which is a known PCR inhibitor. In the study by McCord B. *et al.* [43] it was proven that 0.4 mM EDTA causes only a slight change in Cq values by affecting the DNA binding compared to 0.0 mM EDTA. Due to dilutions, the final concentration of EDTA in PCR reactions are less than 0.1 mM which should not affect the reaction. Normally, primers are prepared to TE buffer but there was shortage of chemicals at that time. 10 µM working solutions from stock solution were prepared to PCR grade water for further experiments.

DNA sequencing

The DNA sequencing was undertaken to verify the qPCR primers. Conventional PCR from the greedy scale (50 adults) and latania scale (one adult) DNA extraction and a non-template control (NTC) was run using the instructions of Q5 Hot Start High-Fidelity 2x Master Mix (NEB, Lot: M0493S). A 25 µl PCR reaction contained 1x Q5 reaction buffer, 200 µM of dNTPs, 0.5 µM of forward and reverse primer (UniversalF & R), 0.02 U/µl of Q5 Hot Start DNA polymerase, 1 x Q5 High GC enhancer buffer, about 10 ng of template and PCR grade water. The annealing temperature was set to 58 °C and the Master Mix protocol was followed for the rest of the PCR program. Cycling conditions were as shown in table 3.

Table 3. PCR program.

	Temperature	**Time**
Hold	98 °C	30 sec
	35 cycles:	
Denaturation	98 °C	10 sec
Annealing	58 °C	30 sec
Extension	72 °C	30 sec
Final extension	72 °C	2 min
Hold	4 °C	

PCR samples were run in 1.5 % AGE with 1x TBE buffer and with low voltage, 50 V, to get a clear band resolution. The DNA bands were extracted from the gel with Zymo-clean™ Gel DNA Recovery kit (Zymo Research, Lot: ZRC184901) following the kit protocol. The centrifuge speed was 10 000 rpm in all the steps and elution was done to 10 µl of elution buffer. Before sending the samples to sequencing, the total volume was adjusted to 20 µl with PCR grade water and also 10 µl of both Universal F & R primers were sent for sequencing to Macrogen.

3.4 Gradient PCR

Gradient PCR was run to find the optimal annealing temperature of the primer pair. A range of annealing temperatures from 55-65 °C was chosen above and below primers Tm (Primerpair13 Tm: 61.7 °C). The LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, lot: 37868800) was used in this experiment. Suitable cycling conditions were experimentally determined with the Rotor-Gene Q qPCR. Table 4. presents the chosen protocol for the gradient PCR run.

Table 4. Gradient PCR protocol

	Temperature	**Time**
Hold	95 °C	5 min
	35 cycles:	
Denaturation	95 °C	10 sec
Annealing	55-65 °C	10 sec
Extension	72°C	5 sec

Extension time of 5 seconds was calculated according to the kit protocol, which defines the extension time by dividing the amplicon length over 25 [44]. For instance, primer-pair13 (Greedy scale ITS2 primers) product size is 130 bp, hence 5 seconds is enough (130 bp / 25= 5.2 seconds) [44, 7]. The kit protocol standard reaction volume is 20 ul. To save reagents the final volume and reagent concentrations were halved. The reaction volume was 10 ul containing 0.5 µM of both forward and reverse primer, 1x SYBR Green I Master buffer, about 10 ng of template and PCR grade water. PCR samples were run in 1.5 % AGE (1x TBE; 50 V) for result analysis.

3.5 Real-time PCR Assay Design

There are no published qPCR assays for the detection of scale insects, thus the assay design for the detection of greedy scale had to be conducted. The goal was to find a specific, sensitive and efficient method that meets the validation requirements. Absolute specificity in this assay design was particularly important as Sybr Green chemistry was used.

The qPCR assay was developed with Rotor-Gene Q (Qiagen) real-time PCR and every run had triplicate DNA samples and non-template controls (NTC). Assay optimization was not necessary at this stage of assay development because of the LightCycler® 480 SYBR Green I Master kit used in this study. The master mix of the kit contains optimal amounts of FastStart Taq DNA polymerase, reaction buffer, dNTP mix, $MgCl_2$ and Sybr Green I dye.

However, some modifications were made to the reaction set up of the kit protocol. The final volume was reduced from 20 µl to 10 µl and reagent amounts were halved to save reagents. Additionally, the template amount was reduced in order to save the samples and to get more efficient results. In the original kit protocol 1/4 of the reaction is the template, but in this assay only 1/10 the reaction is the template. 10 µl reaction contained 1x concentration of Master Mix, 0.5 µM of primers, 0.4-3.4 ng of the template depending on the experiments and PCR-grade water.

Table 5. The Two-step qPCR protocol.

	Temperature	**Time**
Hold	95 °C	5 min
	35 cycles:	
Denaturation	95 °C	5 sec
Annealing/Extension	62 °C	15 sec

The two-step qPCR protocol used in this study can be seen in table 5. The protocol is a modified version of the protocol found from the Rotor-Gene SYBR Green PCR kit. Results improved by elongating the annealing/extension time 5 seconds.

3.6 Assay Specificity

The specificity of the assay was verified in many ways. Upon primer design, the specificity of the primers was tested in silico with Primer-BLAST. Results proved the primers to be specific to only the target species sequences. Then, a specific qPCR protocol was found for greedy scale.

The assay and primers specificity were tested against eleven other common insect pests found from avocado and kiwifruit orchards including the closely related latania scale species. These species are listed in table 1. Also, a test using a different DNA matrix was conducted. One adult latania and greedy scale were soaked in one millilitre of tap water in same and separate tubes for 15 min shaking occasionally. One microliter of this was added to a PCR reaction to test whether the target ITS2 gene region of greedy scale was only amplified, even though the tap water samples contained other eDNA.

4 Results

4.1 DNA Concentration

DNA concentration and purity were measured with Nanodrop 2000 spectrophotometer. Two microlitres of each sample were measured and the average of three replicates was calculated for every sample. Table 6. presents all the average concentration values in ng/µl and both A260/280 and A260/280 values for each measured DNA sample.

Table 6. Nanodrop measurement results.

Common name	Sample name	Concentration (ng/µl)	A260/280	A260/230
Greedy scale	G.S 1	343.4	1.93	1.98
Latania scale	L.S 1	265.6	1.99	1.97
Greenhouse thrips	GHT	78.0	1.89	1.22
Passion vine hopper	PVH	153.4	2.06	1.29
Long-tailed mealybug	LTM	164.4	2.10	1.36
Brown beetle	BB	225.4	2.06	1.46
Black-lyle leafroller	Cnej	127.1	1.92	1.06
Lightbrown apple moth	Epo	95.6	2.00	1.56
Brownheaded leafrollers	Cob	206.0	1.93	1.22
	Cher	267.2	2.04	1.67
Greenheaded leafrollers	Pex	82.6	1.98	1.16
	Poc	127.3	1.90	1.24
Latania scale	L.S (one scale)	28.9	1.87	0.76
Greedy scale	G.S (one scale)	4.10	1.50	0.23

The expected A260/280 value is 1.8 and A260/230 values are commonly in the range of 2.0-2.2. All the extractions, except one greedy scale extraction sample, have A260/280 value above 1.8. Some of the sample results have closer to acceptable RNA values. However, this is not alarming since the results are probably affected by the elution buffer chemical properties. Also, the blanking solution used in this test was PCR grade water instead of elution buffer, which might have affected the results.

In table 6. Starting from Greenhouse thrips the A260/230 ratios are lower than 2.0. This indicates that all the samples might have contaminants absorbing. However, amplification was successful from DNA extraction of one greedy scale, which suggests that low ratios do not necessarily inhibit amplification.

DNA extraction from fifty individual greedy scales, G.S 1 in table 6, gave a reliable quantifiable concentration compared to the concentration of one scale DNA extraction, which is why it was used as a standard sample for the qPCR assay design. L.S 1 sample containing 50 adult latania scales could not be used as a negative control because it was contaminated with greedy scale DNA. Instead, a DNA extraction from one latania scale was used.

4.2 Primer Properties

The sequence, length, melting temperature and GC% information of each selected primer is mentioned in table 7. Primerpair13 primers correspond to qPCR primers and Universal primers to PCR primers that were designed for sequencing. The information of the other qPCR primer pair that was not selected for this study is in appendix 1. Primerpair13 primers have the same length, melting temperature and GC%, which promotes simultaneous binding to the template under optimal conditions. Between UniversalF and -R primers there is differences in length, Tm and GC %, which are acceptable. For instance, the Tm difference between UniversalF and UniversalR primer pair is within 5 °C as it is recommended [24].

Table 7. Primer properties of the selected primers for this assay.

Name	Sequence	Length (bp)	Tm (°C)	GC %
Primerpair13-F	5'-CGT ATA CGG GTC GCG TAG C-3'	19	61.7	63.16
Primerpair13-R	5'-CTG CAC GGT AAC CCA CTC G-3'	19	61.7	63.16
UniversalF	5'-GCA CAA CAG ACC AGA CCG TA-3'	20	60.5	55
UniversalR	5'-TGA TCT GAG GTC GGC CGA-3'	18	58.4	61.11

The PCR product size of Primerpair13 is 130 bp and Universal primers about 660 bp for both species. The GC clamp (presence of G and C bases in the last 5 bases of the 3'-end) is found from every primer. All the primers, except the UniversalR, have no more than three repeats of G and C bases in the first five bases from 3'-end of the primer as recommended. UniversalR primer has more G and C repeats, which makes it more prone to form primer-dimers.

Primer-Dimer Probabilities

The primer-dimer probability by observing delta G (ΔG) values was done with Primer Inspector program [45]. Primerpair13 ΔG value for cross dimers and Primerpair13-R self-dimers is less than -3 kcal/mol, which is an acceptable value. Primerpair13-F can form two internal self-dimers with ΔG values more than -6 kcal/mol, which is above recommended values. [22.] These two possible dimers are shown in figure 8. However, primerpair13-F primer-dimer possibilities are located closer to the 5'-end of the sequence, which makes the hybridization more unlikely, since DNA polymerase adds free nucleotides onto 3'-end of the primer.

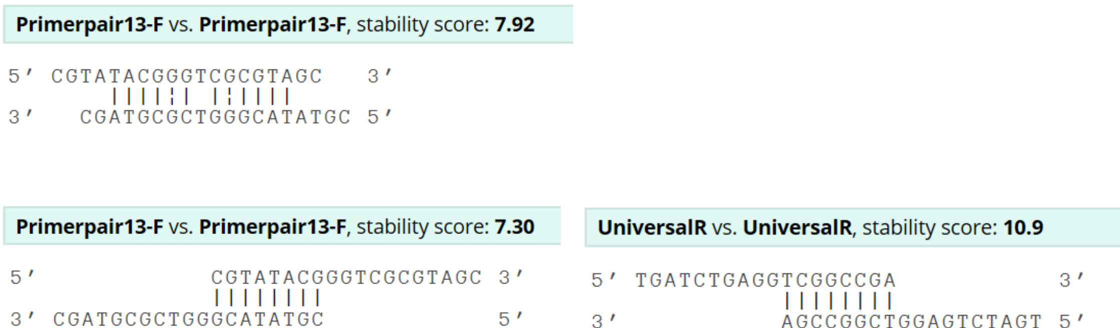

Figure 8. The possible self-dimers of Primerpair13-F and UniversalR primer. The stability score is the opposite value of the predicted delta G value (kcal/mol). [44.]

Universal primers cross dimer and UniversalF self-dimer probability is under recommended -5 kcal/mol value. UniversalR primer can form a self-dimer with a score of -10.9 kcal/mol (figure 8.), which is above recommended -6 kcal/mol. Hybridization is possible at the 3'-end of the primer, thus primer-dimers can form during PCR reactions competing with the target sequence amplification. However, these results are predictions and by finding the right PCR reaction conditions, primer-dimer formations could be prevented.

4.3 qPCR Primer Verification

Sanger sequencing of the ITS2 gene was done to both directions as part of the qPCR primer verification, since there are no ITS2 gene sequences of greedy nor latania scale from New Zealand in GenBank. However, the ITS2 gene is a conserved gene within species, hence the sequences should be all the same. Novel universal primer pair targeting the whole ITS2 gene were designed for this purpose and the Hot Start Q5 Master Mix and corresponding PCR program was used in this part.

The DNA sequencing was only successful with universal forward primer in both cases. Sequence quality is good and the primer pair 13 is found from the sequence as expected. In figure 9. is the whole ITS2 and partial 28s rRNA sequence of greedy scale. It is unsure why the reverse primer failed in sequencing, since the PCR was successful. The reverse primer is located in the 28s gene and between that and the ITS2 gene, the gene region seems to be complicated for sequencing purposes (data not shown, sequences were observed with Geneious program [42]).

```
1        10        20        30        40
TCGATGTTAGCGTGGGTCTTTGGCGGCGTTCCGTTCGCGGGGCCCGT
   50        60        70        80        90
CAAAAGAAACACGAGAGTGTGGCGTTTTCGCGAAACGGTCCGTTTAC
     100       110       120       130       140
TAGCGTATACGGGTCGCGTAGCGTACGCTGAAGTATCGGATCTTGCC
        150       160       170       180
AATCCGTAGCTAAATACGTCTGAGGTGTGAAATACCACCTCGCGACA
 190       200       210       220       230
CTCGGCCAACGGAGCTCGGTCGAGTGGGTTACCGTGCAGCGGCGCGC
    240       250       260       270       280
GTACCTACAGCACAGAGGTCTGCGGTGTTTGGCGACAGCTAACGCTC
       290       300       310       320
CCACACCGTAGAGAGTTGTATGGTAAGCCGCCGGTTCATGCCACTCG
330       340       350       360       370
CTACCGAGTCGCCAAGGTGCTCGATGCGTCTCTCTAAGAGTGCGCGA
   380       390       400       410       420
GTACGTTTGCCTCCGGCCGTTGTCAACGAACCGCACAAACAGAAGAA
      430       440       450       460       470
ATGACGGTATCGACAACGCGCGACCGATGAGGCTAGACGTACACGCG
         480       490       500       510
ACAGGGAGCTAAGACAACGCTCGAACAGCGCCGTTCTCGG
```

Figure 9. The whole ITS2 and partial 28S rRNA sequence of greedy scale (*H. rapax*) captured from the sequence result sent by Macrogen.

Greedy scale sequence matches 100% with the other sequences found from the GenBank database, which further proves that the studied species is correct and that the ITS2 gene is conserved. Interestingly, the ITS2 genes of greedy and latania scale are 90.7% homologous with only a few SNPs found in the sequences.

4.4 Optimal Annealing Temperature

Determining the optimal annealing temperature of the primers is an essential part when designing new assays. Temperature range of 55-65 °C was tested and the best annealing temperature is chosen by observing the gel bands. The temperature that gives a single target sequence band that is the most intense, is the best annealing temperature for the primers.

In figure 10. the gradient PCR gel run using Primerpair13 is presented. Run was done to both scale species to proof primers specificity to greedy scale and as expected no amplification occurred in latania scale samples. The upper row of the gel in figure 10. represents the different temperatures. PCR product size was 130 bp, which is correct and can be seen in figure 10. above 100 bp marker band.

Metropolia University of Applied Sciences

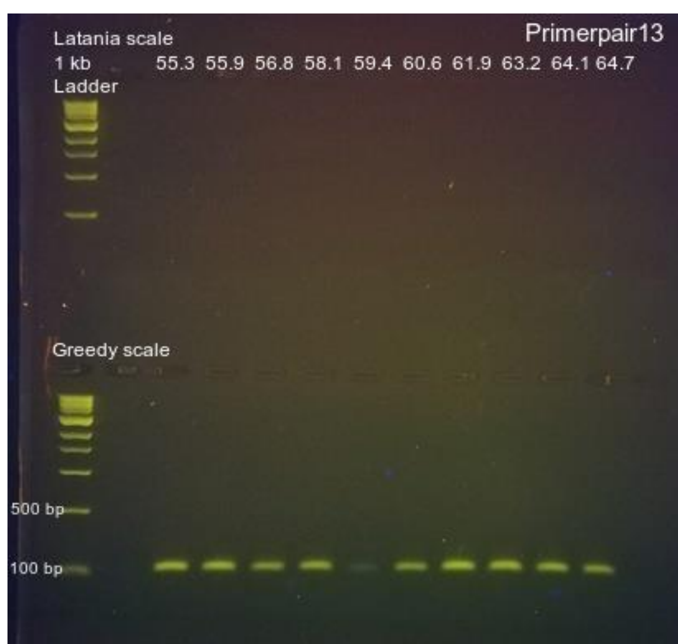

Figure 10. Gradient PCR products of primerpair13 on a 1.5% agarose gel. Upper row shows all the temperatures used in this experiment. Gradient PCR was run against latania scale species also to ensure that only greedy scale species is amplified in every annealing temperature.

As seen from figure 10. the primer pair has a wide range of working annealing temperatures with only desired and intense bands produced, except for a pipetting error that occurred in sample loading at 59.4 °C well. The slightly more intense band is at 61.9 °C compared to the rest, thus the annealing temperature for the qPCR assay was chosen to be 62 °C.

4.5 The Optimal qPCR Method

For data analysis, the instructions of the qPCR machine were followed. The “Comparative Quantitation” option, which is available in Rotor-Gene Software only, was used for evaluating the results. Comparative quantitation compares the relative expression of samples to a control sample when a standard curve is not available. The peaks are presented in second derivative amplification plot (figures 12, 14 and 16). The first peak in the plot presents the second derivative maximum level that corresponds to the maximum rate of fluorescence increase per reaction. The software determines the “take off” point, Cq value, of each reaction. The Cq value is defined as the cycle at which the second derivative is 20% of the maximum level. [46.] The optimal efficiency value of a reaction is 2, which means 100 % efficiency. If the reaction efficiency of a sample is not similar to

Metropolia University of Applied Sciences

other reactions in the experiment or the value is less than 1.4, the reaction is defined as an outlier. In figure 11. is presented how the “Comparative Quantitation Analysis” software option determines the “take off” point, Cq value, and calculates the amplification efficiency of each reaction.

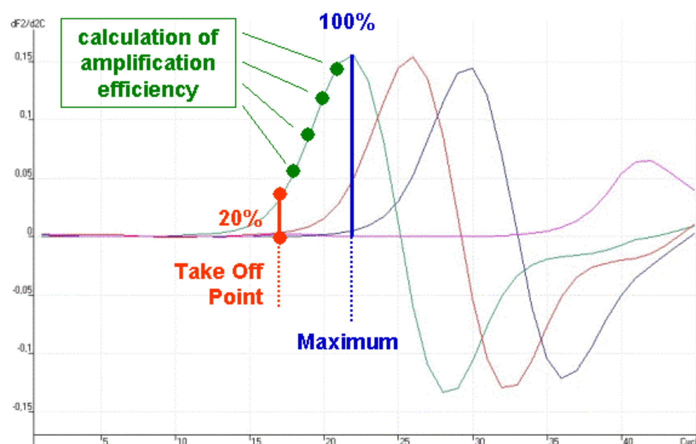

Figure 11. An illustration of the “take off” point, Cq value, and the amplification efficiency calculation of each reaction determined by the “Comparative Quantitation Analysis” software option [47]. Maximum (100%) peak corresponds to the maximum rate of fluorescence increase per reaction [46].

Cq values above 30 cycles were analyzed with suspicion. Non-template control (NTC) samples that had 5 or more cycles difference compared to DNA samples and similar DNA and NTC sample values, were treated as outliers. In this part of assay design, obtained Cq values are less of importance, because the quantification assay could not be finished.

In the qPCR run, 1/10,000 dilution of greedy scale DNA sample containing 50 scale individuals (0.034 ng/µl in one reaction) was used as a control sample and named as G.S 1/10 000. The same greedy scale DNA sample was used as the control sample with different dilutions in every qPCR test. L.S corresponds to latania scale DNA extraction of a single scale adult (2.89 ng/µl in one reaction). The amplification plot is shown in figure 12. where the pink curve is greedy scale DNA sample, light green and blue lines are latania scale DNA and NTC sample respectively.

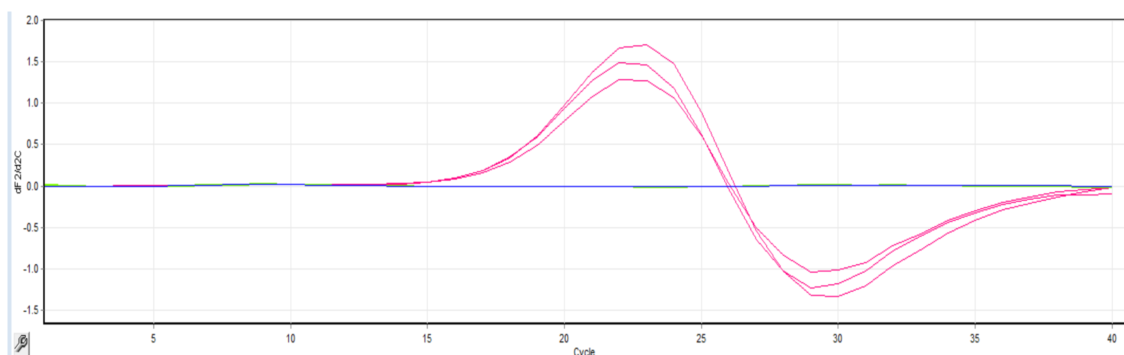

Figure 12. The second derivative amplification plot of the qPCR assay specific to greedy scale. Y-axis corresponds to the second derivative scale and x-axis to the cycle number. The Cq values of each reaction are defined as 20% of the second derivative maximum peak (the pink peak in this case). The pink curve presents the greedy scale DNA sample (1/10,000 dilution). Green and blue lines are latania DNA (undiluted) and NTC sample.

Amplification was successful only in greedy scale sample indicating that this assay is 100% specific to greedy scale. Amplification values were over 1.4, with an average of 1.77 for three replicates. Cq values were similar within three replicates with no more than 0.3 value difference, implying that assay systematic error rate is low. For the rest of the samples, amplification values were under 1.4, thus they are considered as outliers. Amplification and Cq values of each sample and averages of replicates are listed in table 8.

Table 8. Cq and amplification values and average values of the samples.

Name	Cq value	Amplification	Av. Cq value	Av. Amplification
G.S 1/10 000	18.6	1.79	18.4	1.77
G.S 1/10 000	18.3	1.76		
G.S 1/10 000	18.4	1.75		
NTC	6.1	0.0	6.4	0.62
L.S	7.6	0.26	6.9	0.09
L.S	6.8	-0.27		
L.S	6.4	0.29		
NTC	6.8	1.24		

Melting curve analysis was conducted after the qPCR run to assess the specificity of the assay. The result is shown in figure 13. where green and blue lines correspond to latania scale and NTC sample. The pink curve is equivalent to greedy scale sample.

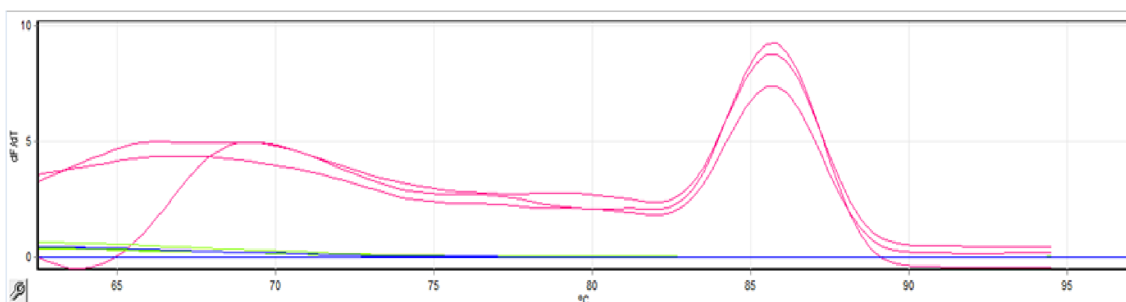

Figure 13. Melting curve analysis result of the assay, where pink curve presents greedy scale DNA sample, blue and light green are NTC and latania scale DNA sample.

Only one product was amplified with a melting temperature of 85.7℃. This temperature was consistent in other run conditions, with less than 0.3℃ difference between samples, when finding the optimal assay (data not shown).

Specificity Tests

Even though Primer Blast gave a 100% specific result of primers binding only to the target ITS2 gene section of greedy scale, the working assay was tested against eleven other common insect pest species (table 5.) found from avocado and kiwi orchards to further proof the assay's specificity. The amplification plot is shown in figure 14. in which violet curve presents the greedy scale sample. In the run, one greedy scale sample failed, resulting in later Cq value and can be seen as the latter curve in figure 14. After

Metropolia University of Applied Sciences

30 cycles there are several curves from different species that seem to be amplifications but are treated with suspicion.

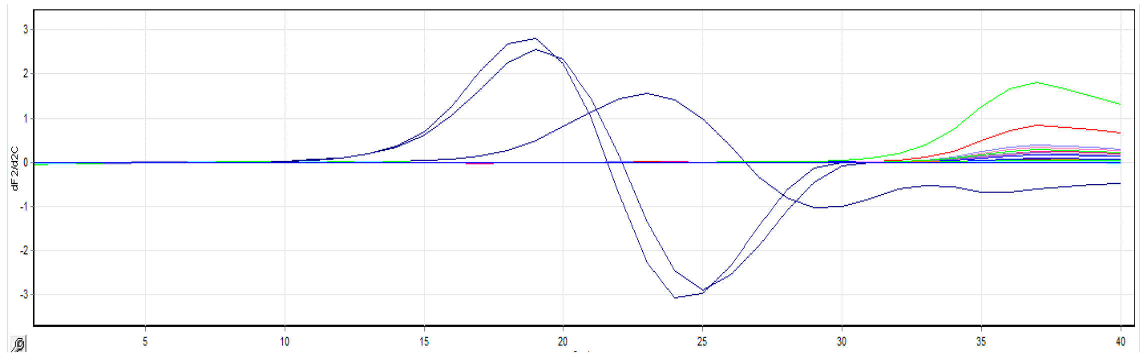

Figure 14. The second derivative amplification plot of the specificity test against other common insect pests. Y-axis corresponds to the second derivative scale and x-axis to the cycle number. The Cq values of each reaction are defined as 20% of the second derivative maximum peaks. The violet curve corresponds to greedy scale DNA sample. The rest of the curves are fluorescent after 30 cycles and efficiencies are differing, which is why they are false positive results.

Cq and amplification value average of the two successful greedy scale samples are 15.35 and 1.8. Assessing the other curve's Cq values that are seen in figure 14. the results are unreliable, as the values differ between replicates and most of them Cq values are under 1.4. Some values are above 2.0, which might be an indication of a primer dimer formation. This would be possible as the excess amount of primers cannot bind to the sequences. Hence, they might form self-dimers, which can be formed according to primer-dimer check result. All the species, Cq and amplification values and averages of replicates are listed in appendix 2.

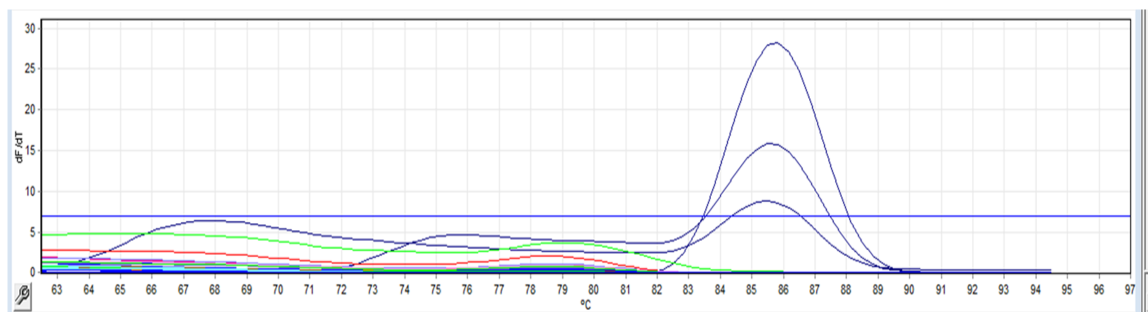

Figure 15. Melt curve analysis result of the specificity test. Violet melting curve is greedy scale DNA sample and the rest of color lines are different insect pest species.

The melt curve analysis result is shown in figure 15. where it can be seen that only greedy scale target ITS2 gene section was amplified with correct temperature of ~85.7 ℃. This further proves that the other curves seen in figure 14. are false positive results.

Furthermore, an assay specificity and sensitivity test were conducted with a different DNA matrix too, to assess the possibility of using eDNA, which contains only small traces of pest DNA in later studies. One adult scale of both greedy and latania scale was soaked in one millilitre of tap water in the same and each species in separate Eppendorf tubes, and from those samples, one microliter was added to the PCR reaction straight. The amplification plot can be seen in figure 16.

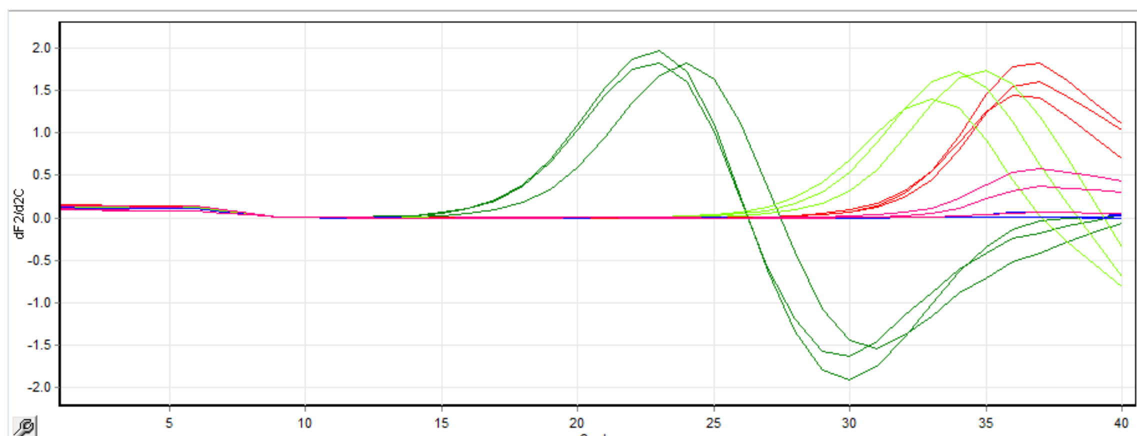

Figure 16. The second derivate of the amplification plot of the test using a different DNA matrix to assess assay's specificity and sensitivity. Y-axis corresponds to the second derivative scale and x-axis to the cycle number. The Cq values of each reaction are defined as 20% of the second derivative maximum peaks. The dark green curve is greedy scale DNA sample, light green curve is both scale species in water, red curve is greedy and pink curve is latania scale in water. The blue line corresponds to NTC sample.

The dark green curve presents a greedy scale DNA extraction sample, the light-green curve is both scale species in water, the red curve is greedy and the pink curve is latania scale in water. The Blue line presents the NTC sample. Amplification was successful for the samples containing greedy scale DNA as expected. In figure 16. the pink curve containing latania scale DNA seem to be amplified but the Cq and amplification values are not reliable, since the values differ a lot from each other. Also, as seen from table 9. the Cq value of one NTC sample is 34 and the latania scale sample (L.S test) values are close to that. Thus, no real amplification happened with latania scale sample.

Table 9. The Cq and amplification values and averages of the samples, where G.S 1/100, G.S test, G.S+L.S test and L.S test are a greedy scale control sample dilution of 1/100, an adult greedy scale, adults of both latania and greedy scale and an adult latania scale in water respectively.

Name	Cq value	Amplification	Av. Cq value	Av. Amplification
G.S 1/100	19.7	1.74	18.9	1.76
G.S 1/100	18.5	1.78		
G.S 1/100	18.6	1.77		
NTC	6	-0.73	13	0.41
G.S test	33.1	1.85	32.9	1.84
G.S test	33	1.88		
G.S test	32.5	1.8		
NTC	6	0		
G.S+L.S test	28.9	1.76	29.9	1.76
G.S+L.S test	30.8	1.76		
G.S+L.S test	29.9	1.77		
NTC	6	0		
L.S test	6	0	24.6	1.46
L.S test	34.1	2.4		
L.S test	33.6	1.97		
NTC	34	2.37		

The rest of the sample values demonstrated in table 9. are similar to each other. However, there are differences between the G.S test and G.S+L.S test replicate values. The DNA extraction was not done for these samples, thereby the DNA is not spread out equally within the samples and the concentrations are low. Hence the high Cq and amplification values. One of the greedy scale control samples (G.S 1/100 sample) failed also, likely due to a pipetting error, when the replicate Cq values were compared. It can be seen as the latter curve in figure 16. Specificity was assessed with melting curve analysis, which is shown in figure 17.

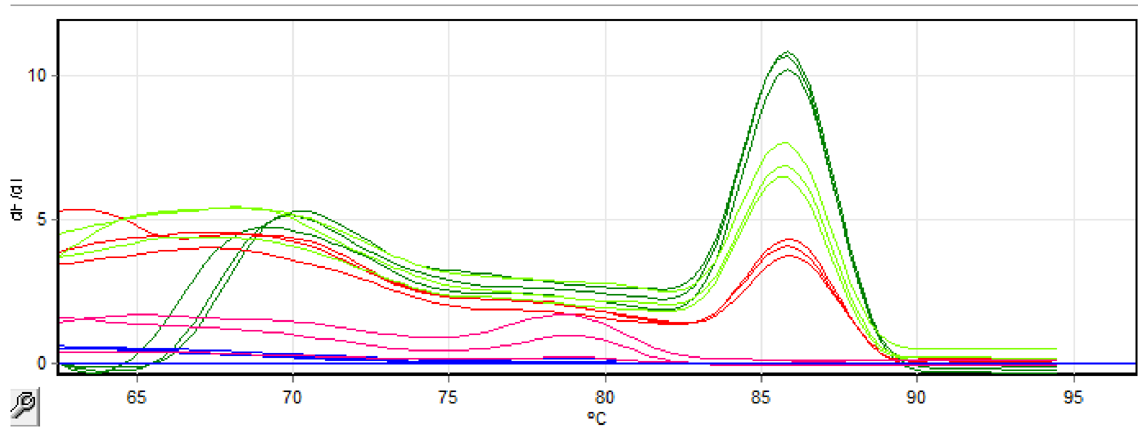

Figure 17. Melting curve analysis result when using a different DNA matrix. Dark green curve represents greedy scale DNA sample, red curve greedy scale in water sample and light green curve greedy and latania scale in water sample. The pink line is latania scale in water sample.

Melting curves were formed at ~85.7℃ as expected. Earlier suspected result of latania scale test (pink line) is a false positive, as there is no melting curve at the product point. There are little curves before 80℃, which might be an indication that primer-dimers were formed during the PCR reaction. The light green melting curve is at the greedy scale's ITS2 product melting point, which also proves that only greedy scale DNA was amplified even though latania scale DNA was present in the same sample.

5 Discussion & Conclusion

There is interest for new pest monitoring tools. Pest identification and DNA quantification from environmental DNA with a suitable machine rather than manual inspection offers faster and potentially more reliable results. This can lead to prospective economical savings and more ecological fruit orchards management. The goal of this project was to design a novel qPCR assay for the detection of greedy scale pest as a proof of concept that could be used in future research to improve pest monitoring in New Zealand.

The designed qPCR assay proved to be only specific to greedy scale species. Specificity was proven with testing the assay against ten other common insect pests and using a different DNA matrix. Results were analysed by treating samples with Cq values above 30 and similar sample and NTC values with suspicion. Final specificity assessment was done with melting curve analysis, where the target PCR product melting point is at

~85.7℃. Melting curve analyses of the specificity tests showed that suspected amplifications in other insect species were false-positive results.

Apart from high specificity, the assay seems to be sensitive too, since it is possible to detect even small traces of greedy scale DNA without doing DNA extractions of the tap water samples. Amplification values for greedy scale DNA samples were ~1.8 out of the optimal 2.0, which suggests that the assay is also efficient enough for validation. However, assay validation was not completed as the copy number of the target ITS2 gene could not be determined. The genome size of greedy scale is not known to date and plasmids could not be prepared due to lack of time and permissions.

Designing a completely new qPCR assay was not effortless. Many complications were faced with DNA extractions qualities and finding suitable PCR run conditions for the primers. Sybr Green chemistry was used in this study, which makes finding the right assay affordable but more complicated and time-consuming. On the other hand, it is recommended to start assay design with Sybr Green chemistry first to assure specificity to the target before moving on to other fluorescence chemistries.

Novel designed primers targeted the ITS2 gene, which has been used as a barcoding gene for insects in some studies. Although, mitochondrial genes as COI or COII, has been more widely used in animal barcoding. This gene is extremely A-T nucleotide rich in scale species, which makes it complicated for stable primer design and amplification purposes. The whole ITS2 gene was sequenced of both greedy and latania scale species for primer verification, making the sequences the first ones from New Zealand so far. Results proved that the ITS2 gene can be used to differentiate between the two closely related species, even though the genes are highly homologous. Genetically the ITS2 genes of greedy and latania scale are 90.7% similar (Geneious).

Furthermore, the designed qPCR species-specific primers and assay for the detection of greedy scale is the first one designed until now. The results suggest that designing qPCR assays as specific and sensitive as this one for greedy scale, they could be used to identify and quantify pest DNA amounts found from environmental DNA. Thereby, similar assays using species-specific tags could be applied to pest monitoring and biosecurity as genetic identification tools.

References

1 Clarke L.J., Soubrier J., Weyrich L.S. & Cooper A. Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias [serial online]; 17 April 2014. Molecular Ecology Resources;14(6),1160–1170. URL: https://onlinelibrary.wiley.com/doi/full/10.1111/1755-0998.12265. Accessed 30 November 2018.

2 Turner C.R., Barnes M.A., Xu C.C.Y., Jones S.E., Jerde C.L. & Lodge D.M. Particle size distribution and optimal capture of aqueous macrobial eDNA [serial online]. Methods in Ecology and Evolution; 16 May 2014;5(7). URL: https://besjournals.onlinelibrary.wiley.com/doi/10.1111/2041-210X.12206. Accessed 2 October 2018.

3 Nielsen K.M., Johnsen P.J., Bensasson D. & Daffonchio D. Release and persistence of extracellular DNA in the environment [serial online]; 12 September 2007. Environmental Biosafety Research; 2007;6(1-2):37–53. URL: https://www.ebr-journal.org/articles/ebr/abs/2007/01/ebr0608/ebr0608.html. Accessed 2 October 2018.

4 SPYGEN. What is eDNA. [online]; 2019. URL: http://www.spygen.com/technologies/what-is-environmental-dna-edna/. Accessed 3 October 2018.

5 Kondo T. & Munoz J.A. Scale insects (Hemiptera: Coccoidea) associated with avocado crop, Persea americana Mill. (Lauraceae) in Valle del Cauca and neighboring departments of Colombia [online]. Florida: University of Nebraska-Lincoln; 2016. URL: http://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1973&context=insectamundi. Accessed 8 October 2019.

6 New Zealand Avocado. Growers' Manual. Pests. October 2004. pdf. p.6-7. Accessed 15 May 2019.

7 PestWeb. UnivarSolutions. Pest information. Greedy Scale [online];2019. URL: https://pestweb.com/pests/a23b33/greedy-scale. Accessed 14 May 2019.

8 PestWeb. UnivarSolutions. Pest information. Latania Scale [online]; 2019. URL: https://pestweb.com/pests/a44303/latania-scale. Accessed 14 May 2019.

9 Zespri Group Limited & Plant & Food Research. Kiwitech bulletin N1. Armoured Scales. October 2017. Pdf. Accessed 1 November 2018.

10 Eaton E.R. Family Diaspididae- Armored Scale Insects [serial online] Iowa State University; 23 October 2011. URL: https://bugguide.net/node/view/43499. Accessed 16 May 2019.

11 Assured AvoGreen® New Zealand. AvoGreen® Manual. February 2018. Version 6.3. pdf. Accessed 31 May 2019.

12 Hwang Ui-Wook & Kim Won. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics [serial online]. In: The Korean Journal of Parasitology; 31 December 1999; 37(4): 215-228.
URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2733198/. Accessed 23 September 2019.

13 Polanco C., González A.I., De la Fuente Á. & Dover G.A. Multigene Family of Ribosomal DNA in Drosophila melanogaster Reveals Contrasting Patterns of Homogenization for IGS and ITS Spacer Regions: A Possible Mechanism to Resolve This Paradox [serial online]. Genetics 1998 ;149 (1):243-256.
URL: https://www.genetics.org/content/149/1/243. Accessed 23 September 2019.

14 Edger P.P., Tang M., Bird K.A., Mayfield D.R., Conant G., Mummenhoff K., et al. Secondary Structure Analyses of the Nuclear rRNA Internal Transcribed Spacers and Assessment of Its Phylogenetic Utility across the Brassicaceae (Mustards) [online]; 1 July 2014. PLoS ONE 9(7): e101341.
URL: https://doi.org/10.1371/journal.pone.0101341. Accessed 23 September 2019.

15 Von Der Heyden S. A Review of Molecular Approaches for Investigating Patterns of Coevolution in Marine Host-Parasite Relationships [serial online] In: Elsevier. Advances in Parasitology; January 2014; 84: 209-252.
URL:https://www.researchgate.net/publication/259987431_A_Review_of_Molecular_Approaches_for_Investigating_Patterns_of_Coevolution_in_Marine_Host-Parasite_Relationships Accessed 23 September 2019.

16 Toju H. & Baba Y.G. DNA metabarcoding of spiders, insects, and springtails for exploring potential linkage between above- and below-ground food webs. Zoological Lett. 2018; 4:4. Published 15 February 2018.
URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5815251/. Accessed 24 September 2019.

17 Yao H., Song J., Liu C., Luo K., Han J., Li Y. et al. Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals [online]. PlosOne; 1 October 2010.
URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0013102. Accessed 24 September 2019.

18 Bustin S. & Huggett J. qPCR primer design revisited [serial online]. Biomolecular Detection and Quantification; December 2017; 14:19-28.
URL: https://www.sciencedirect.com/science/article/pii/S221475351730181X. Accessed 15 April 2019.

19 MERCK. Assay Optimization and Validation [online]. A technical guide to PCR technologies.
URL: https://www.sigmaaldrich.com/technical-documents/articles/biology/assay-optimization-and-validation.html. Accessed 12 April 2019.

20 Dieffenbach C.W., Lowe T.M.J & Dveksler G.S. General Concepts for PCR Primer Design [serial online]. Manual supplement. Cold Spring Harbor Laboratory Press. In: PCR methods and applications; December 1993;3(3):S30-S37.
URL: https://genome.cshlp.org/content/3/3/S30.full.pdf. Accessed 11 April 2019.

21 Prediger E. Consider SNPs when designing PCR and qPCR assays [serial online] Integrated DNA Technologies; 2019.
URL: https://sg.idtdna.com/pages/education/decoded/article/considering-snps-when-designing-pcr-and-qPCR-assays. Accessed 11 April 2019.

22 Premier Biosoft. PCR Primer Design Guidelines [online].
URL: http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html. Accessed 17 April 2019.

23 Khan Academy. Polymerase chain reaction (PCR). DNA analysis methods [online].
URL: https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/polymerase-chain-reaction-pcr. Accessed 7 October 2019.

24 Prediger E. How to design primers and probes for PCR and qPCR [online]. Integrated DNA Technologies; 20 August 2018.
URL: https://sg.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes. Accessed 16 April 2019.

25 Biocompare. Primers, by Design - Tips for Optimal DNA Primer Design [online]. Life Science Articles; 25 March 2013.
URL: https://www.biocompare.com/Bench-Tips/133581-Primers-by-Design-Tips-for-Optimal-DNA-Primer-Design/. Accessed 15 April 2019.

26 Genetic Education. “Primer Dimer”: Zones DNA amplification by pairing with foe oligo [online]. 15 April 2019.
URL: http://geneticeducation.co.in/primer-dimer-zones-dna-amplification-by-pairing-with-foe/. Accessed 7 October 2019.

27 Addgene. Primer Design for PCR [online].
URL: https://www.addgene.org/protocols/primer-design/. Accessed 12 April 2019.

28 Thornton B. & Basu C. Real-time PCR (qPCR) primer design using free online software [serial online]. IUBMB Journals; 28 March 2011. In: Biochemistry and Molecular Biology Education; 39(2).
URL: https://iubmb.onlinelibrary.wiley.com/doi/full/10.1002/bmb.20461: Accessed 17 April 2019.

29 Gollmick F.A., Lorenz M., Dornberger U., Von Langen J., Diekmann S. & Fritzsche H. Solution structure of dAATAA and dAAUAA DNA bulges [online]. Nucleic Acids Research; 15 June 2002:30(12):2669–2677.
URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC117287/. Accessed 9 April 2019.

30 Čepin U. Real-Time PCR (qPCR) technology basics [online]. BioSistemika; 4 July 2017.
URL: https://biosistemika.com/blog/qPCR-technology-basics/. Accessed 8 April 2019.

31 Whitman M. qPCR terminology—what does it mean? [online]. Integrated DNA Technologies; 15 June 2013.

URL: https://sg.idtdna.com/pages/education/decoded/article/qPCR-terminology-what-does-it-mean-. Accessed 8 April 2019.

32 Merck. Quantitative PCR and Digital PCR Detection Methods. A Technical Guide to PCR Technologies.
URL: https://www.sigmaaldrich.com/technical-documents/articles/biology/quantitative-pcr-and-digital-pcr-detection-methods.html. Accessed 6 October 2019.

33 Roche. LightCycler 480 Instrument. Operator's manual, Software Version 1.5, Hard cover manual. Accessed 21 November 2018.

34 Oswald N. What Is a Cq (Ct) Value? [online]. BiteSizeBio; May 2017
URL: https://bitesizebio.com/24581/what-is-a-ct-value/. Accessed 3 December 2018.

35 Bustin S.A. Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments [online]. ClinChem; 26 February 2009.
URL: http://clinchem.aaccjnls.org/content/55/4/611.long. Accessed 4 March 2019.

36 Downey N. Interpreting melt curves: An indicator, not a diagnosis [online]. Integrated DNA Technologies; 20 January 2014.
URL: https://sg.idtdna.com/pages/education/decoded/article/interpreting-melt -curves-an-indicator-not-a-diagnosis. Accessed 4 March 2019.

37 Pryor R.J. & Wittwer C.T. Real-Time Polymerase Chain Reaction and Melting Curve Analysis. In: Humana Press. Methods in Molecular Biology 336, Clinical Applications of PCR. Totowa, New Jersey; February 2006. p. 19-32. Accessed 26 August 2019.

38 Downey N. Understanding melt curves for improved SYBR Green assay analysis and troubleshooting [online video]. Integrated DNA Technologies; 3 April 2015.
URL: https://www.youtube.com/watch?v=qLAGD-Wy0D8. Accessed 20 August 2019.

39 Cha R.S. & Thilly W.G. Specificity, Efficiency, and Fidelity of PCR [serial online]. Manual Supplement. Cold Spring Harbor Laboratory Press. In: PCR Methods and Applications 1993;3(3):S18-S29.
URL: https://genome.cshlp.org/content/3/3/S18.full.pdf. Accessed 22 April 2019.

40 T042-Technical Bulletin Nanodrop Spectrophotometers. 260/280 and 260/230 Ratios [online]. Wilmington, Delaware USA: Thermo Scientific.
URL:https://www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/nanodrop.pdf. Accessed 20 August 2019.

41 Untergasser A., Cutcutache I., Koressaar T., et al. Primer3--new capabilities and interfaces [online]. Nucleic Acids Res. 2012;40(15): e115. doi:10.1093/nar/gks596. Accessed 12 March 2019.

42 Geneious® 8.1.2 [software] Build 2015-03-03 08:51 Java Version 1.7.0_51-b13 (64 bit); Biomatters. Accessed 18 March 2019.

43 McCord B., Pionzio A. & Thompson R. Analysis of the Effect of a Variety of PCR Inhibitors on the Amplification of DNA using Real Time PCR, Melt Curves and STR Analysis. U.S. Department of Justice; September 2015. Pdf.

44 LightCycler® 480 SYBR Green I Master [Kit protocol]. Roche Applied Science; June 2005. Pdf. Accessed 13 March 2019.

45 Čermák V. Primer Inspector [computer program]. Molbiotools; 2019. URL: http://www.molbiotools.com/primerinspector.php Accessed 23 September 2019.

46 Rotor-Gene® Q User Manual. Qiagen; December 2015. Pdf. Accessed 14 March 2019.

47 Efficiency.gene-quantification.info [online]. URL: https://www.gene-quantification.de/efficiency1.html. Accessed 13 November 2019.

The Other qPCR Primer Pair

The primer pair name, sequence and properties are listed in the table below. This primer pair was not selected for this study.

Name	Sequence	Length (bp)	Tm (°C)	GC %
Primerpair12-F	5'-GTT GTC AAC GAA CCG CAC AA-3'	20	58.4	50.0
Primerpair12-R	5'-TCG AGC GTT GTC TTA GCT CC-3'	20	60.5	55.0

Specificity Test qPCR Results

The resulted values are listed below of the specificity test that was done against eleven other common insect pests found from avocado and kiwifruit orchards.

Species	Name	Cq value	Amplification	Av. Cq value	Av. Amplification
Greedy scale	G.S 1/10	15.3	1.84	16.5	1.78
	G.S 1/10	15.4	1.75		
	G.S 1/10	18.8	1.75		
	NTC	7.3	-0.18	24.9	0.63
Latania scale	L.S	11.1	0.44	22.4	0.68
	L.S	34.2	2.08		
	L.S	21.8	-0.47		
	NTC	34.2	2.57		
Greenhouse Thrips	GHT	34.3	2.21	28.2	0.74
	GHT	22.3	0		
	GHT	28.1	0		
	NTC	11.1	0.09		
Passion vine hopper	PVH	34.8	2.04	26.8	1.59
	PVH	11.3	0.64		
	PVH	34.2	2.1		
	NTC	11.9	0		
Brown beetle	BB	35.3	0.39	27	0.81
	BB	11.3	0.16		
	BB	34.4	1.88		
	NTC	28.4	0.03		
Black-lyle leafroller	Cnej	22.1	-0.14	21.4	-0.19
	Cnej	7	-0.69		
	Cnej	35.1	0.28		
	NTC	34	2.17		
Light brown apple moth	Epo	34.3	2.45	25.3	1.51
	Epo	34.1	2.12		
	Epo	7.6	-0.03		
	NTC	22.1	-1.48		
Brown headed leafrollers	Cob	9.4	-0.01	16.5	0.72
	Cob	6	0		
	Cob	34.1	2.16		
	NTC	33.2	1.43		
	Cher	11.3	-0.14	23.2	0.74

	Cher	24.3	0.23		
	Cher	34	2.13		
	NTC	34	2.18		
Green headed leafrollers	Pex	22.1	-0.4	21.2	0.23
	Pex	7.1	-0.67		
	Pex	34.5	1.78		
	NTC	34.5	2.3		
	Poc	11.3	1.02	19.3	0.97
	Poc	11.6	0.5		
	Poc	35	1.39		
	NTC	14.1	-0.76		
Long-tailed mealy-bug	LTM	35.1	1.15	19.3	0.32
	LTM	11.6	0.01		
	LTM	11.1	-0.21		
	NTC	30.1	0		