Possibility of using High Resolution Melting (HRM) Analysis to identify the genetic purity of vegetable seed samples

-- Thesis report in Genetic Purity lab, Syngenta

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Preface and Acknowledgement

As a fourth year of International Horticulture of marketing course student, it is very important to be able to apply the theory into practice, organize and manage a project in a whole process by planning, conducting and finding the solutions.

This four month thesis research was done from February till June, 2010 in Syngenta Seeds to learn how to define a research objective, analyze and solve the problem.

I would like to express my gratitude to my course coordinator Albertien Kijne, she helped me to contact with my thesis company. I would like to thank my supervisor from Syngenta Tony de Rover, the leader of Genetic Purity Lab. He gave me the opportunity to conduct my thesis research. I would like to thank Remco Kwakman, who is another supervisor of my thesis. He not only taught me a lot of background knowledge on the molecular maker but also helped me with experiment set up and gave comments for my report writting. I also want to thank my colleagues from genetic purity lab, Rimco Smit, Maartje Grippeling, Marianne Govers, Bernadette Heidotting, Dragana Zivanovic, Julian Campfens and Kirsten Bruinings. They helped me a lot during my research experiments.

In particularly, I would like to thank my supervisor of Van Hall Larenstein, Mr Bernard Gildermasher. He spent time to visit me in the company Syngenta during I was doing my thesis and give me a lot of suggestions concerning my situation.

I had an unforgettable time at Syngenta. I really appreciate for all the people who helped me with my thesis research as well as life aspect. I will keep in mind the great help from all of you. Thank you very much!

Abstract

The thesis research objective is to find out the possibility of using High Resolution Melting (HRM) Analysis to identify the genetic purity of vegetable seed samples. Pepper, Melon and Squash DNA samples were taken to run tests by HRM machine to see if it is possible to discriminate female, male and hybrid DNA samples from the curves plotted by Melting Curve program. The current lab protocol and Bio-Rad company protocol were used to see if they work for HRM Analysis. Primers were chosen to amplify different length of amplicons. The HRM dye, SYBR Green and Eva Green were used to compare which one gives stronger signal. The HRM machine was run with resolution 0.1 ℃/s, 0.3 ℃/s and 0.5 ℃/s to see the differences of results. The Precision Melt Analysis™ Software was also used in the data analysis.

The test results showed that HRM can be used for inbred test for Pepper, Melon and Squash seed samples. The current protocol of Syngenta genetic purity works better. Eva Green shows less PCR inhibitory and gives stronger signals. It can be an alternative choice. Shorter products amplify with higher efficiency. 0.3 ℃/s balances the high resolution and efficiency. Precision Melt Analysis™ Software can analyze the data form melting curves and cluster the samples' genotype.

Table of content

1. Introduction

‘Syngenta is one of the world's leading companies with more than 25,000 employees in over 90 countries dedicated to our purpose: Bringing plant potential to life.[1]. ’ Syngenta has two main product lines, Crop Protection and Seeds. Both lines have further subdivisions. In Crop Protection, Syngenta is a global leader. In high value commercial Seeds, it ranks third. Within the Crop Protection segment, Syngenta also offers Professional Products include flowers and vegetables. The product portfolio covers the entire spectrum of growers' needs.

Production and distribution of high-quality seeds is fundamental to modern agricultural system. The majority of annual crops are established each season from seeds and seed quality can have a major impact on potential crop yield. Seeds carry the genetic traits incorporated by years of breeding and selection to create varieties that are adapted to specific production environments and will produce high yields and product quality. The genetic purity of seeds (the percentage of contamination by seeds or genetic material of other varieties or species), their physiological quality (or vigor), and the presence of weed seeds, diseases or other materials (dirt or plant residues) contribute to overall seed quality. The genetic purity of seed stocks is important to ensure that growers, processors, and consumers receive the crop varieties and products that they expect.

Plant hybrid[2] seeds, especially, are often stronger than either parent variety, a phenomenon which when present is known as hybrid vigor (heterosis) or heterozygote advantage. Plant breeders make use of a number of techniques to produce hybrids, including line breeding and the formation of complex hybrids[3]. Heterosis is a term used in genetics and selective breeding. The term heterosis, also known as hybrid vigor or out breeding enhancement, describes the increased strength of different characteristics in hybrids. It is the possibility to obtain a genetically superior individual by combining the virtues of its parents.

Syngenta aims to provide high quality hybrid seeds with high germination percentage, pest and disease resistance, genetic purity, good tasty and high yield traits. Within the Supply Chain of vegetable seeds, the quality control departments are responsible for testing the samples from produced commercial seed batches on these subjects and report testing results to logistic department through an online database system.

As a part of quality control department, the Genetic Purity lab is working on determination of the level of inbreds and off-types by using the molecular biological

[1] http://www2.syngenta.com/en/about_syngenta/

[2] Plant hybrid: In biology and specifically genetics, hybrid has several meanings, all referring to the offspring of sexual reproduction. (Rieger, R., A. Michaelis, and M.M. Green (1991). *Glossary of Genetics*, Fifth Edition. Springer-Verlag. ISBN 0-387-52054-6 page 256)

[3] http://en.wikipedia.org/wiki/Hybrid_(biology)

techniques. RFLP[4], PCR[5] and Gel-Electrophoresis[6] are used in our Lab at this moment. Inbred and off-type tests are the major Quality Control (QC) tests. Inbred test is performed on commercial hybrid-productions to confirm that the hybrid we sell is real hybrid. Off-type test is to determine the level of true to type seeds.

Tests should be done effectively, accurately as well as cost efficient. In genetic purity lab, there is a strong focus on increase of sample throughput and cost-efficiency. Therefore, I need to conduct a research and find out the possibility of moving inbred test from the current protocol to a more automated platform, such as High Resolution Melting (HRM) Analysis[7]. This is also the goal of the Genetic Purity lab 2010, remove all gel-activities from the processes.

In this report, you can find more about the research objective in the chapter 2. In this part, I will clarify the research objectives and define the research questions. The chapter 3 of this report is about the research method, you can find more information about the protocol in use at Syngenta, PCR. Also a new technique we are about to apply, HRM. In the chapter 4, the tests will be described, which is planed based on the research questions. Analysis and conclusion will be drawn for each test and next step will be planed. In the chapter 5, an analysis and comparing for all the tests will be done. Then the research question will be answered and a recommendation will be given for this research project in the end. The literature quotations can be found in the reference part. Pictures and figures which are relevant to this report are in the appendix.

[4] RFLP, Restriction Fragment Length Polymorphism (RFLP), as a molecular marker, is specific to a single clone/restriction enzyme combination.-http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechRFLP.shtml

[5] PCR, polymerase chain reaction. Please turn to chapter 3.1 of this report.

[6] Gel-Electrophoresis

[7] HRM, High Resolution Melting (HRM) is a novel, homogeneous, close-tube, post-PCR method, enabling genomic researchers to analyze genetic variations (SNPs, mutations, methylations) in PCR amplicons. Please turn to chapter 3.2 of this report. More information on http://www.gene-quantification.de/hrm.html.

2. Research objectives

2.1 Research background

The molecular technique in use for inbred test in our lab is PCR (Polymerase Chain Reaction) and Gel- Electrophoresis. The whole procedure is time consuming and too much labor work. There is a DNA contamination risk during transferring and pipetting sample DNAs from plate to Agarose Gel. Additionally, the Ethidium Bromide (EtBr) [8]dye which is commonly used as a non-radioactive marker for identifying and visualizing nucleic acid bands in electrophoresis and in other methods of nucleic acid separation. EtBr is a dark red, crystalline, non-volatile solid, moderately soluble in water, which fluoresces readily with a reddish-brown color when exposed to ultraviolet (UV) light. Although it is an effective tool, its hazardous properties require special safe handling and disposal procedures. There is probably a better choice which is High Resolution Melting (HRM) Analysis. These two protocols will be described later on in chapter 3, research materials and methods. Theoretically, HRM will perform quicker with results getting with less labor work steps than the current protocol. Workers can also avoid dealing with Ethidium Bromide. Therefore, the project is to investigate the feasibility of introducing HRM to genetic purity lab.

2.2 Research questions

The research question is: is it possible to use HRM to do inbred test?

The Sub-questions are:

1. Is it possible to discriminate female, male and hybrid DNA samples from the curves plotted by Melting Curve program?
2. What kind of chemicals has to be added in? Can we keep using the current recipe for HRM Analysis? Like the primers, PCR mix which are used in inbred test currently.
3. What is the appropriate reaction volume? The current protocol is 12 micro liters with 10 micro liters mixture and 2 micro liters DNA samples.
4. Does the amplicons length influence the test results?
5. Which HRM dye works better, SYBR Green or Eva Green?
6. What is the ideal resolution to visualize the difference of male, female and hybrid DNA? Is it necessary to increase temperature from 65 ℃ to 95 ℃ by heating 0.1 ℃/s continuously to allow HRM machine capture every slight curves' changes? Or it can be done by applying heating 0.5 ℃/s to shorten reaction time?
7. How to analyze the data from the outcomes of HRM curves and data files? Can we see the differences between female, male and hybrid DNA samples directly or we need specific software?

[8] http://www.ehs.berkeley.edu/pubs/factsheets/47ethidiumbromide.html

3. Research materials and Methods

3.1 Research materials

The research materials are showed in Appendix 1, information list of chemicals and materials on page 47.

3.2 The current protocol in use, PCR

The research was done to look at Melon, Pepper and Squash DNA (genotype) of seeds by using PCR technique. There are 7 steps, first step is sampling taking from young leaves, and then we can isolate DNA from samples we took. After this step, we get DNA from young plant leaves. Then, we add primer, PCR mix and Taq[9] into DNA samples, let them react in PCR machine to amplify target DNA sequence. The following step is to add Cresol-red loading dye and transfer amplicons to Agarose Gel-Electrophoresis. The final step is staining gels into Ethidium Bromide for 30 minutes then taking pictures by putting dyed gels onto a UV lighter. From the picture, we can see the differences between female, male and hybrid DNA fragments. By comparing with female and male, we can determine hybrid and inbred seeds.

Step 1, Seeds sowing and samples taking

The first step of the inbred test is seeds sowing. We could not get plant DNA directly from seeds but young leaves. After seeds sowing for 2 weeks, we can get young seedlings for tomatoes and peppers. Melon is growing faster about 1 week only.

There 2 status for samples taking, status A and status B. Status A is high value seeds harvested from greenhouses, farmers require higher price. Therefore, more samples need to take to guarantee high percentage of hybrid, 382 samples need to take. Status B is the seeds harvested from open field, 192 samples need to take.

Step 2, DNA extraction

DNA isolation process comes after we have dry, grinded leaves material. In short, this is a process to separate DNA from the other parts of the leaf material.

[9] Taq is a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated by Thomas D. Brock in 1965. It is often abbreviated to "Taq Pol" (or simply "Taq"), and is frequently used in polymerase chain reaction (PCR), methods for greatly amplifying short segments of DNA. These information comes from: http://en.wikipedia.org/wiki/Taq_polymerase.

Step 3, DNA samples preparing

In this step, 384 wells plate are filled with samples. For Pepper and Melon, the reaction components include 5.38 microliters water, 3.94 microliters PCR mix, 0.6 microliters forward and reverse primers, 0.09 microliters Taq and 2 microliters sample DNA.

Step 4, PCR

The polymerase chain reaction (PCR) is a technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.[10] There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 94 ℃:
During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

Annealing at 54 ℃:
The primers are jiggling around. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. Each primer theoretically has its own optimal temperature. Since we are using multiple markers we choose to use one temperature. Consequence of this is that some markers might be further from their optimal annealing temperature than others, creating differences in yield of the PCR reaction.

Extension at 72 ℃:
This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

[10] http://en.wikipedia.org/wiki/Polymerase_chain_reaction

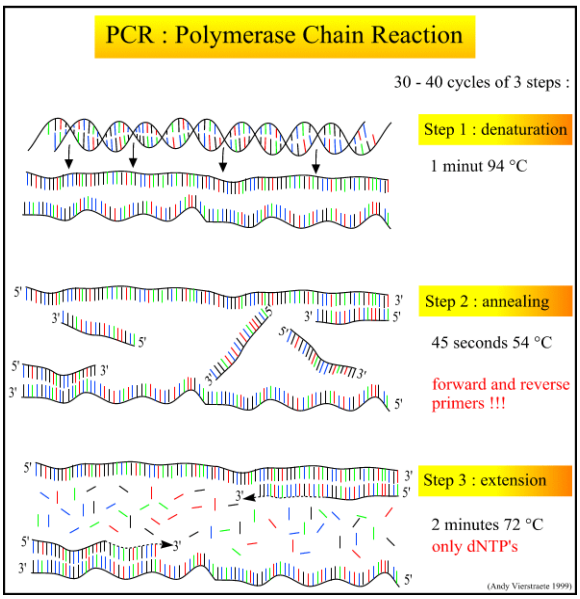


Figure 3: The different steps in PCR

Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on.

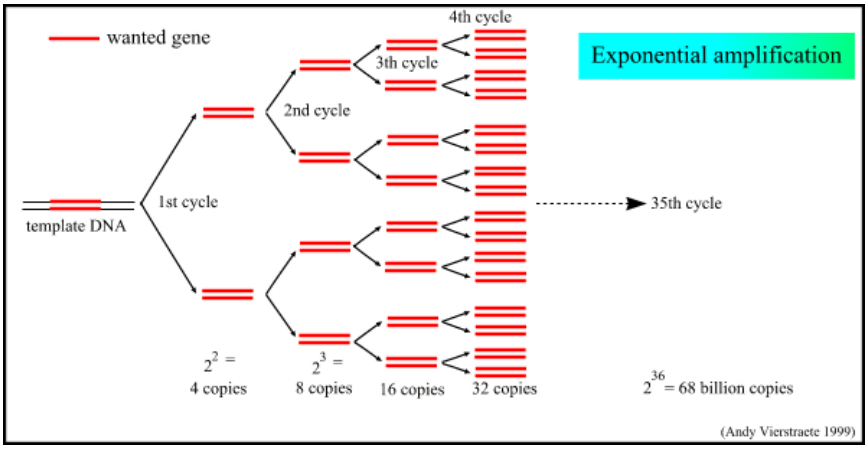


Figure 4: The exponential amplification of the gene in PCR

Step 5, Cresol-red dye loading

It is one extra manual step in which the plates are opened and there is Loading Dye pipetted into the wells to make material get into the bottom of wells. Laborious and risk on contamination will be increased.

Step 6, Gel- Electrophoresis

Agarose Gel- Electrophoresis is a method used in biochemistry and molecular biology to separate DNA, or RNA molecules by size. [11]This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones. In this step, Amplified DNAs are transferred to agarose matrix and let it run for 2 and half hours.

Step 7, Ethidium Bromide staining and picture taking

The DNA is visualised in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. [12] By soaking an gel with DNA samples into ethidium bromide solution for 30 minutes, the DNA band becomes distinctly visible. We can compare and identify the DNA types easily and save it for long time by take a digital picture.

3.3 HRM

High Resolution Melting (HRM) Analysis is a new, post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Simple and fast, this method is based on PCR melting (dissociation) curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate DNA sequences based on their composition, length, GC content, or strand complementarities[13].

HRM analysis starts with PCR amplification of the region of interest in the presence of a dsDNA binding dye. This binding dye has a high fluorescence when bound to dsDNA and low fluorescence in the unbound state. Amplification is followed by a high resolution melting step using instrumentation capable of capturing a large number of fluorescent data points per change in temperature, with high precision. When the dsDNA dissociates

[11] http://en.wikipedia.org/wiki/Agarose_gel_electrophoresis

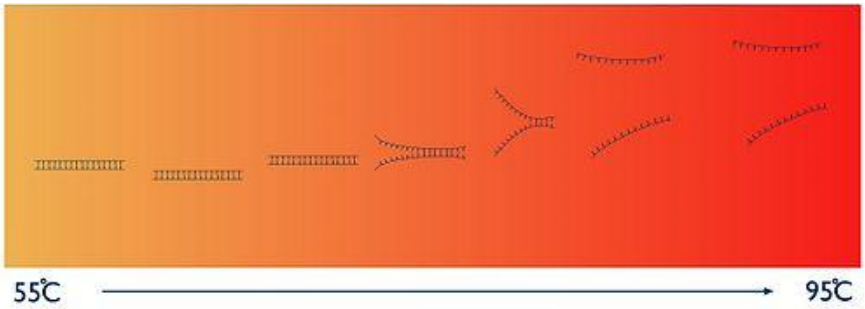
[12] http://www.methodbook.net/dna/agarogel.html

[13] Reed GH, Kent JO, and Wittwer, CT (2007) High-resolution DNA melting analysis for simple and efficient molecular diagnostics, *Pharmacogenomics* **8(6):**597–608.

(or melts) into single strands, the dye is released, causing a change in fluorescence. The result is a melt curve profile characteristic of the amplicon.

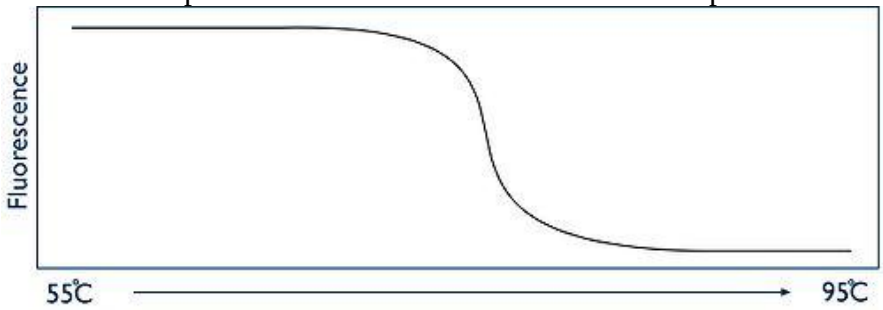
Step 1, Melting double-stranded DNA

After the PCR process the HRM analysis begins. The process is simply a precise warming of the amplicon DNA from around 65˚C up to around 95˚C. At some point during this process, the melting temperature of the amplicon is reached and the two strands of DNA separate or "melt" apart.[14] This procedure is showd by picture 1 below.



Picture 1, Double-stranded DNA melt apart with the temperature rising up

HRM can this process happening in real-time. This is achieved by using a fluorescent dye. The dyes that are used for HRM are known as intercalating dyes and have a unique property. They bind specifically to double-stranded DNA and when they are bound they fluoresce brightly. In the absence of double stranded DNA they have nothing to bind to and they only fluoresce at a low level. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions of copies of the amplicon. But as the sample is heated up and the two strands of the DNA melt apart, presence of double stranded DNA decreases and thus fluorescence is reduced. The HRM machine has a camera that watches this process by measuring the fluorescence. The machine then simply plots this data as a graph known as a melt curve, showing the level of fluorescence and the temperature. Picture 2 below shows an example.

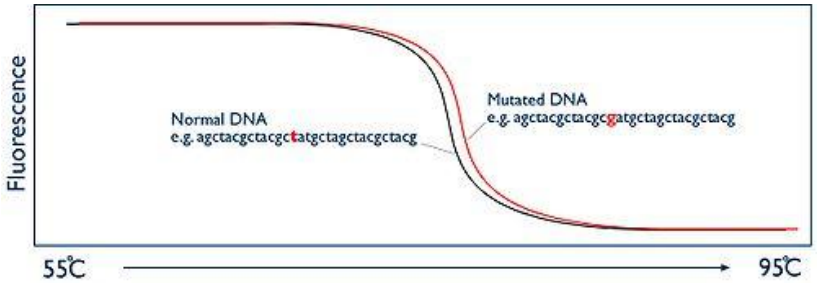


Picture 2, A melt curve plotted by HRM machine

[14] http://en.wikipedia.org/wiki/High_Resolution_Melt

Step 2, Spot the difference

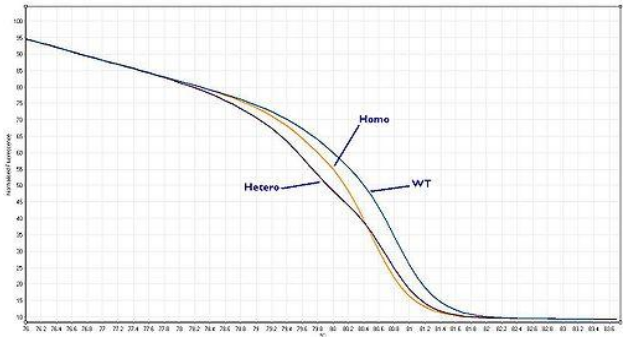
The melting temperature of the amplicon at which the two DNA strands come apart is entirely predictable. It is dependent on the sequence of the DNA bases. If you are comparing two samples from two different people, they should give exactly the same shaped melt curve. However if one of the people has a mutation in the DNA region you have amplified, then this will alter the temperature at which the DNA strands melt apart. So now the two melt curves appear different. Picture 3 shows the curves of a normal DNA and a mutated DNA. The difference may only be tiny, perhaps a fraction of a degree, but because the HRM machine has the ability to monitor this process in "high resolution", it is possible to accurately document these changes and therefore identify if a mutation is present or not.



Picture 3, The curves of a normal DNA and a mutated DNA

Step 3, Wild type, heterozygote or homozygote

Things become slightly more complicated than this because organisms contain two copies of each gene, known as the two alleles. So, if a sample is taken from a patient and amplified using PCR both copies of the region of DNA (alleles) of interest are amplified. So if we are looking for mutation, there are now three possibilities: neither allele contains a mutation, one or other allele contains a mutation, both alleles contain a mutation. These three scenarios are known as "Wild –type", "Heterozygote" or "homozygote" respectively. Each gives a melt curve that is slightly different. With a high quality HRM assay it is possible to distinguish between all three of these scenarios.



Picture 4, melting curves of Wild –type, Heterozygote and homozygote

According to this theory, we can consider that females and males are homozygote and hybrids are heterozygote. Inbred is supposed to be same with female.

4. Tests and Results

4.1 Test 1, Volume test focusing

The seeds technology lab in our company they use HRM to do seeds disease test and they use 25 micro liters reaction volume. To define the reaction volume of inbred test by using HRM, a test was set up. The experiment was done by using pepper 4396 DNA samples from 205 plants by random as our test sample.

4.1.1 Reaction volume and layout

The test materials are 1 female and 1 male DNA and 190 test samples. The reaction components are same as what is the lab currently using at this moment. It shows by table 1. Therefore, 12 micro liter reaction volumes are prepared for PCR reaction in the end. The plate used for HRM is 96 wells. I just simply transfer the DNA plate which is used for QC test with 384 wells. The layout shows in table 2 and 3.

Reaction volume for PCR	
Water	5.38 µl
PCR mix	3.94 µl
Primer	0.60 µl
Taq	0.09 µl
DNA	2.0 µl
Total	12.0 µl

Table 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
B	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
E	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
F	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
G	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
H	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid

Table 2, plate 1 of test 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
B	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
E	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
F	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
G	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
H	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid

Table 3, plate 2 of test 1

- PCR reaction

The program set up can be found in appendix 2 on page 48.

-SYBR Green adding

SYBR green is an asymmetrical cyanine dye , used as a dye for the quantification of double stranded DNA in some methods of real time PCR. The resulting DNA-dye-complex absorbs blue light (λ_{max} = 488 nm) and emits green light (λ_{max} = 522 nm). The stain preferentially binds to double-stranded DNA, but will stain single-stranded DNA with lower performance. It is also used to visualize DNA in gel electrophoresis.[15]

After the PCR program, 12 microliters SYBR green are added to each sample. So there are 24 micro liters reaction component are used for HRM reaction. The program for HRM can be found in Appendix 3 on page 48.

[15] http://en.wikipedia.org/wiki/SYBR_Green_I

4.1.2 Results

- Overview of the result.

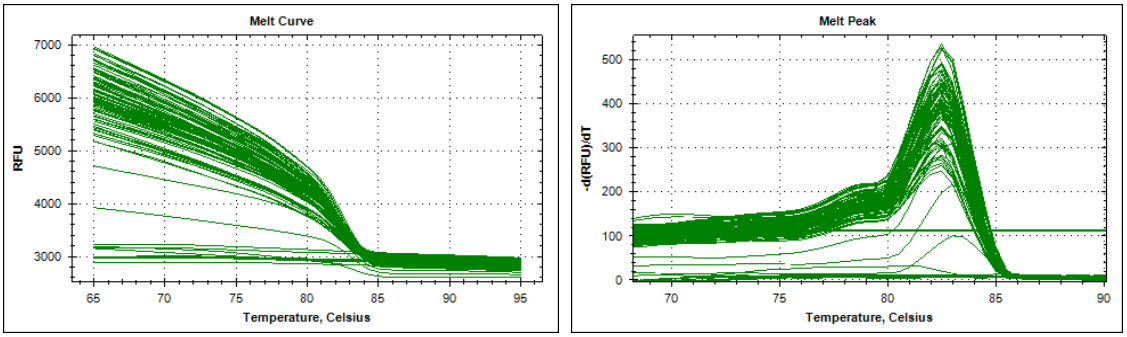


Figure 1, Curves of hybrid DNA

The figure1 shows the curves of hybrid DNA which are samples from plate 1. Because I only simply transfer DNA samples from QC test plate to HRM plate, there are no female and male DNA in plate 1. The left picture of figure 1 shows the Melting curve of hybrid DNA. At the beginning, the DNA are double stranded when the temperature is 65 Celsius, the SYBR Green dye are binding on the 2-strand DNA, so strong signals are given. With the temperature rising, double stranded DNA start melting, the 2-strand DNA start melting to single DNA strands where the SYBR green banding dye start releasing, signals decreased. From the left picture, we can see that the melting temperature is between 80-85 Celsius. To get closed to real melting temperature, the picture on the right of figure 2 shows the melting temperature of DNA samples.

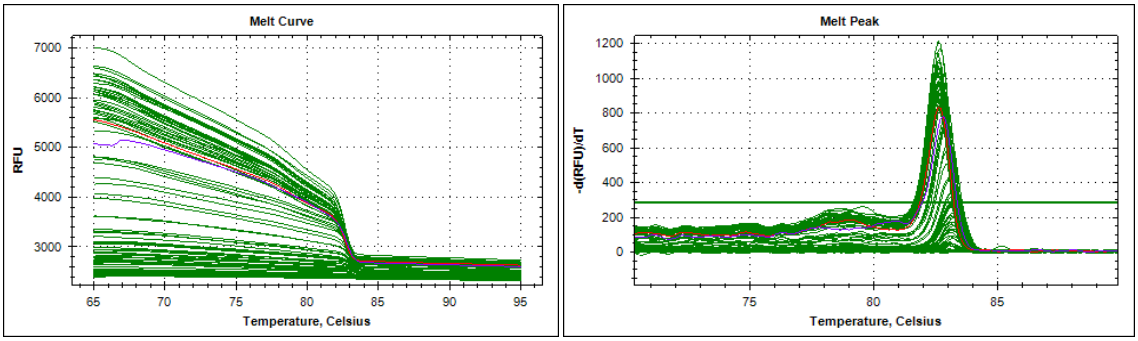


Figure 2, Curves of female, male and hybrid DNA

Figure 2 shows the curves of female, male and hybrid DNA which are samples from plate 2. The purple line shows female DNA and a red one shows male DNA. To see the differences between different DNA types, a zoom-in picture shows 1 hybrid, 1 female and 1male DNA in figure 3.

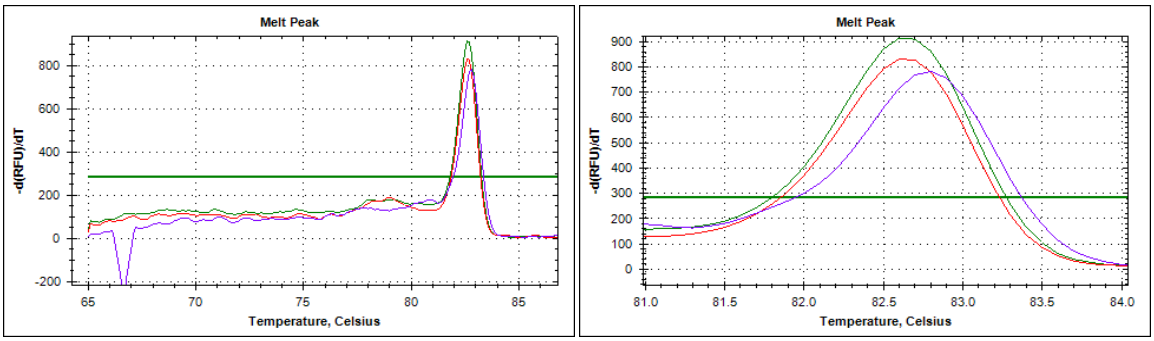


Figure 3, zoom-in picture with 1x hybrid, 1x Male, 1x female

The left picture of figure 3 shows the different curve shapes of 3 types of NDA. The female DNA (purple line) is different from others with 1 extra downwards peak. The picture from right side of figure 3 shows the slight difference in melting temperature. The melting temperature for female is 82.8 Celsius and male is 82.6 Celsius.

4.1.3 Conclusion and continuance

The test result shows some differences, but the difference is not sufficient to define samples' genotype. We expect all DNAs to have the same melting temperature which is different to female's melting temperature, unless the sample is a Inbred. The curve shapes are not same for each type of DNA. So we can distinguish the inbred from hybrid, female and male.

Next to do: For the next experiment, we can make more replication for female and male DNA instead of only 1 for control to see if the differentiation is reproducible.

4.2 Test 2, differentiation test

Normally, there is only one female and male DNA in a plate for one inbred test. From test 1, we can see the differences with only one female and male DNA samples. The purpose of this test is to find out the variation among female, male and hybrid by enlarge the number of testing samples.

4.2.1 Plate layout

There are 16 replications of DNA form the same female, male and hybrid respectively. To eliminate contamination, one column was left empty between each treatment. The plate layout is showed by table 4 below. Each treatment has 24 micro liters for PCR reaction. Afterwards, I separated samples into 2 plates with 12 micro liters for each. Then I add SYBR Green into one plate for melting curve and another is processed further Gel-Electrophoresis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	female	female		male	male		hybrid	hybrid		water		
B	female	female		male	male		hybrid	hybrid		water		
C	female	female		male	male		hybrid	hybrid		water		
D	female	female		male	male		hybrid	hybrid		water		
E	female	female		male	male		hybrid	hybrid		water		
F	female	female		male	male		hybrid	hybrid		water		
G	female	female		male	male		hybrid	hybrid		water		
H	female	female		male	male		hybrid	hybrid		water		

Table 4, layout of test 2

4.2.2 Results

- Result from melting curve:

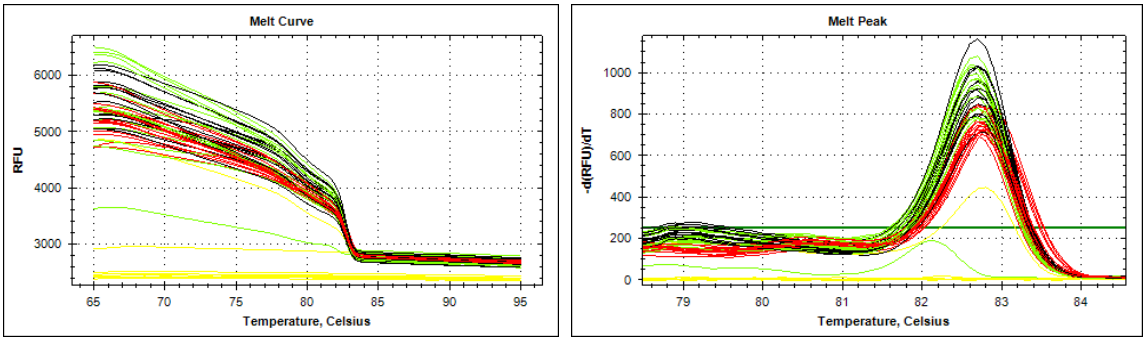


Figure 4, overview of all the DNA samples

The left picture from figure 4 shows changes of signals and the right picture show DNA curve shapes and melting temperature peaks. The red lines shows female DNA curves, the black ones are male, the green ones are from hybrid and the yellow ones are water.

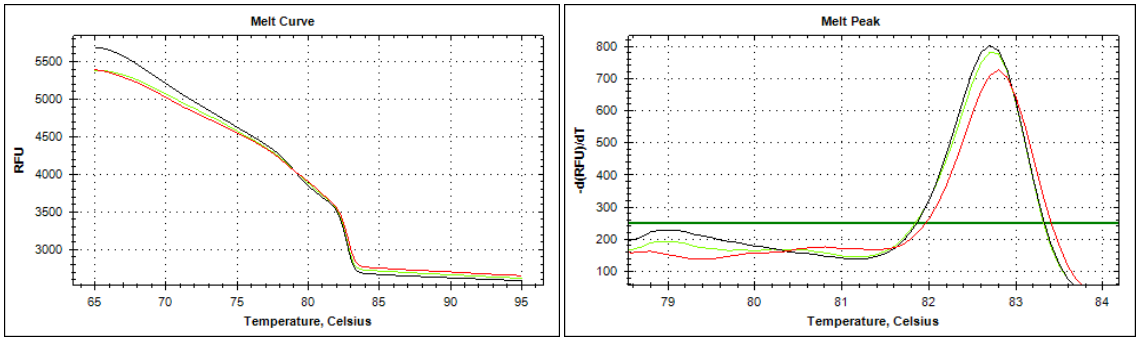


Figure 5, zoom-in picture with 1x hybrid, 1x Male, 1x female

Figure 5 show a zoom-in picture of one hybrid, 1 male and 1 female DNA curves. From the right picture, the melting temperature for male and hybrid DNA are same with 82.7 Celsius and the melting temperature for female DNA is 82.8 Celsius.

4.2.3 Conclusion and continuance

Differences in curves are there and can be seen if we only take small amount of sample at one time, like what figure 5 shows. But we cannot use them for a QC test (too small and no software to indicate these differences between samples).

Problems:

1. Small difference can be visible (DNA fragments are too closed).
2. Data file analysis. Is there any research method or software can be used for analyze this kind of curves to see the significant difference?

Next to do to find solutions: Eva Green instead of SYBR Green?

To improve the results by answering these questions, I did some desk research by searching for the relevant information on the internet. There is another option, a new HRM banding dye which is called Eva Green. It can give better results than SYBR Green does. Figure 6 below shows 2 examples by using SYBR Green and Eva Green to PCR amplification and Melting curve analysis.[16]

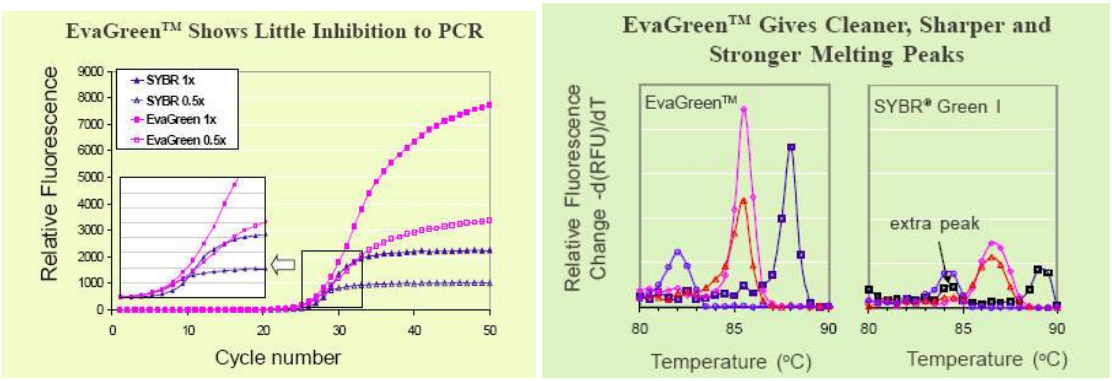


Figure 6, comparing SYBR Green and Eva Green

The left picture of figure 6 shows a PCR amplification plots using Eva Green and SYBR Green I at two different concentrations. SYBR Green I exhibits significant PCR inhibition at1x concentration (0D~0.05) while Eva Green does not (See inset)

The right picture shows DNA melting curve analysis using Eva Green and SYBR Green I respectively, with 4 different amplicons: 1) TBP (--); 2) SDHA (--); 3) RPL4 (--); and 4) HMBS (--).SYBR Green I exhibits significant PCR inhibition, resulting in occasional formation of an extra melting peak.

[16] http://www.gencompare.com/qpcr.htm

4.3 Test 3: Test for Comparing SYBR Green and Eva Green by using Bio-Rad protocol

The purpose of this test is to find out if the Eva Green can give a better result which means larger differences from HRM outcomes. Compared with the widely used SYBR Green I, Eva Green dye is generally less inhibitory toward PCR and less likely to cause nonspecific amplification. As a result, Eva Green dye can be used at much higher dye saturation than SYBR Green I, resulting in more robust PCR signal.

4.3.1 Reaction volume and layout

As the Eva Green dye from Bio-Rad Company is a mixture with Taq and PCR-mix, so we set up a reaction component according to the current Genetic Purity protocol for inbred testing, we replace the Taq and PCR-mix by SsoFast™ EvaGreen® Supermix, provided by Bio-Rad, in a certain percentage and get 20 microliters reaction volumes in total. All the reagents are added before PCR. By this way, all the programs are conducted in same closed tubes. Table 4 shows the reaction component by using Bio-Rad protocol. Table below shows the layout of SYBR Green and Eva Green test by using Bio-Rad protocol. Table 4, reaction component

Reaction for PCR and HRM	
Water	8.97 µl
primer	1.00 µl
DNA template	3.33 µl
SYBR or Eva Green	6.72 µl
Total volume	20 µl

Table 4

	Biorad protocol, SYBR green						Biorad protocol, EVA green					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Female + SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
B	Female+ SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
C	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
D	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
E	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid+ SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
F	Female+ SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
G	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
H	Female + SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva

Table 5, layout of SYBR Green and Eva Green test by using Bio-Rad protocol

4.3.2 Results

Overview of results

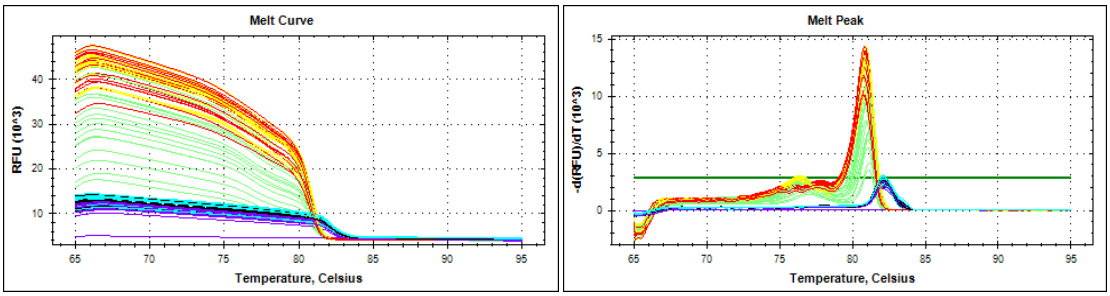


Figure 7, overview of curves plotted by SYBR and Eva Green

Figure 7 shows the outcomes by using SYBR Green and Eva Green with Bio-Rad protocol respectively. The red, yellow and green curves were plotted by using Eva Green. The blue, purple and black curves were plotted by using SYBR Green. Eva Green gives sharper peaks and stronger signals by plotting higher peak.

Results from Eva Green

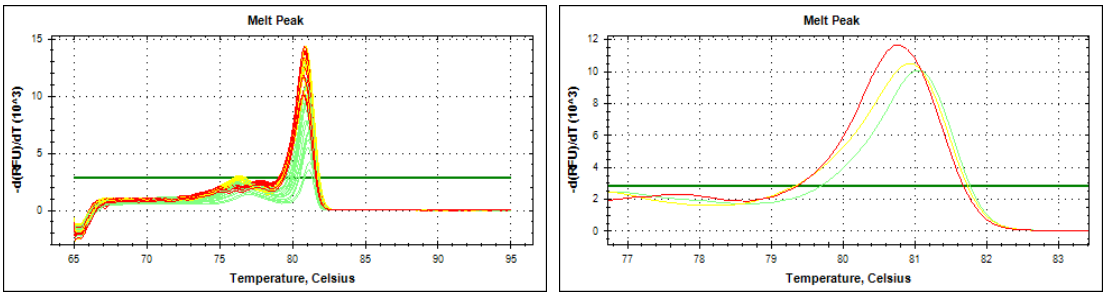


Figure 8, Curves plotted by Eva Green

Figure 8 shows the curves plotted by Eva Green. The red lines are female DNA samples, the yellow lines are male samples and the green ones are hybrid. The melting temperature for female DNA is 80.7 Celsius, for male DNA is 80.9 Celsius and for hybrid DNA is 81.1 Celsius.

Results from SYBR Green

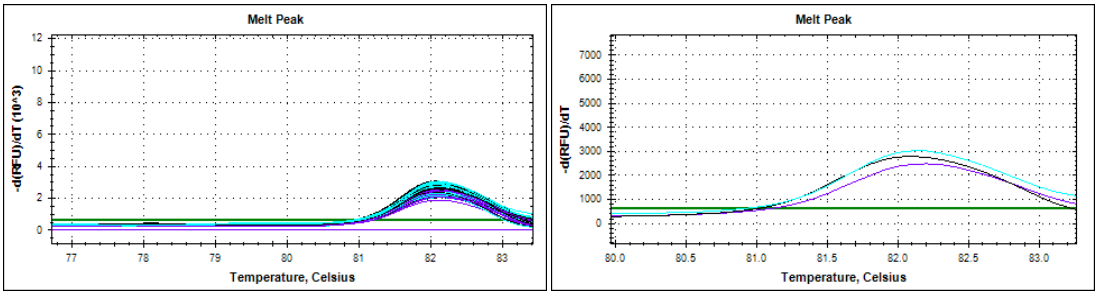


Figure 9, Curves plotted by SYBR Green

Figure 9 shows Curves plotted by SYBR Green the blue lines on top are female DNA samples, the black lines are male samples and the purple ones are hybrid. A zoom-in picture on the right shows only one sample for each DNA type and the melting temperature. It is 82.1 Celsius for female and male DNA, 82.2 Celsius for hybrid DNA.

4.3.3 Conclusion and continuance

Eva Green gives higher signal by plotting higher peaks. The differences among 3 genotype DNAs are also enlarged because it has less inhibition. The differences with melting temperature are also larger. Resolution of EVA green is higher so the small differences that we detected before can be observed better.

Next to do: I am going to use current lab protocol to see what we can get.

4.4 Test 4, Comparing SYBR Green and Eva Green by using the current protocol of Syngenta genetic purity lab

4.4.1 Reaction volume and layout

My literature study and test 3 show that SYBR Green dye exhibits very high PCR inhibition. Eva Green is a better choice to improve our research from test 3. The objective of this experiment is to find out if we can get even better results by using the current lab protocol. Table 6 on the right shows the ingredients for PCR reaction.

Reaction volume for PCR	
Water	5.38 µl
PCR mix	3.94 µl
Primer	0.60 µl
Taq	0.09 µl
DNA	2.0 µl
Total	12.0 µl

Table 6

After PCR, 10 micro liters SYBR Green dye and Eva Green dye are added into post-PCR products. In the end, 22 micro liters reaction volume is used for HRM. Table 7 on the right and table 8 below show the reaction component for HRM and plate layout respectively.

Reaction volume for HRM	
PCR product	12.0 µl
Post-PCR SYBR and Eva Green	10.0 µl
total	22.0 µl

Table 7

	Genetic Purity Protocol + POST-PCR Sybrgreen						Genetic Purity Protocol + POST-PCR EVA gr.					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Female + SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
B	Female+ SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
C	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
D	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
E	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid + SYBR	Hybrid+ SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
F	Female+ SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
G	Female + SYBR	Female + SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva
H	Female + SYBR	Female+ SYBR	Male + SYBR	Male + SYBR	Hybrid+ SYBR	Hybrid + SYBR	Female + Eva	Female + Eva	Male + Eva	Male + Eva	Hybrid + Eva	Hybrid + Eva

Table 8, layout of SYBR Green and Eva Green test by using current lab protocol

4.4.2 Results

- Overview of results

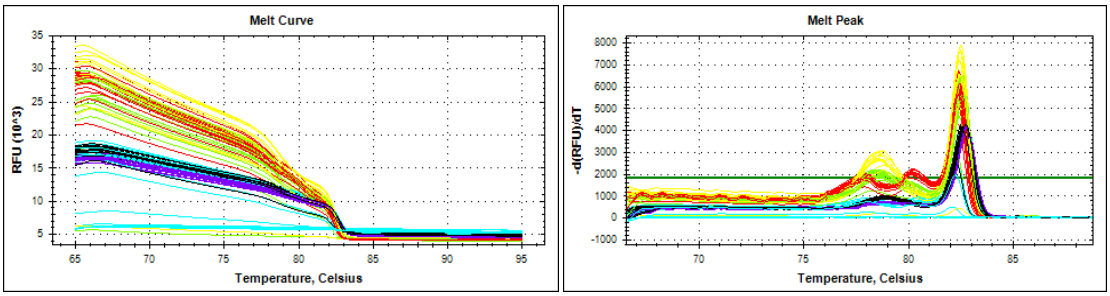


Figure 10, overview of curves plotted by SYBR and Eva Green

Figure 10 shows the outcomes by using SYBR Green and Eva Green respectively with current lab protocol. The red, yellow and green curves were plotted by using Eva Green. The blue, purple and black curves were plotted by using SYBR Green. Same results from test 3, Eva Green gives sharper peaks and stronger signals by plotting higher peak.

- Results from Eva Green

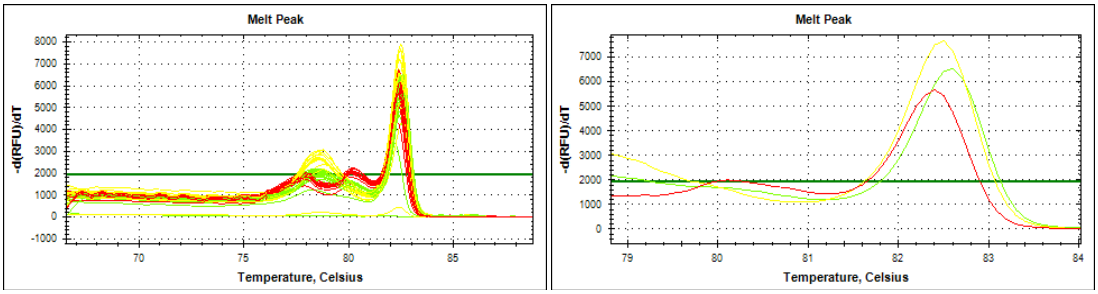


Figure 11, Curves plotted by Eva Green

Figure 11 shows the curves plotted by Eva Green. The red lines are female DNA samples, the yellow lines are male samples and the green ones are hybrid. The melting temperature for female DNA is 82.4 Celsius, for male DNA is 80.5 Celsius and for hybrid DNA is 82.6 Celsius.

-Results from SYBR Green

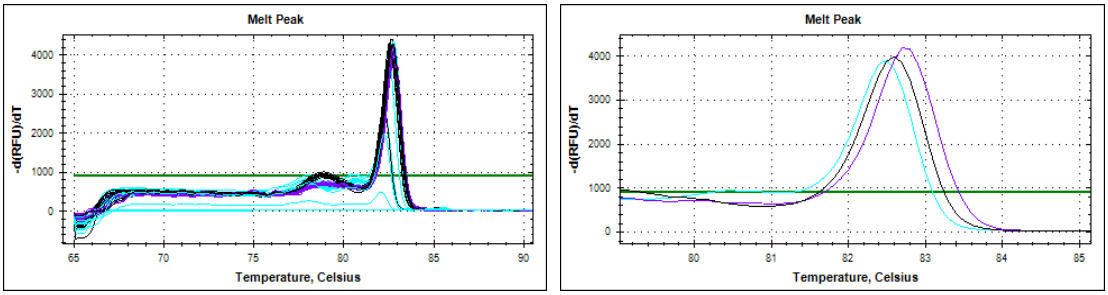


Figure 12, Curves plotted by SYBR Green

Figure 12 shows Curves plotted by SYBR Green. The blue lines on top are female DNA samples, the black lines are male samples and the purple ones are hybrid. A zoom-in picture on the right shows only one sample for each DNA type and the melting temperature. It is 82.5 Celsius for female 82.6 Celsius for male DNA and 82.7 Celsius for hybrid DNA.

4.4.3 Conclusion and continuance

Test 3 and test 4 indicated that Eva Green gives better results no matter using Bio-Rad or current lab protocol. To comparing Bio-Ra.d protocol and current lab protocol, I would like to put the curves plotted by using these 2 protocols. Figure 13 shows the results of Bio-Rad protocol, Eva Green and figure 14 shows the results of current lab protocol, Eva Green.

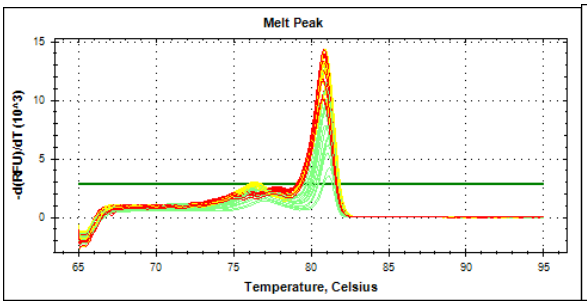


Figure 13, results of Bio-Rad protocol, Eva

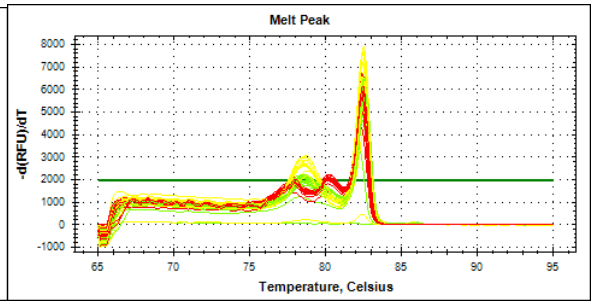


Figure 14, results of current lab protocol,Eva

The red curves are female DNA, the yellow curves are male DNA and the green curves are hybrid DNA. From figure 14, we can see that the red curves give one more extra downwards peak, this can enlarge the differences between female from male and hybrid DNA.

From the comparing we can conclude that the current lab protocol with Eva Green can get better results than the protocol of Bio-Rad Company. Therefore, we are going to continue our protocol and switch SYBR Green to Eva Green for further tests.

Next to do:

1. Primer changes

A primer is a strand of nucleic acid that serves as a starting point for DNA or RNA synthesis. They are required because the enzymes that catalyze replication, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand.[17]

[17] http://en.wikipedia.org/wiki/Primer_(molecular_biology)

The information I found shows that primer is a element can influence HRM results significantly. Theses information come from my literature study on the material, *A Guide to High Resolution Melting (HRM) Analysis*[18], it is available to download from the website below. And an instruction manual I got from Seeds Health lab of Syngenta, *REAL-TIME PCR: FROM THEORY TO PRICTICE*. From the literature, it is said that designing primers to amplify a segment ranging from 60 base pair [19](bp) to 150 bp will enhance reaction efficiency.

The previous 4 tests were used primer NP0055 which can amplify 284 bp female amplicons[20] and 264 bp male amplicons. It is longer according to the literature. Therefore, I am going to choose other primers which can produce shorter amplicons and use Eva Green dye for further test.

2. Test software with more analysis options.

I also contacted with Rodger Kuhlman and Eddy van Collenburg from Bio-Rad Laboratories B.V., they are the supplier of the HRM machine which I am using for my project at this moment. From the information they gave, Eva Green does give less inhibition, shaper peaks and stronger signals. Additionally, they do have software called Precision Melt Analysis™ Software which can be used for melting curve data file analysis. They also sent me an instruction manual for using it.

[18] http://hrm.gene-quantification.info/

[19] In molecular biology, two nucleotides on opposite complementary DNA or RNA strands that are connected via hydrogen bonds are called a base pair (often abbreviated bp). In the canonical Watson-Crick base pairing, adenine (A) forms a base pair with thymine (T), as does guanine (G) with cytosine (C) in DNA. More information on http://en.wikipedia.org/wiki/Base_pair

[20] Amplicons are pieces of DNA formed as the products of natural or artificial amplification events. More information on http://en.wikipedia.org/wiki/Amplicons

4.5 Test 5: Primers test

In this test, I am going to find out if using primers which can produce shorter PCR products (amplicons) can enhance reaction efficiency.

The test used pepper variety 4P396, lab number 4032, primer NP0055 and primer NP0017. Primer NP0055 can amplify female PCR amplicons with length 284 bp and male PCR amplicons length 264 bp. Another primer NP0017 can amplify female PCR amplicons with length 168 bp and male PCR amplicons length 180 bp.

4.5.1Reaction volume and Layout

The reaction component and volume are just same as the current lab protocol. As table 8 and table 9 show below.

.

Reaction volume for HRM	
PCR product	12.0 µl
Post-PCR SYBR and Eva Green	10.0 µl
total	22.0 µl

Table 8, Reaction volume for HRM

Reaction volume for PCR			
PCR Mixture		1	**140**
	Water	5.38 µl	753.2µl
	PCR mix	3.94 µl	551.6µl
	Primer	0.60 µl	84µl
	Taq	0.09 µl	12.6µl
	total	10 µl	1401.4µl
DNA		2.0 µl	
Total		12.0 µl	

Table 9, Reaction volume for PCR

There 8 replications for female and male respectively. For the rest are different samples instead of repeat 1 sample which are supposed to be hybrid.

Pepper 4032, Primer NP0055 or NP0017, Eva Green												
	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
B	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
E	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
F	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
G	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
H	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid

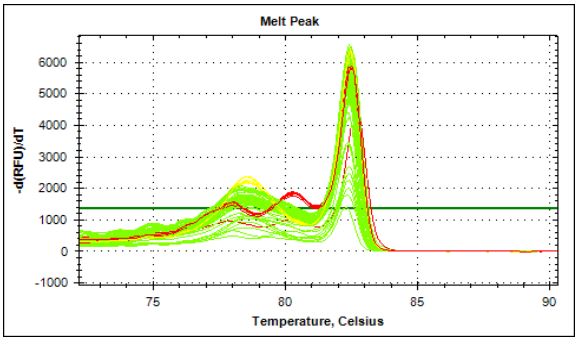
Table 10, plate layout of primer test

4.5.2 Result

- Results from Melt Curve

Figure 15 shows the results of using primer NP0055, The red curves are female, yellow curves are male and green ones are hybrid DNA samples. It is just same as the results we have got from previous tests.

Figure 16 shows the results of using primer NP0017, The red curves instead of female, and the yellow curves are male and the green curves are hybrid DNA samples. By this picture, it is clearly shows by green curves which are instead of hybrid DNA samples that there are 2 peaks. The female and male DNA only shows 1 peak but with big different melting temperature and different melting peak height.



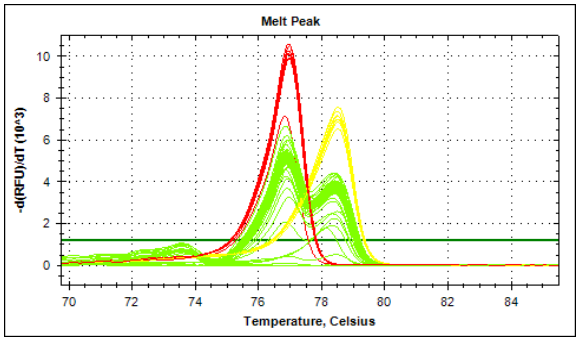
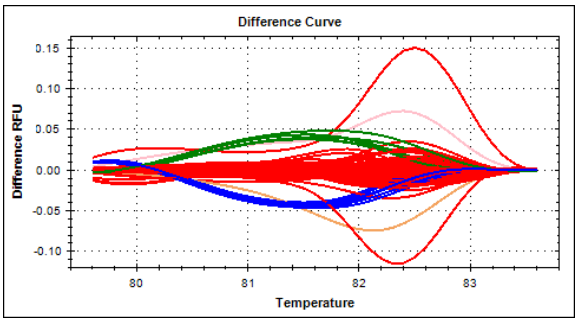


Figure 15, pepper 4032, primer NP0055 **Figure 16, pepper 4032, primer NP0017**

- Results from Precision Melt Analysis™ Software

Figure 17 and 18 show the outcomes by using Precision Melt Analysis™ Software. The green lines are male DNA, the red lines are hybrid and the blue lines are female DNA.

By comparing with 2 pictures, figure 18 gives better result. 3 types of DNA samples are clustered nicely.



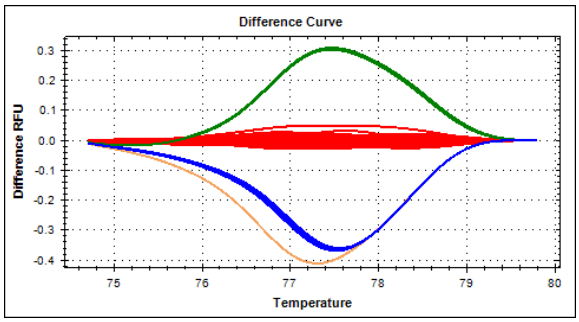


Figure 17, pepper 4032, primer NP0055 **Figure 18, pepper 4032, primer NP0017**

4.5.2 Conclusion and continuance

The test 5 results show that using primer to amplify shorter amplicons gives better result. Precision Melt Analysis™ Software brings a blue sky for our test. The curves and data files can be analyzed then get clear discrimination for different types of DNA.

Next to do:

Theoretically, the curves plotted by HRM for inbred should be as same as female curves. For the next test, I am going to choose a seeds batch with inbred DNA to see what is the hybrid curves look like.

At the same time, I am going to continue primer test by keep primer NP0055 and choose other one or two primers which can produce shorter amplicons.

4.6 test 6: Primers and inbred test

The purpose of this test is to find out if inbred DNA gives same curve as female DNA. In a same way, I choose another pepper variety PM815, lab number 4259 to do one more test about primers. Primer NP0055 will be kept to use in this test for comparing. Other 2 primers are primerNP0129 which can amplify female amplicons with length 207 bp and male amplicons length 192 bp. Primer NP0436 can produce female PCR products with length 120 bp and male PCR products length 138 bp.

4.6.1 Reaction volume and layout

The reaction volume is same as test 5. The layout is showed by table 11 below. From the result of Gel-electrophoresis (see Appendix 4 on page 49, gel pictures for pepper 4259), there is 1 inbred. Therefore, this inbred sample was taken into HRM inbred test and 8 replications were made. For the hybrid, different samples were used not replication of one sample.

Pepper 4259, Primer NP0055, NP0219 or NP0436, Eva Green												
	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
B	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
E	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
F	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
G	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
H	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid

Table 11, layout of primer and inbred test

4.6.2 Results

- Results from Melt Curve

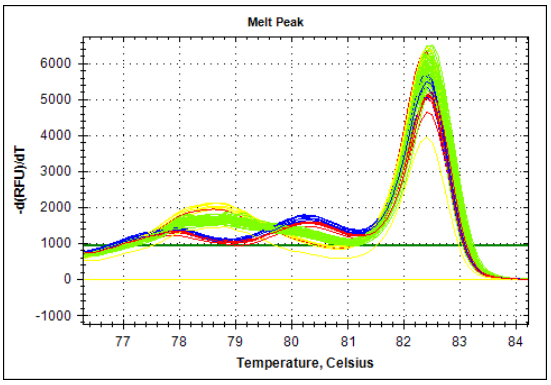


Figure 19, Pepper 4259, primer NP0055

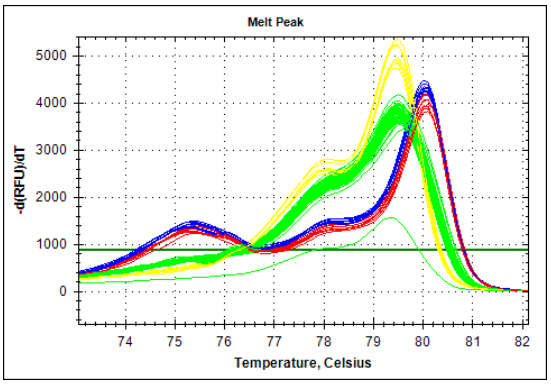


Figure 20, Pepper 4259, primer NP0219

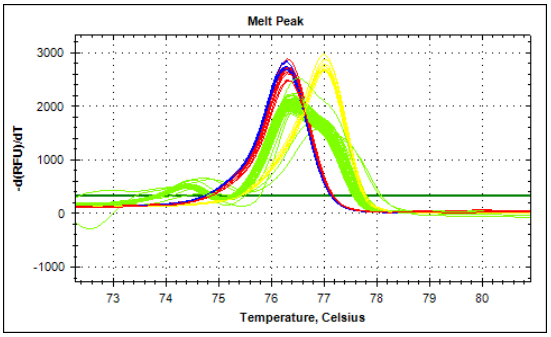


Figure 21, Pepper 4259, primer NP0017

The pictures above show the results of HRM pepper 4259 by using different primers. The red lines are female DNA, yellow lines are male, green lines are hybrid and blue ones are inbred. Figure 19 shows the outcome by using primer NP0055. Figure 20 shows the result by using primer NP0219. Figure 21 shows the result by using primer NP0017.

Result 1: from figure 19, there is no big difference for 4 colored curves in melt peak and melt temperature. Figure 20 and figure 21 show differences in melt peak and melt temperature. This means that shorter amplicons have better reaction efficiency and result in a less complex melting pattern where it becomes easier to distinguish between the different sequences variants.

Result 2: 3 pictures show that the red curves and blue curves are totally coincident. It means that the curves of female and inbred plotted by HRM program are exactly same.

- Results from Precision Melt Analysis™ Software

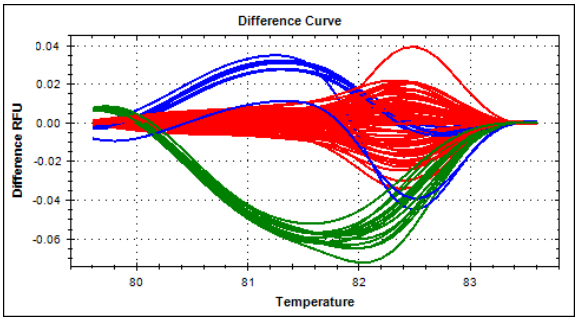


Figure 22, Pepper 4259, primer NP0055

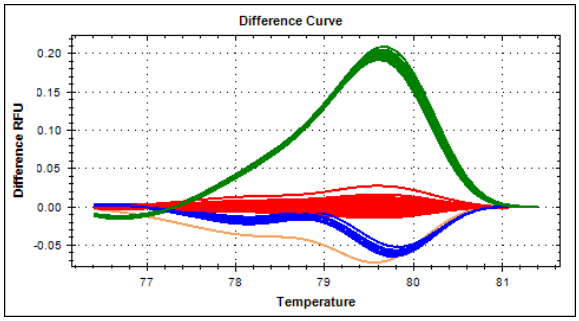


Figure 23, Pepper 4259, primer NP0219

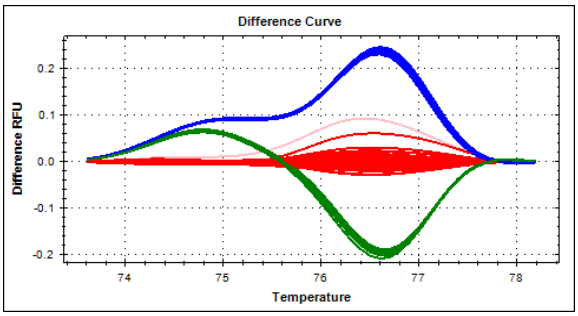


Figure 24, Pepper 4259, primer NP0436

With Precision Melt Analysis™ Software, we can see the difference better. As the figures show below, the green lines are female and inbred DNA, the red lines are hybrid and the blue lines are male DNA. The software works based on the data file of HRM. Consequently, better result from HRM performs better under the software. By comparing 3 pictures, figure 23 and figure 24 give more clear results than figure 22 does.

Result 1, the primer produced shorter amplicons works better in PCR and result in clear discrimination in HRM and Precision Melt Analysis™ Software. Result 2, like the result from HRM Female DNA curves and inbred curves overlap by showing with green curves from pictures.

4.6.3 Conclusion and continuance

Both software shows that female curves and male curves are coincident with each other. We can consider that inbred DNA is performance same with female DNA on HRM. On the other hand, the test using primer NP0219 and NP0436 which produce shorter amplicons than NP0055 does show larger difference among 4 types of DNA.

Next to do:

Previous tests show that with the usage of Precision Melt Analysis™ Software, HRM program indeed performs well in inbred test for Pepper. We are going to move the test to Melon to find out if HRM also works for other varieties.

4.7 HRM test for Melon

4.7.1 Reaction volume and layout

From previous tests for Pepper, we get very positive results. So we want to switch to Melon to see if the new technique also works for Melon.

Firstly, an inbred test on Gel-Electrophoresis for Melon, variety ML675 was done to find out the inbred from Gel pictures (please turn to Appendix 5 on page 50, gel pictures for Melon ML675). Afterwards, HRM will be used to test again to see if inbreds can find as well. The layout is showed by table 12. The reaction component and volume are same as Pepper.

3 Primers also be chosen to test if there is any influence on Melon. Primer SE0222 can amplify female DNA amplicons with 228 base pair and male DNA with 211 base pair. Primer SE0032 can amplify female DNA amplicons with 166 base pair and male DNA with 189 base pair. Primer SE0426 can amplify female DNA amplicons length with 109 base pair and male DNA with 95 base pair.

Pepper 4259, Primer SE0222, SE0032 or SE0426, Eva Green												
	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	hybrid	hybrid	inbred	hybrid	hybrid	hybrid	hybrid	inbred	hybrid	water
B	female	male	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	female	male	hybrid	hybrid	hybrid	inbred	hybrid	hybrid	hybrid	inbred	hybrid	hybrid
E	female	male	hybrid	hybrid	hybrid	hybrid	inbred	hybrid	hybrid	hybrid	hybrid	hybrid
F	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
G	female	male	hybrid	inbred	hybrid	hybrid	hybrid	hybrid	inbred	inbred	inbred	hybrid
H	female	male	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	inbred	hybrid	hybrid

Table 12, HRM test for Melon

4.7.2 Results

- Results from Melt Curve

The picture below shows the result from Melting Curve. The red curves stand for female DNA, the blue curves stand for inbred, the yellow curves stand for male DNA and the green ones are hybrid DNA.

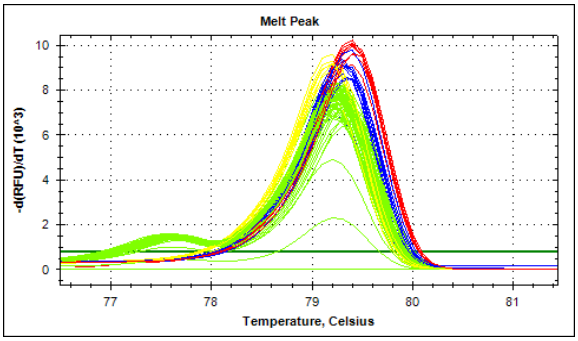


Figure 25, ML675, primer SE0222

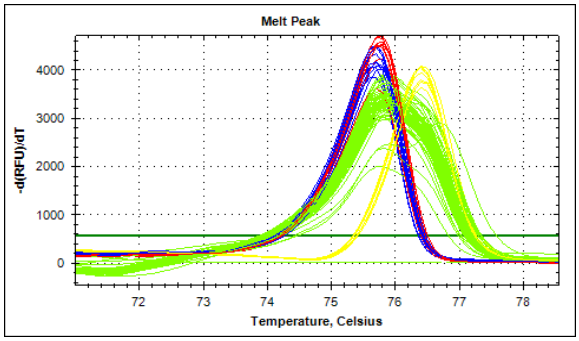


Figure 26, ML675, primer SE0032

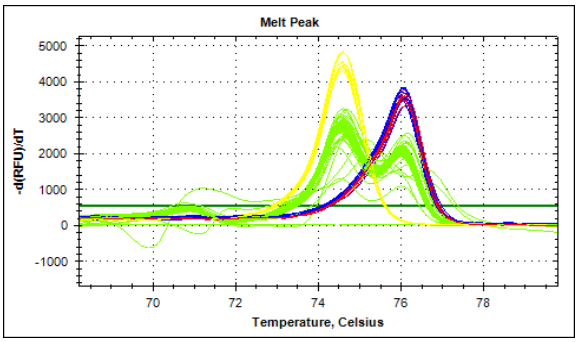


Figure 27, ML675, primer SE0426

Figure 26 give more clear discrimination for 4 types of DNA, whereas figure 27 is more clear than figure 26. In figure 27, the melt peaks apart from each other since their melt temperatures quite differ from each other except female and inbred DNA, they have same shapes of melt curves and same melt temperature.

- Results from Precision Melt Analysis™ Software

With Precision Melt Analysis™ Software, we can see the difference better. The green curves are female and inbred DNA, the red curves are hybrid and the blue curves are male DNA. There is also a orange line which is unknown type. Interpreting the curve-shape we can be sure that this is not a inbred.

Same as the software works on data file of HRM from Pepper, The primer produced shorter amplicons works better in PCR and result in clear discrimination in HRM and Precision Melt Analysis™ Software. Figure 30, ML675, primer SE0426 shows high clustered curves clearly.

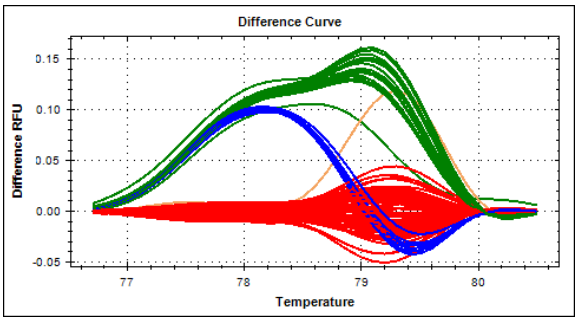


Figure 28, ML675, primer SE0222

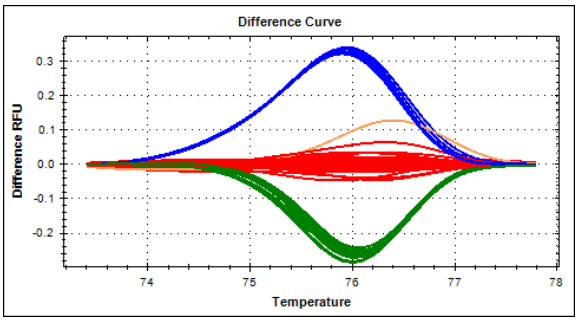


Figure 29, ML675, primer SE0032

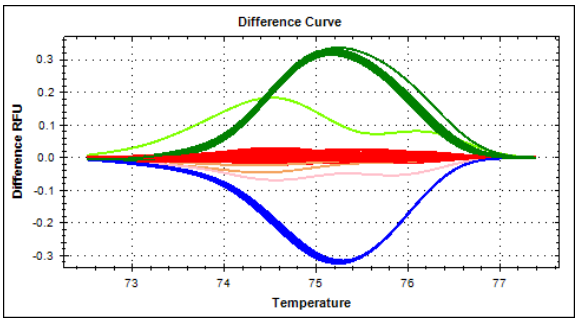


Figure 30, ML675, primer SE0426

4.7.3 Conclusion and continuance

The results of HRM test for Melon is as good as Pepper. With the usage of the software, 4 types of DNA can be discriminated like what we expected.

Next to do:

HRM test for Squash?

4.8 HRM test for Squash

4.8.1 Reaction volume and layout

For Squash inbred test, there 2 PCR program, PMm and ZMm (Please turn to Appendix 6 on page 51, PCR program for Squash, PMm and Appendix 7 on page 51, PCR program for Squash, ZMm). The test material is Squash 4046. Reaction components are showed by table 12, PCR reaction component of PMm and table 13, PCR reaction component of ZMm.

PMm PCR	
PMm-reaction	8.49 µl
ROG 308	0.11 µl
ROG 312	0.11 µl
ROG 320	0.11 µl
Taq	0.17 µl
Sample DNA	2.0 µl
Total	11.0 µl

Table 12

ZMm PCR	
ZMm-reaction	7.75 µl
HiNK 1496	0.23 µl
HiNK 1515	0.23 µl
HiNK 1639	0.23 µl
HiNK 1540	0.23 µl
Taq	0.33 µl
Sample DNA	2.0 µl
Total	11.0 µl

Table 13

From gel picture (please see Appendix 8 on page 52, gel pictures for Squash 4046), there are 3 inbreds in DNA plate 2. Therefore, these 3 inbred samples were taken into HRM inbred test and 8 replications were made. For the hybrid, different samples were used not replication of one sample. The plate layout for Squash HRM test is showed by table 14 below.

Squash 4046, Primer PMm or ZMm												
	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	hybrid	hybrid	inbred	hybrid						
B	female	male	hybrid	hybrid	hybrid	hybrid						
C	female	male	hybrid	hybrid	hybrid	hybrid						
D	female	male	hybrid	hybrid	hybrid	hybrid						
E	female	male	hybrid	hybrid	hybrid	hybrid						
F	female	male	hybrid	hybrid	hybrid	inbred						
G	female	male	hybrid	hybrid	hybrid	hybrid						
H	female	male	inbred	hybrid	hybrid	hybrid						

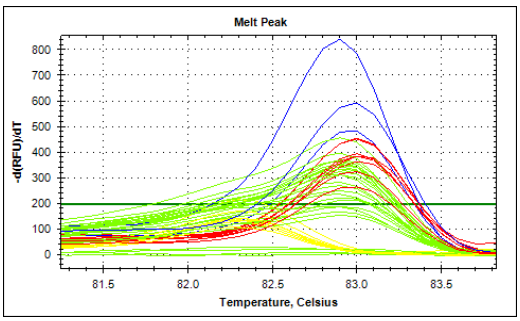
Table 14, HRM test for Melon

4.8.2 Results

- Results of PMm

Figure 31 and 32 show the results of Squash HRM test by using primer PMm. Figure 31 shows the outcomes of HRM program, red curves stand for female DNA, yellow curves are male DNA, green curves are hybrid DNA and the 3 blue curves are inbreds. 4 types of DNA give different curves. Especially, the result is different from precious tests that inbred DNA gives exactly same curves with female DNA. Therefore, identification of samples' genotype becomes complex.

Figure 32 shows the outcomes by Precision Melt Analysis™ Software. Green curves are female and inbred. Blue curves are hybrid and red stand for hybrid. Female and inbred DNA gives exactly same curves by software.



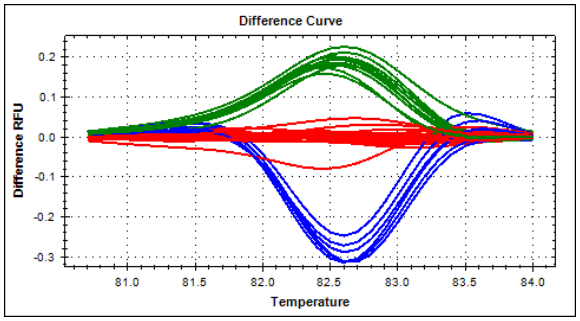
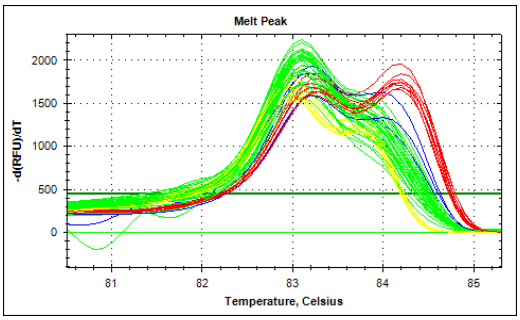


Figure 31, results from Melt Curve, PMm **Figure 32, from Software, PMm**

- Results of ZMm



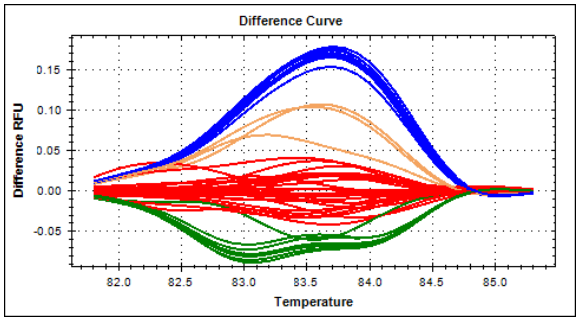


Figure 33, results of Melt Curve, ZMm **Figure 34, from Software, ZMm**

Figure 33 and 34 above show the results of Squash HRM test by using primer ZMm. Figure 33 shows the outcomes of HRM program, red curves stand for female DNA, yellow curves are male DNA, green curves are hybrid DNA and the 3 blue curves are inbreds. 4 types of DNA give different curves. Same as the result of using primer ZMm, female DNA and inbred DNA give different curves.

Figure 32 shows the outcomes by Precision Melt Analysis™ Software. Blue curves are female DNA. Green curves are male, red cuves are hybrid and the 3 orange curves stand for inbred. Although female and inbred DNA gives different curves by software, difference can be discriminated.

4.8.3 Conclusion and continuance

From the rest for Squash 4046 with PMm and ZMm PCR program on HRM machine, the discrimination can be defined by Precision Melt Analysis™ Software. One thing we should pay more attention when we apply this technique into further Quality Control test is that the result for Squash HRM differs from Pepper and Melon. The HRM for Squash does not show same curves for female and male DNA.

Next to do: Resolution test

To capture every slight curves' changes, the previous tests were used the setting which increase temperature from 65 ℃ to 95 ℃ by heating 0.1 ℃/s continuously for 2 and half hours. In the real QC test, it takes too much time. The next test, the program will be run to increase temperature from 65 ℃ to 95 ℃ by heating 0.3 ℃/s and 0.5 ℃/s to see if the DNA types still can be discriminated.

4.9 resolution test

The purpose for this test is to to find out if the DNA types still can be discriminated by running the program to increase temperature from 65 ℃ to 95 ℃ by heating 0.1 ℃/s, 0.3 ℃/s and 0.5 ℃/s respectively.

4.9.1 Reaction volume and layout

The test material is Squash 4046, PMm, the reaction component and PCR program are same as test 4.8. The HRM program shows in Appendix 2, but with increment 0.1 ℃/s, 0.3 ℃/s and 0.5 ℃/s respectively. The layout is showed by table 15 below.

There is 1 female and 1 male DNA samples. There are 3 inbreds which are determined from Gel pictures. For the rest are hybrids DNA samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	female	male	hybrid	hybrid	hybrid	hybrid	inbred	hybrid	hybrid	hybrid	hybrid	hybrid
B	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
C	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
D	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
E	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
F	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	inbred
G	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid
H	hybrid	inbred	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid	hybrid

Table 15, Resolution test for Squash, PMm

4.9.2 Results

- Results from Melt Curve

The figures below show the results from Melt Curve. red curve stands for female DNA, yellow curve (not obvious from the figures) is male DNA, green curves are hybrid DNA and the 3 blue curves are inbreds.

The figures also show the differences by applying 3 HRM programs. Figure 37 gives most smooth curves. Figure 35 shows less smooth curves since the temperature was increased slowest with 0.1 ℃/s, with the changes of temperate, every tiny changes were appeared.

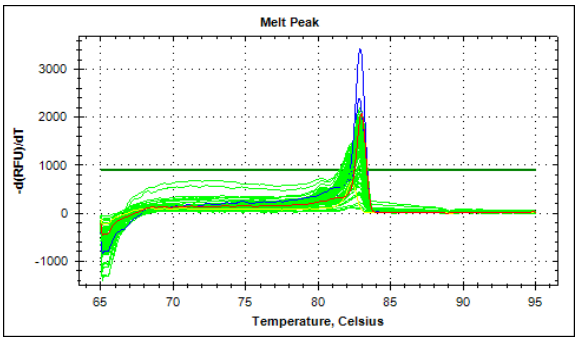


Figure 35, results from Melt Curve, 0.1 ℃/s

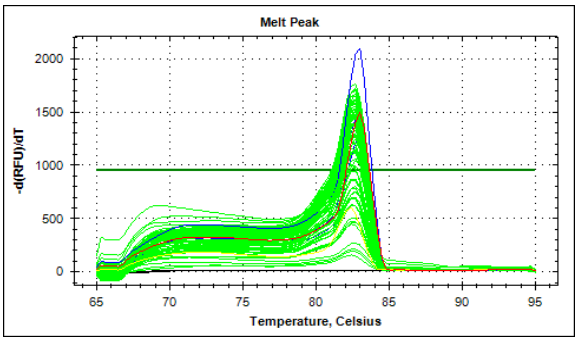


Figure 36, results from Melt Curve, 0.3 ℃/s

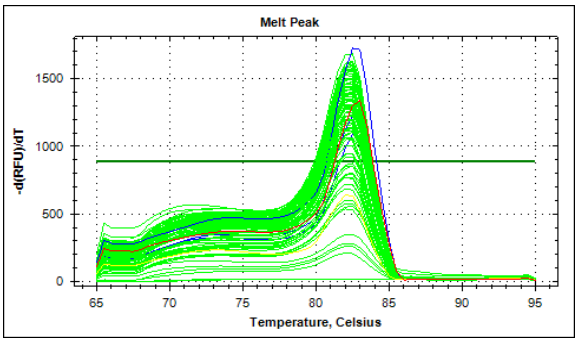


Figure 37, results from Melt Curve, 0.5 ℃/s

- Results from Precision Melt Analysis™ Software

Figures below show the outcomes by Precision Melt Analysis™ Software. In figure 38, Green curves are female and inbred. Red curves are plotted by hybrid DNAs. The male DNA is not showed. An orange curve is unknown DNA type. In figure 39, 4 green curves are plotted by 1 female and 3 inbred DNAs. A blue curve stands for male DNA. Red curves are hybrid DNA. Figure 40 shows exactly same results with figure 39 from the software.

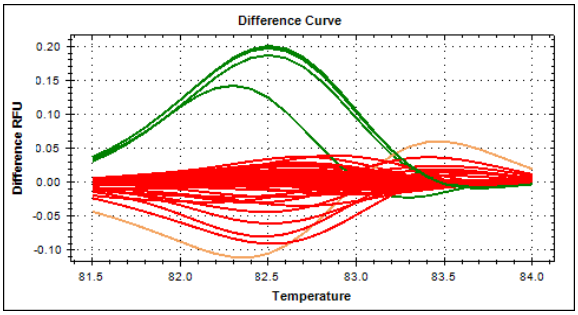


Figure 38, from Software, 0.1 ℃/s

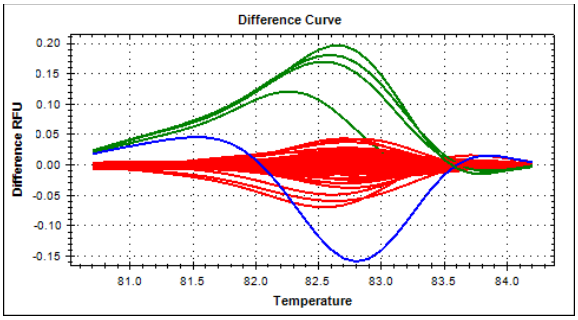


Figure 39, from Software, 0.3 ℃/s

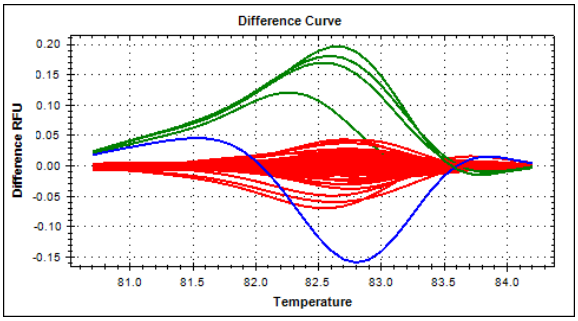


Figure 40, from Software, 0.5 ℃/s

4.9.3 Conclusion

The program with higher temperature increment variable (0.5 ℃/s) do not show more detailed changes on curves than less temperature increment (0.1 ℃/s) variable. With the Precision Melt Analysis™ Software, differences can be determined.

5 Conclusion

It is proved that HRM can be used for inbred test from the previous tests. It is time efficient, environment friendly and results accurate. Additionally, it reduces laborious risk on contamination. Since HRM analysis requires no manual post-PCR processing, is performed in a closed-tube system—no need to handle samples after loading. Workers can also avoid dealing with a carcinogen dye, Ethidium Bromide. Moreover, it has a low reaction cost relative to other methods used to study genetic variation.

For successful analysis, care should be taken in assay development to ensure that the experiment is optimized for HRM. Small differences in melt curves can be caused by DNA quality, primers (amplicons length), dye selection and PCR reagent choice. The following sections clarify these factors based on previous tests.

5.1. Reaction components and volume

By comparing with the current protocol of Syngenta genetic purity lab and the protocol of Bio-Rad Laboratories, the previous test 3 and test 4 indicate that the lab current protocol performs better.

5.2. Layout

The plate for HRM program is 96 wells. Therefore, 1 female DNA and 1 male DNA should be added in for positive control and one well for water added for negative control. The rest are unknown samples for inbred test.

5.3. HRM dyes selection

HRM analysis uses double-stranded DNA- binding dyes that, at relatively high concentrations, do not inhibit PCR. The amount of fluorescent dye bound to double-stranded DNA can vary with amplicon length, composition, and PCR conditions.

There are multiple HRM dye products available. By comparing with SYBR Green I and Eva Green in inbred test in usage together with the current lab protocol, Eva Green shows less PCR inhibitory and gives stronger signals. It can be an alternative choice.

5.4. Primer (amplicon length)

Amplicon lengths of 100–300 bp are generally recommended for HRM analysis of different sequence variants. Unless there is a reason to amplify longer targets, choose a product length preferably less than 200 bp. shorter products amplify with higher efficiency.

5.5. Ideal resolution

The ideal resolution means the difference of male, female and hybrid DNA can be visualized efficiently. A processing for one cycle to increase temperature from 65 ℃ to 95 ℃ by heating 0.1 ℃/s continuously takes 2 and half hours to allow HRM machine capture every slight curves' changes. Whereas one running cycle by heating 0.5 ℃/s only takes half hours, but the tiny difference may not be captured. 0.3 ℃/s keeps balance of the high resolution and efficiency.

5.6. Instrument and Software

-HRM Analysis Instrumentation

HRM analysis requires a PCR thermal cycler and an instrument with optics capable of collecting the numerous fluorescent data points. It is best when one instrument can perform both functions. The tests described in this report were conducted by Bio-Rad CFX96™ Real-Time PCR System.

-HRM Analysis Software

Software for HRM analysis needs to be capable of handling the large amounts of data generated during an HRM experiment. Most HRM specific software use new algorithms and display plots which help discriminate DNA variants in HRM data. Precision Melt Analysis™ Software provided by Bio-Rad is one of the choice, it is a data analysis method based on the PCR-melt data file by first, normalize melt curves. Second, apply an optional temperature shift. Third, plot curves in a difference graph for easy visualization. Forth, clusters curves into groups representing different genotypes/sequences.

6. Recommendation

Based on the previous test, the High Resolution Melting Analysis can be used for inbred test of vegetable seeds, especially for Pepper, Melon and Squash. Reaction components, reaction volumes, dye selection, primer chose and resolution setting are tested and compared. Besides, there is one problem with the melting results. There are sometimes strange curves, which should be hybrid samples. But it could not be found the same results by HRM Analysis. These could be off-types, but it is not been proved. Another problem which was found from the tests is that there are few samples dropped out during HRM program. This might because of the DNA samples quality or the problem with plate seals.

It is recommended that genetic purity lab can work on these two problems in the further research. They also can repeat once more the tests to see if they get same results. Afterwards, they can start on purchasing the HRM program and the software for data file analysis.

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2. http://www.gene-quantification.de/ab-hrm-guide.pdf, HRM guide. Accessed in 2nd of March, 2010
3. http://www.gencompare.com/qpcr.htm, Eva Green dye. Accessed in on 3rd of March, 2010
4. http://www.gene-quantification.de/hrm.html, 3rd of March, 2010

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10. http://en.wikipedia.org/wiki/Taq_polymerase.

11. http://en.wikipedia.org/wiki/Polymerase_chain_reaction

12. http://en.wikipedia.org/wiki/Primer_(molecular_biology)

13. http://hrm.gene-quantification.info/

14. http://en.wikipedia.org/wiki/Base_pair

15. http://en.wikipedia.org/wiki/Amplicons

16. http://en.wikipedia.org/wiki/High_Resolution_Melt

17. http://en.wikipedia.org/wiki/SYBR_Green_I

18. http://www.gencompare.com/qpcr.htm

19. http://en.wikipedia.org/wiki/Agarose_gel_electrophoresis

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21. DNA Fingerprinting in Plants, Principles, methods, and Application, Second Edition. By Kurt Weising, Hilde Nybom, Kirsten Wolff, and Gunter Kahl.

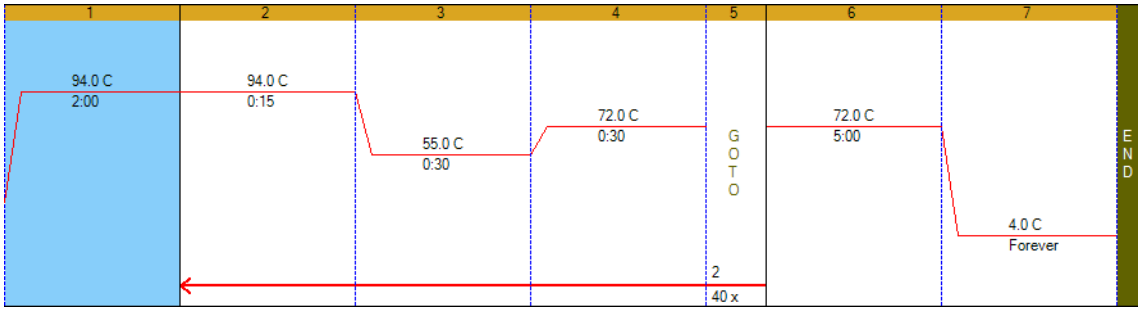
Appendix

Appendix 1, information list of chemicals and materials

INFORMATION CHEMICAL list

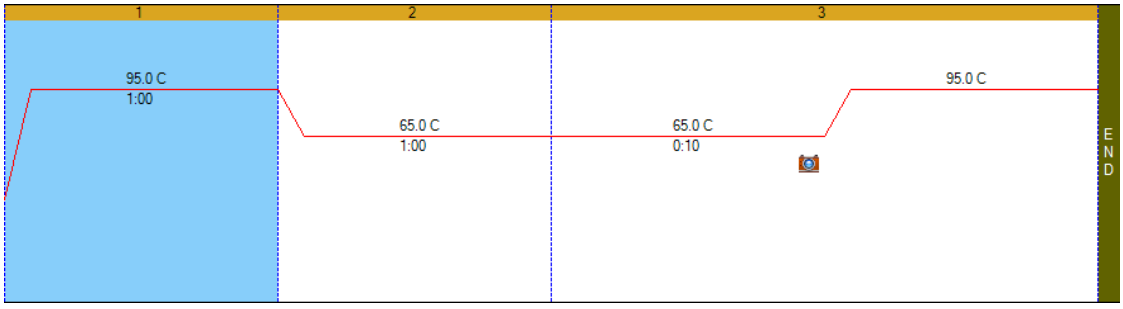
Item	Process	Supplier	Name @ Supplier	Cat.number
Cresol Red Sodium Salt	PCR	Sigma	Cresol Red	C-9877
dNTP's PCR grade 4x 250	PCR	Invitrogen	100 mM dNTP Set (4X 250 umol)	10297-117
Ethidium Bromide	PCR	Sigma	Ethidium Bromide liquid 10 mg/ml	E 1510-10ml
KB+ DNA Size standard 1000ul (0.1 ug/ul)	PCR	Invitrogen	1 Kb plus DNA size standard	10787-026
Primer non-labeled 50 nmol large	PCR	Invitrogen	Price per bp	geen
Taq-boxes (TAq + Mg2+ buffer) 1500U	PCR	Sigma	JumpStart Taq DNA Polymerase	D6558-1.5KU
Agarose UltraPURE	divers	Invitrogen	Life technologies	15510027
TBE Buffer 10*	divers	Invitrogen	Life technologies, 10* TBE Buffer	15581-028
Bags for disposal	PCR	Baas business market	LDPE zakken transp. 18/4 * 35 cm 0,02mm	122635
Tubes 4 ml	PCR	Greiner	Cellstar met speciaalstop 12-75 5 ml	115262
Deepwell plates 0.8 ml	PCR	VWR	Abgene PCR plates	ADVAAB-0765
Comb 67-wells	PCR	Sigma aldrich	Combs 67-wells	Z340960
Cover-seal 384-plate	PCR	VWR	Adhesive pcr film	ADVASP-0027
PCR plates 384-wells Bioké Red	PCR	Bioké	FrameStar 384 (TF-0384), 50 plates, red frame	4Ti-0384/R-BC
Pipettips (finntip 384-format 20 ul, purple box)	PCR	VWR	Tip, Pipette, 0,2-20 ul, Finntip 20	613-2604
Simport PCR foil	PCR	VWR	Secureseal Therm. Adhesive Sealing film	SIMPT329-1
Rainin LTS SR-L1200 , 768 pcs	divers	Mettler-Toledo	Rainin LTS SR-L1200 , 768 pcs	17007086
LTS Pipettip, <250 µl 960 tips in 10 racks FILTER	divers	Mettler-Toledo	LTS Pipettip, <250 µl 960 tips in 10 racks FILTER	17002927
LTS Pipettip, <20 µl, 960 tips in 10 racks FILTER	divers	Mettler-Toledo	LTS Pipettip, <20 µl, 960 tips in 10 racks FILTER	17002928
Tape to close trays 50m x 25 mm	divers	Govers	Schilderstape	516612F
Epjes 1.5 ml Eppendorf	divers	VWR	Eppendorf	EPPE0030120.086

Appendix 2, Set up of PCR



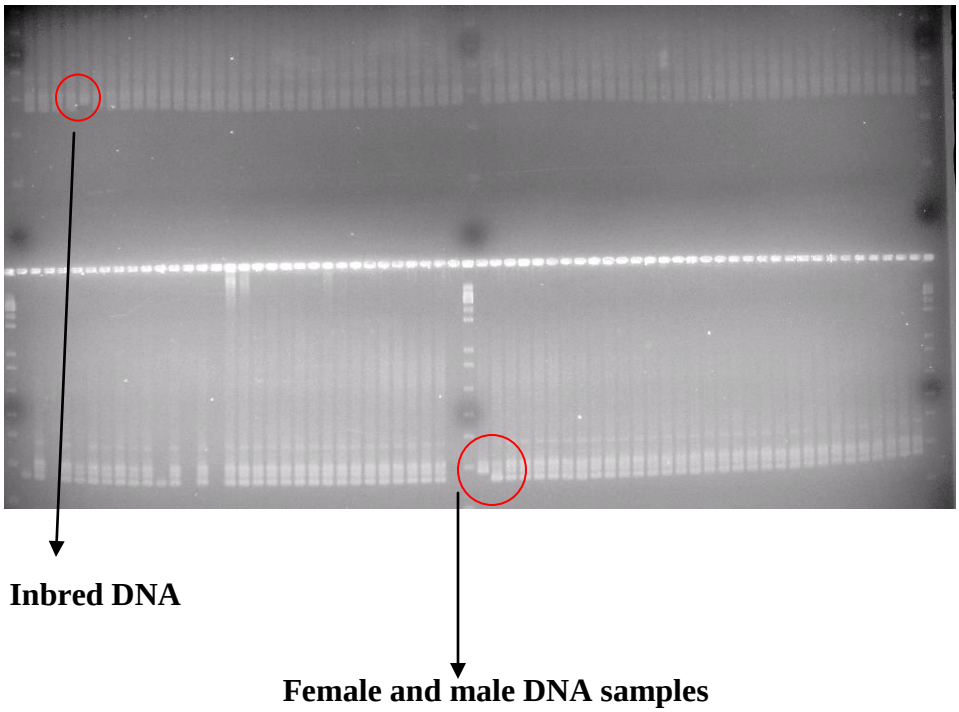
Step 1, 94.0 ℃ for 2:00
Step 2, 94.0 ℃ for 0:15
Step 3, 55.0 ℃ for 0:30
Step 4, 72.0 ℃ for 0:30
Step 5, GOTO 2, 40 more times
Step 6, 72.0 ℃ for 5:00
Step 7, 4.0 ℃ Forever

Appendix 3, Set up of HRM



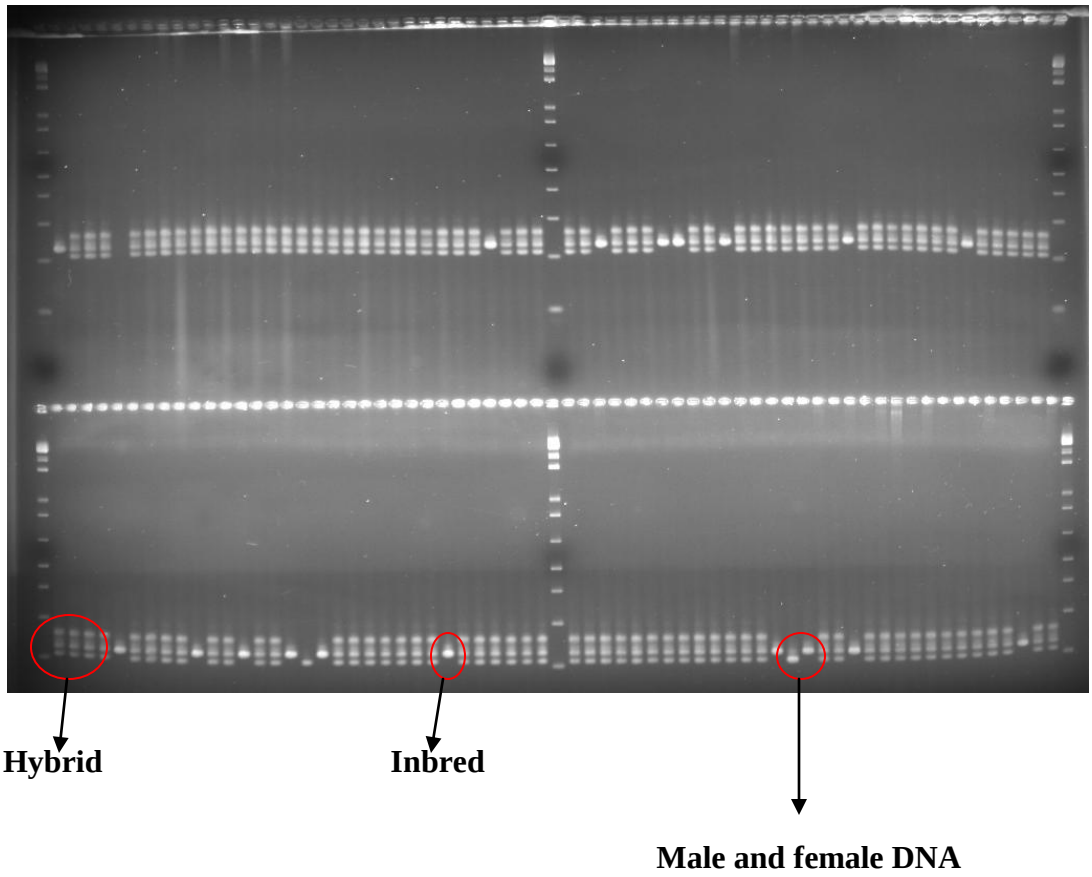
Step 1, 95.0 ℃ for 1:00
Step 2, 65.0 ℃ for 1:00
Step 3, Melt ℃ curve 65.0 to 95.0 ℃, increment 0.1 ℃, 0:10 + Plate Read

Appendix 4, Gel pictures for pepper 4259



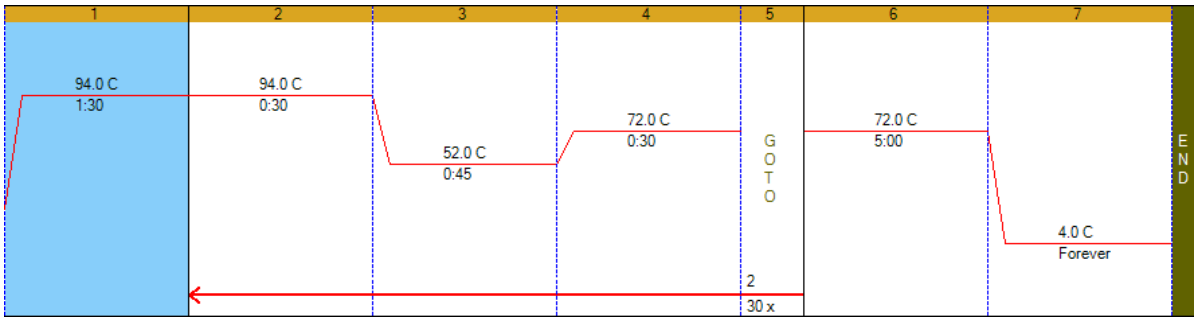
The picture shows the results from gel-electrophoresis for Pepper 4259. By comparing with female can male DNA samples, it is can be found that there is 1 inbred sample in this picture. It is same as female DNA without male band.

Appendix 5, Gel pictures for Melon ML675



The picture shows the results from gel-electrophoresis for Melon ML 675. By comparing with female can male DNA samples, it is can be found that the hybrid includes both female and male bands. The inbred only has 1 band.

Appendix 6, PCR program for Squash, PMm



Step 1, 94.0 C for 1:30

Step 2, 94.0 C for 0:30

Step 3, 52.0 C for 0:45

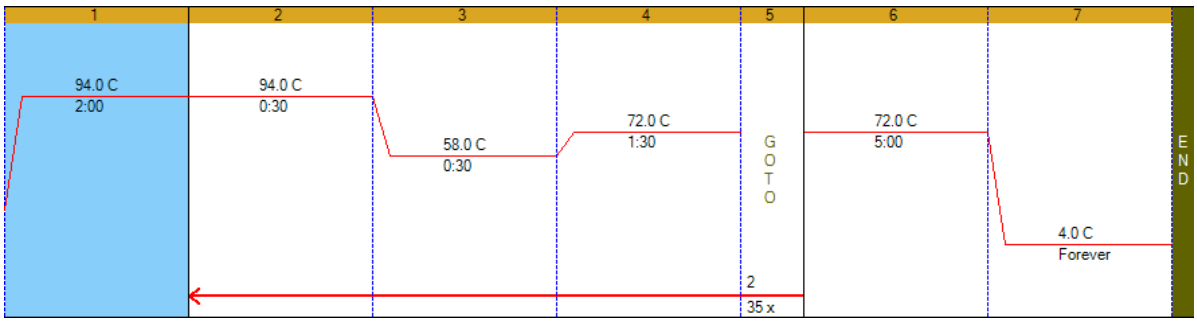
Step 4, 72.0 C for 0:30

Step 5, GOTO 2, 30 more times

Step 6, 72.0 C for 5:00

Step 7, 4.0 C Forever

Appendix 7, PCR program for Squash, ZMm



Step 1, 94.0 C for 2:00

Step 2, 94.0 C for 0:30

Step 3, 58.0 C for 0:30

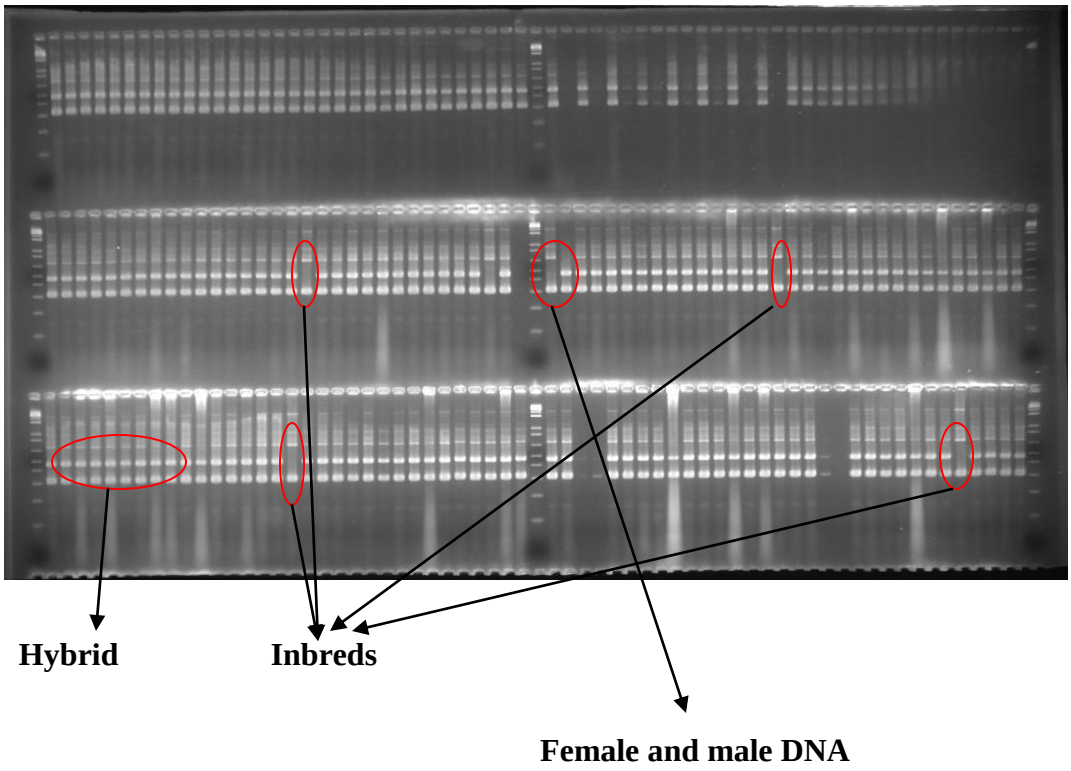
Step 4, 72.0 C for 1:30

Step 5, GOTO 2, 35 more times

Step 6, 72.0 C for 5:00

Step 7, 4.0 C Forever

Appendix 8, Gel pictures for Squash 4046



The picture shows the results from gel-electrophoresis for Squash 4046. By comparing with female can male DNA samples, it is can be found that the hybrid includes both female and male bands. The inbred only has 1 band. There 4 inbreds in total from the picture.