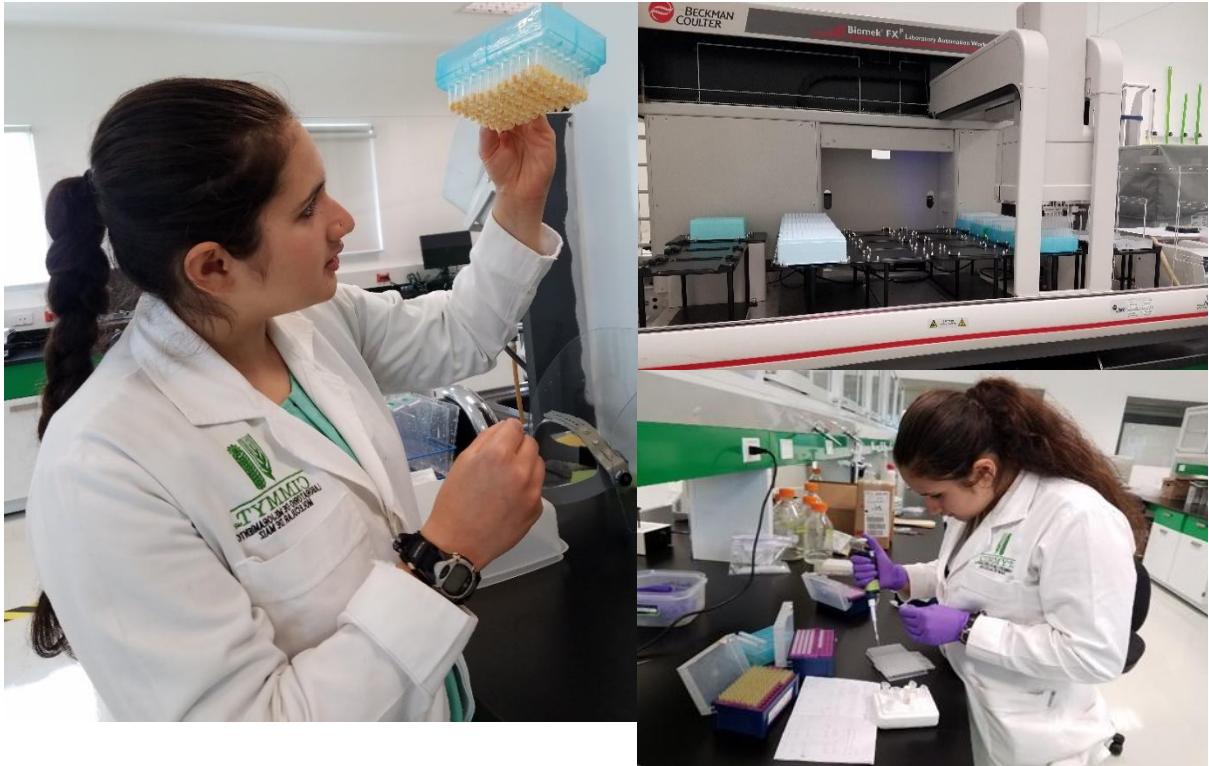


Expediting the Breeding Process for Increasing Provitamin A in *Zea mays*: Advancing a Semi-Automated Crude DNA Extraction Protocol



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I would like to extend a final thank you to anyone I have not mentioned directly but have played a part in making my internship an experience I will never forget.

Personal Introduction

Growing up in cities and suburbs, I was not always very aware of the importance of agriculture, or even what it was. I thought I would never be interested in agriculture because I had only stepped foot on a farm less than a dozen times in my life. I was drawn into agricultural community when I joined the Regional Agricultural Science and Technology program at Glastonbury High School because I was interested in animals. Being an "Ag Student", as we are called, opened my eyes to the complex field that is agriculture. What I once thought as just farmers and ranchers now included farmers, florists, dog breeders, park rangers, researchers, teachers, extension workers and so many more. I began to find my place in this diverse community, and felt tremendous pride in being a part of the ag-science program. It set me apart from many students in high school, but in a positive way. On the other hand, it also introduced me to a motivated community of likeminded students and allowed me to become a member of the National FFA Organization (FFA), one of the largest student organizations in the nation. No matter where I go, the FFA is like a family. I have struck up conversations and connections with absolute strangers just by wearing my FFA t-shirt or official dress. The FFA has also offered me countless opportunities from developing my leadership and public speaking skills to attending state and national conventions.

The ag-science program is partially responsible for my introduction to The World Food Prize and the Global Youth Institute. Every student in the program is required to complete a supervised agricultural education project, which allows us to combine our classroom instruction and leadership education to further our interests and experiences in agriculture. As I was looking for a new SAE, my father came across some information on the Global Youth Institute. After a bit of exploring I became very excited to possibly participate in this wonderful opportunity. As I spent my summer researching and writing my paper, I had no idea that in just a few months, the World Food Prize would inspire me to change the trajectory of my life.

I have always been aware of the issue of food security, at least on a personal and domestic level. After knowing that one in five kids in the United States suffers from food insecurity, I embarked on a campaign to raise awareness in my school about the issue and promote opportunities to fight back against food insecurity. I volunteered personally and worked with my FFA chapter and other organizations to organize fundraising and food packaging events. It was my participation in the Global Youth Institute that broadened my focus to food security on a global level. Attending the 2015 World Food Prize Global Youth Institute provided me an opportunity to learn from world leaders and agricultural scientists. Before writing my research paper last summer, I knew that food insecurity existed in many parts of the world, but it was at the symposium where I learned how complex and compelling the issue is. After hearing from many distinguished speakers and former Borlaug-Ruan interns, I became motivated to play my part in ending food insecurity through scientific research and discovery.

CIMMYT: Turning Research into Impact

The International Maize and Wheat Improvement Center (CIMMYT) was formally established in 1966. It developed from a program to increase farm productivity in Mexico established in the 1940s with funding from the Government of Mexico and the Rockefeller Foundation. It was in this program that renowned wheat breeder Dr. Norman Borlaug and Mexican scientist and farmers worked to develop the improved varieties of wheat that would eventually lead to the “Green Revolution.” For fifty years, CIMMYT has been conducting research for improved maize and wheat varieties, developing better agricultural practices, and training thousands of scientists and breeders. CIMMYT works with various non-government organizations, national research institutes, other CGIAR centers, and farmers to transform research into large-scale farm level impacts. With projects and offices in over forty countries around the world, the work of CIMMYT impacts millions of lives and benefits those in the most disadvantaged groups (CIMMYT. n.d.).

CIMMYT is also a member of the Consultative Group on International Agricultural Research (CGIAR), a consortium of non-profit, agricultural research centers across the globe. CGIAR centers work closely with each other and various private and public organizations to conduct research to reduce food insecurity, improve human lives through nutrition and health, and promote sustainability.

The Global Maize Program

The Global Maize program, as its name suggests, works to develop improved maize varieties with increased yields, resistance to environmental stresses, and higher micronutrient content. The research is focused on a wide variety of traits from drought tolerance to disease resistance to enhanced provitamin A and zinc concentrations in maize kernels. From using double haploid technology to developing hybrid plants, the work of the Global Maize program helps to provide improved seeds that benefit subsistence farmers in developing countries.

The main objectives of the program are to:

- "Develop genetically diverse, high-yielding maize germplasm, combining abiotic and biotic stress resilience, nutrient use efficiency, enhanced nutritional quality, and desirable seed production properties, for target production zones.
- Increase genetic gains and breeding efficiency through integrated application of modern tools and technologies
- Deploy CIMMYT-derived improved maize varieties in the tropics through strong public-private partnerships, and strengthen local maize seed systems
- Build local capacity, with particular focus on women and youth, in maize breeding and seed systems through formal and informal training and knowledge exchange”

(CIMMYT. n.d.)

HarvestPlus

While at CIMMYT, I worked in the Global Maize Program on a project originally funded by HarvestPlus. HarvestPlus began as the “biofortification challenge program” of CGIAR, which caught ground in 2002 (HarvestPlus, n.d.a). The goal of HarvestPlus is to fight “hidden hunger,”

a lack of vitamins and minerals in the diet, through the development and distribution of staple crops with higher micronutrient contents. This is accomplished through conventional breeding, in a process known as biofortification. HarvestPlus also works to provide global leadership on biofortification evidence and technology to increase the distribution and scale of biofortified crops so that they will be available to those in need (HarvestPlus. n.d.b).

Introduction to the Project

The idea of this project is to assist the goals of the Global Maize Program and Harvest Plus by developing a crude DNA extraction protocol that can decrease the time required to extract DNA from seed chips and analyze it using Molecular markers. Two very large constraints for breeding programs are time and funding. Crude DNA extraction is both faster and cheaper than existing DNA extraction methods. Our goal was to test and optimize crude DNA extraction protocol so that it could yield DNA of sufficient quality to function in a KASP (Kompetitive Allele Specific PCR) SNP assay that detects a favorable allele for provitamin A production in maize.

Abstract

Vitamin A deficiency is an issue that affects millions of people worldwide and often leads to blindness and even death in children. Maize is a staple crop in many developing countries where vitamin A deficiency is widespread. While most maize lines are low in provitamin A, the great genetic diversity of maize makes it possible to increase the provitamin A content through breeding. This would provide a long-term solution to vitamin A deficiency. To make the process of breeding for pro-vitamin A more efficient and cost-effective, we tested a crude DNA extraction protocol to determine whether it can yield DNA of sufficient quality to function in a KASP (Kompetitive Allele Specific PCR) SNP assay that detects a favorable allele for provitamin A production in maize. The original protocol worked very well for five of seven markers, and the modified semi-automated protocol with the Biomek FXP liquid handler worked well enough with two of the markers to be able to determine favorable homozygotes and heterozygotes. The amplification of the crude DNA was comparable to DNA extracted using the traditional CTAB preparation. This semi-automated crude DNA protocol would allow for many samples of maize endosperm to be extracted and screened for predicted provitamin A content using KASP markers quickly and efficiently. While the selected marker does not distinguish clearly between the heterozygotes and positive homozygotes, this method can still be used to eliminate negative homozygotes before planting.

Background

Vitamin A deficiency is an issue that affects millions of people, especially in developing countries in Africa and South-East Asia. Even though many people associate vitamin A with eyesight, vitamin A is also essential to growth and a healthy immune system. Vitamin A deficiency can cause vision loss, night blindness, total blindness, and stunted growth in children (Suwarno, Pixley, Palacios-Rojas, Kaeppler, & Babu, 2014). Up to half a million children each year lose their eyesight due to Vitamin A deficiency (Yan et al., 2010). A lack of vitamin A can also compromise the immune system, leading to a greater chance of death from disease. (Suwarno et al., 2014). Vitamin A either obtained from animal foods such as meat and dairy or produced in the body through processes that convert provitamin A into vitamin A. Provitamin A

can be obtained from a wide variety of plant foods, however, most staple crops contain very little to no provitamin A carotenoids (Wurtzel, Cuttriss, & Vallabhaneni, 2012).

Current options for alleviating vitamin A deficiency include supplementation and fortification of foods during processing. The ideal way to increase vitamin A intake is through a balanced and varied diet, unfortunately, this is not a possibility for people who live in poverty and lack access to foods high in various micronutrients. These people are often dependent on staple foods that are high in calories, easily obtained, and inexpensive, but also low in essential micronutrients. Although supplementation and fortification are working to reduce micronutrient deficiencies, these methods are not always efficient and not all people in remote areas have access to supplements or fortified foods. A more sustainable solution to this issue would be biofortification, or increasing the micronutrient content of staple crops through breeding (Babu, Rojas, Gao, Yan, & Pixley, 2013).

Maize is a staple crop in many regions where vitamin A deficiency is a serious issue, especially in Africa. The problem is that most elite corn lines have a very low provitamin A content because these lines were bred for other favorable traits (Zhang et al., 2012). Fortunately, there is also considerable genetic variation in corn plants for provitamin A content, which makes conventional breeding for increased provitamin A content possible. Some corn germplasm has been found to contain up to 36 ug of provitamin A carotenoids (Pixley et al., 2013). By crossing these varieties into current varieties, maize can be biofortified, a method that increases the nutrient content of staple crops. This would provide a long-term solution to vitamin A deficiency that is more cost-effective than conventional supplements.

Objectives

A challenge to biofortification programs is the time and cost of breeding and growing plants in the field to analyze provitamin A content within the kernel or the DNA of the plant. The purpose of this research project is to make the process of breeding for pro-vitamin A more efficient and cost-effective by developing a cheap and fast crude DNA extraction method that can be automated. This would allow for many samples of DNA from seed chips to be extracted relatively quickly and analyzed using KASP markers. Screening for predicted provitamin A content based on seed DNA would allow for preliminary selections to be made before time and resources are lost testing plants in the field. There are three sequential specific objectives of the project:

- The first objective of this project was to finalize a working protocol for crude DNA extraction using Sodium Hydroxide (NaOH) that will provide DNA of a high enough quality to be used with KASP SNP assays.
- The second objective was to adjust the protocol to function with the Biomek FXP Liquid Handler and design programs using the Biomek software for DNA extractions from 1, 2, 4, and 8, 96 tube plates.
- The third objective of this project was to determine which of the KASP SNP markers will function the best with the NaOH protocol using the Biomek FXP Liquid Handler and be suitable for large scale screenings for a variation of the crtRB1 gene related to provitamin A content.

Hypothesis

Our hypothesis is that a crude DNA extraction protocol using the Biomek *FX^P* can be developed to yield DNA of sufficient quality to function in a KASP (Kompetitive Allele Specific PCR) SNP assay that detects a favorable allele for a gene related to higher production of Provitamin A in maize. The quality of the DNA from the extractions can be determined by the dispersion of the data points in the graphs generated by data from the plate readings using the Pherastar Plate reader compared to the controls, which are DNA extracted using the CTAB prep. If the crude DNA is of sufficient quality, it is expected that the data points will segregate and form tight clusters around either the positive, negative, or heterozygous control, and that the generated graphs will look very similar to the graphs of DNA extracted using the CTAB prep.

Literature Review

Genetic Background

While there are many carotenoids in Maize, provitamin A carotenoids are beta-carotene, alpha-carotene, and beta-cryptoxanthin. Beta-carotene is considered to have the highest provitamin A activity, therefore, natural mutations that increase the beta-carotene content have been the focus of biofortification programs (Yan et al., 2010). The carotenoid pathway in maize plants has been well studied, and a variety of genes that control carotenoid content have been discovered. *LycE* and *crtRB1* are two genes that have been most closely associated with beta-carotene levels, (Suwarno et al., 2014). (Figure 1.a)

A mutation in the *lycE* gene increases the ratio of beta-carotene to alpha-carotene by apportioning more Lycopene into the beta-carotene branch of the carotenoid pathway. A mutation in the *crtRB1* gene leads to the reduced function of a protein that converts beta-carotene to beta-cryptoxanthin, which causes an accumulation of beta-carotene (Suwarno et al., 2014). Three polymorphisms were associated with the *crtRB1* gene that led to a greater beta-carotene concentration: 5'TE, InDel 4, and 3'TE (Figure 1b). 5'TE was determined to have the greatest effect on beta-carotene concentration (Yan et al., 2010). The focus of this study was on a set of seven KASP markers that identify polymorphism 5'TE.

Identification of the genes and alleles associated with provitamin A concentration makes it possible to implement Marker Assisted Selection (MAS) in a breeding program. Traditional breeding relies on phenotypic information to select plants for breeding and the best offspring in each generation. High Performance Liquid Chromatography (HPLC), which is used to determine provitamin A content, is costly and time consuming (Babu et al., 2013). This makes it especially unsuitable for major breeding programs. There are alternative methods to determine provitamin A content, however, they are not as precise as HPLC. This makes MAS a preferable method in large breeding programs, which are more efficient and cost effective, that require a more effective screening method for a certain trait (Zhang et al., 2012). HPLC will still be necessary to verify the final provitamin A content of lines, but MAS can be used to predict the provitamin A content when creating maize lines.

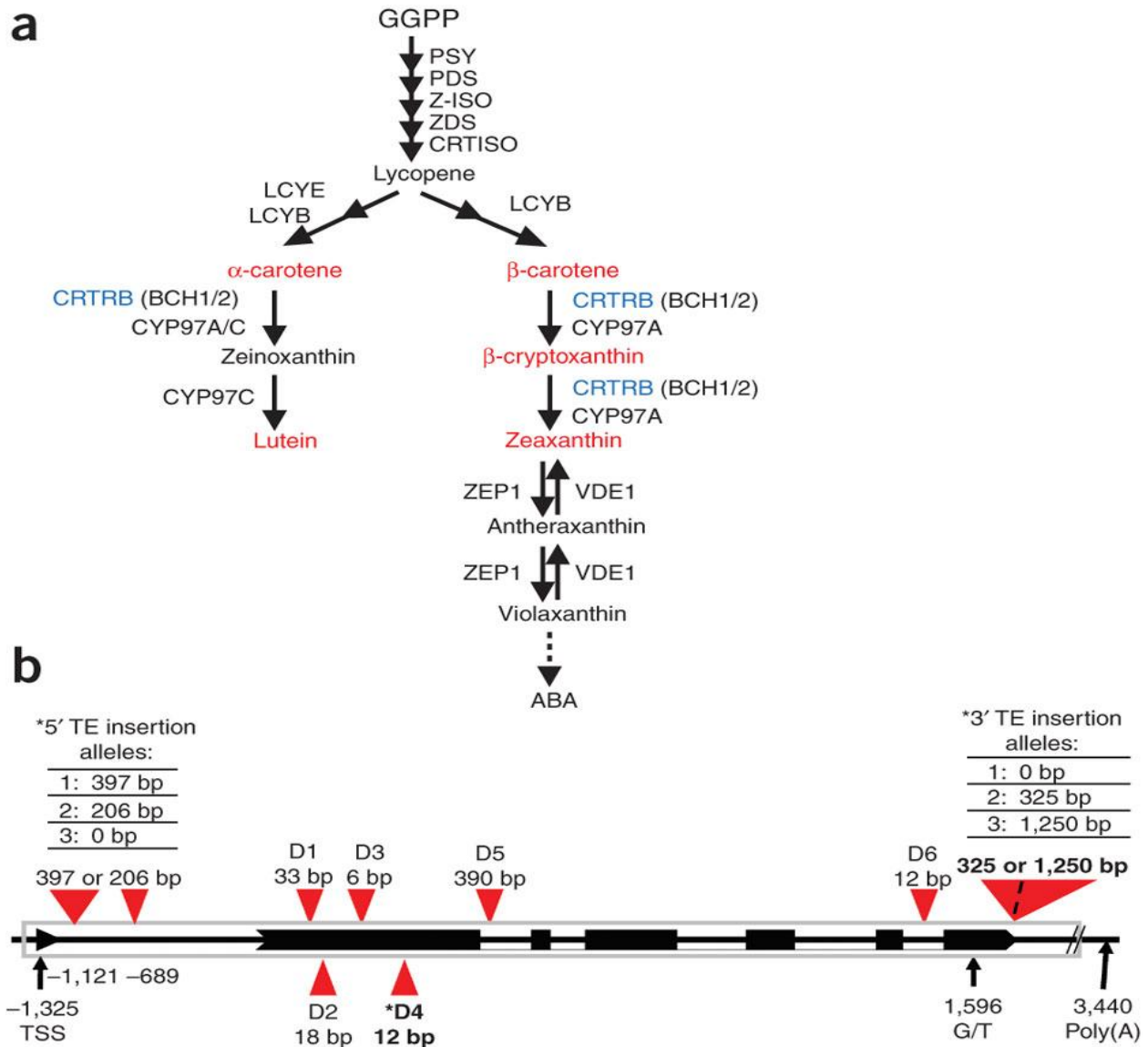


Figure 1: Carotenoid biosynthetic pathway and *Zea mays crtRB1* gene structure. (a) Simplified carotenoid biosynthetic pathway in maize and *Arabidopsis*5,6,12,13 (b) *Zea mays crtRB1* is the target gene in the present study. (adopted from Yan *et al.*, 2010)

Molecular Markers

There are a variety of molecular markers that can be utilized in a MAS breeding program to identify certain alleles. A majority of the traditional markers are Simple Sequence Repeats (SSR) markers, also known as microsatellites. These are sequences of two to five nucleotides that repeat anywhere from just a few to possibly fifty times in a row. SSRs are utilized as genetic markers because they often have many polymorphisms, such as insertions, and differences between the lengths of these sections can indicators of a specific allele (White, Adams, & Neale, 2007).

More recently, there has been a shift towards using Kompetitive Allele Specific PCR (KASP) assays for genotyping in MAS breeding programs. The focus of this study was utilizing KASP

assays along with the crude DNA extraction method. KASP assays are developed using Single Nucleotide Polymorphisms (SNP), a change in a single base pair in the DNA, as identifiers of a specific allele. In each KASP assay there are 2 different forward primers that are specific to one allele based on the SNP and one reverse primer. Depending on the allele and its SNP, only one of the primers will bind to the DNA and allow for transcription to occur during. These two primers are each bound to a different fluorescent dye, HEX or FAM, and when the primer binds to the DNA, the dye molecule is released and the fluorescence can be read by a microplate reader. In a heterozygous sample, both primers will bind to their respective alleles, and both fluorescent molecules will be released, which can also be read by a microplate reader (LGC Genomics, 2013).

Molecular markers are identified by amplifying the region of interest using the Polymerase Chain Reaction (PCR). PCR is a simple yet complex process that amplifies DNA using primers, nucleotide bases, and a DNA polymerase. There are three steps or stages to PCR: denaturing, annealing, and extension. Denaturing is when the DNA is separated into two separate strands by high heat, which breaks the hydrogen bonds that hold the two stands together. Annealing is when the temperature is reduced and the primer, a short length of single stranded DNA that is complementary to a specific region in the DNA, attaches to one strand of the DNA, allowing DNA polymerase to begin synthesizing the second strand. Each PCR requires a forward and a reverse primer to amplify a specific and relatively short region of DNA. The third step, extension, is the synthesis of the complementary strand of DNA by DNA polymerase. The most common polymerase is known as Taq polymerase and was used in this experiment (Integrated DNA Technologies, 2011).

In the first cycle of PCR, if there is only one molecule of double stranded DNA, there are only two templates for DNA synthesis. DNA synthesis is semi-conservative, meaning that one strand of each DNA replication is a template strand while the other is newly synthesized. With each subsequent cycle of PCR, the number of double stranded DNA molecules increases exponentially because each DNA molecule serves as a template for two new DNA molecules (Integrated DNA Technologies, 2011). PCR using SSR markers generally takes two to three hours, but PCR using KASP markers takes only two hours or less.

The products of PCR using SSR markers are analyzed using gel electrophoresis, which separates products by size because the smaller segments of DNA will move much faster through the gel. This is time consuming because it takes roughly two hours to run one gel, and the agarose gel must be made fresh. After the gel has been run, it must also be soaked in ethidium bromide or another dye so that the location of the DNA samples can be visualized by using an ultraviolet light. In comparison, reading one 384 well microplate using a microplate reader takes roughly three and a half minutes.

DNA Extractions

There are various DNA extraction protocols that can be used with leaves and seed chips, but the most common for genotyping at CIMMYT is the CTAB protocol. This DNA extraction method requires about two to three days from start to finish, including PCR and analysis on gel or with KASP assays. The chemicals involved in this extraction, such as chloroform and phenol, are also hazardous to human health, and extraction must be performed under a hood and with higher levels of personal safety equipment. The benefit of using the CTAB DNA extraction is that it yields a high concentration of quality DNA that can be used for multiple tests. While this may be

suitable for some purposes, this amount and quality is not necessary when screening seeds for a certain trait using only one or two efficient molecular markers (CIMMYT Biotechnology Lab, 2015).

Crude DNA extractions, on the other hand, produce a lower amount of DNA with more contaminants. However, these methods are usually much cheaper and safer than standard DNA extractions using chloroform and other relatively toxic chemicals. A crude DNA extraction can also be performed much more quickly than a CTAB DNA extraction, because the time for removing contaminants and drying and rehydrating the DNA is required. Decreasing the cost of materials and time for DNA extraction would be highly beneficial for MAS breeding programs that often screen thousands of seeds each season. A crude DNA extraction using a relatively harmless chemical such as sodium hydroxide would also lessen the risk to lab technicians.

Materials and Methods

Manual NaOH DNA Extraction

Before the start of this project, a functioning NaOH DNA extraction protocol had already been developed by the work of a past intern and Dr. Martha Hernandez. The original protocol used at the start of this project is as follows:

1. Place ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in a water bath
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 116 ul of water to each sample to reach 1:1 dilution. Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 2 ul of dilution for PCR.

*The full protocols for each test can be found in appendices A1-A8.

A total of 7 tests were performed by hand using the NaOH extraction protocol before moving on to tests using the Biomek FXP Liquid Handler. The seeds were sampled by using a dog nail clipper to slice off small chips of endosperm so as not to damage the embryos. The first test we performed by hand was to validate the original protocol using marker SNP-10_137904716. The DNA was then frozen and stored at -20°C until the next day, when it was defrosted and used in the second test to determine the quality of the extracted DNA when the extraction is not fresh. 116ul of water was also added to the extracted DNA to make a ratio of DNA preparation to water of 1:2. This was to test whether the DNA extraction could be increased to a volume that the Biomek can handle without excessively diluting the DNA. In the third test, we extracted DNA from a new set of seeds and diluted it to various ratios to test which combination of dilutions and volume of preparation taken for PCR would yield the best results. In the fourth through seventh tests, we continued to test various combinations of dilutions of the crude DNA preparation and volumes of DNA taken for PCR with all seven of the SNP markers for the crtRB1 gene.

The KASP mix setup was as follows for all except marker S10_136072513:

- 2.5 ul of 2x KASP reaction mix
- 2.73 ul of ddH₂O if DNA is dried, 0.73 ul if 2 ul of DNA was taken and not dried

- 0.07 ul of Assay mix
- For marker S10_136072513, 0.03 ul of 50 mM Mg₂Cl was added

The basic PCR protocol used for all markers except 10_134583972 was as follows:

- 95°C x 15 min
- 10 cycles of 95°C x 20 sec, 61°C x 1 min, decreasing -0.6°C per cycle until achieving 55°C
- 30 cycles of 95°C x 20 sec, 55°C x 1 min.
- For marker 10_134583972 the cycles were the same, however the temperature was 65 °C instead of 61 °C and 57 °C instead of 55 °C

After these tests, we compared the performance of each marker and determined the optimal dilution and volume of DNA for PCR for each marker. The Pherastar plate reader was used to determine the fluorescence of each sample after PCR with the KASP assays. We first analyzed the data produced from the readings using excel to get a general sense of the genotypes and then later used the KlusterCaller software to further analyze the data and assign genotypes to each sample based on the position of its data point. The general distance between the data points (clustering), the ability of the software to “call” the genotype of each data point, the amplification of each sample and the non-template controls (water), and the number of outlying points were all considered when concluding whether the quality of the DNA was sufficient for use with KASP markers and which markers would be suitable for use with the Biomek. The amplification of the samples was compared to the controls, which were extracted using the CTAB method.

Semi-automated Biomek Liquid Handler NaOH DNA extraction

A total of four tests were performed with the Biomek Liquid Handler. We sampled all the seeds by hand, which was the most time-consuming process in the DNA extraction using the liquid handler. All pipetting and mixing by vortexing was completed by the Biomek, with human supervision. The DNA extraction using the Biomek was only semi-automated, as there were certain steps that required human intervention, such as placing caps and transporting the samples to the water bath or centrifuge. The first protocol that was used with the Biomek is as follows:

Protocol:

1. Sample ~20 mg of seed chips into 1.1 Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in water bath
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 232 ul of water to each sample to reach 1:2 dilution. Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 4 ul of dilution for PCR.
9. Spin down and dry samples, along with controls, at 65°C for 1 hour.

*The full protocols for each test can be found in appendices B1-B3

Test 3 was carried out using DNA that had been extracted one and two days earlier and stored at -20°C before being defrosted and used in this trial. The DNA was also diluted further by adding

108ul of water and 6ul of this dilution was taken for PCR instead of 4ul. Test 4 was performed using DNA extracted using the NaOH protocol and DNA extracted from the same seeds using the CTAB protocol. 2ul of the DNA extracted using the CTAB preparation and 4ul of the DNA extracted using the NaOH preparation were taken for PCR with four different KASP assays.

Results and Discussion

Crude DNA extraction with NaOH for KASP genotyping

A total of seven tests were done for the validation of the original crude DNA extraction protocol for KASP genotyping. Results from the tests are explained in the following pages.

KASP marker SNP-10_137904716

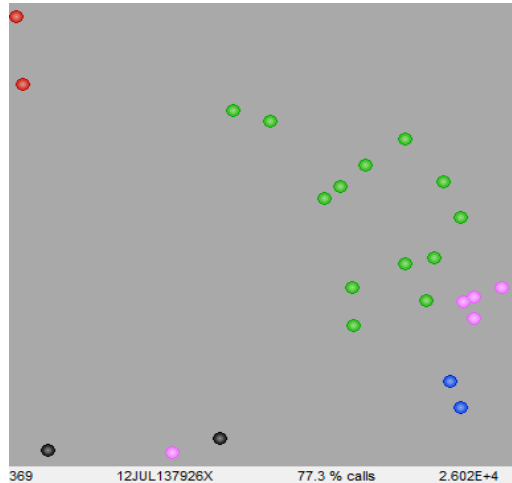


Figure 2a: Clustering and genotypes of marker SNP-10_137904716

The original protocol functioned very well with KASP marker SNP-10_137904716 in our first test. Most of the samples amplified very well, and only one of the non-template controls (NTC) amplified. The negative and positive controls were well located; however, the heterozygous control was inclined towards FAM (blue), the negative samples in this case (Figure 2a). All the samples were identified as heterozygous, which is plausible considering that most of the population was heterozygous. We noticed that the data points were also very dispersed, so we are not yet confident in the results of the genotyping. Even so, the results of this test allowed us to continue with the project and focus on optimizing the protocol.

In the second test, the same marker *SNP-10_137904716* was used with DNA that had been extracted the day before, stored at -20°C , defrosted, and then further diluted for this test. The genotypic results of the second test were very similar to the first test, even though the DNA preparation was diluted and not freshly extracted. All the samples were identified as heterozygous, but the dispersion of the data points was much greater and in a diagonal line towards the top right corner due to over-amplification (Figure 2b). Even though the samples had been defrosted and diluted, they over amplified during the PCR. This indicated that it would be possible to dilute the preparations even more to reach a volume that could be handled by the Biomek Liquid Handler.

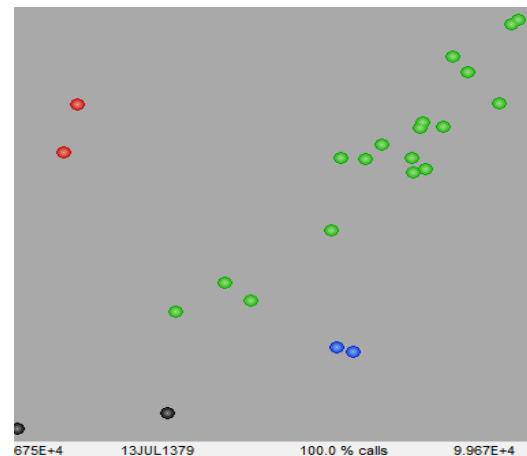


Figure 2b: Clustering and genotypes of marker SNP-10_137904716 with defrosted DNA

All seven KASP markers

In tests 3-7, we determined that each KASP marker had slightly different optimal dilutions and quantity of DNA necessary for PCR. All but two of the molecular markers could be diluted to a 1:2 ratio of DNA extraction to water, and required at least 3ul of this diluted extraction for amplification during PCR. (See table 1) All of the tests with the different markers showed some degree of clustering, but markers S10_136072513 and S10_137904716 showed the least dispersion between data points (Figure 3).

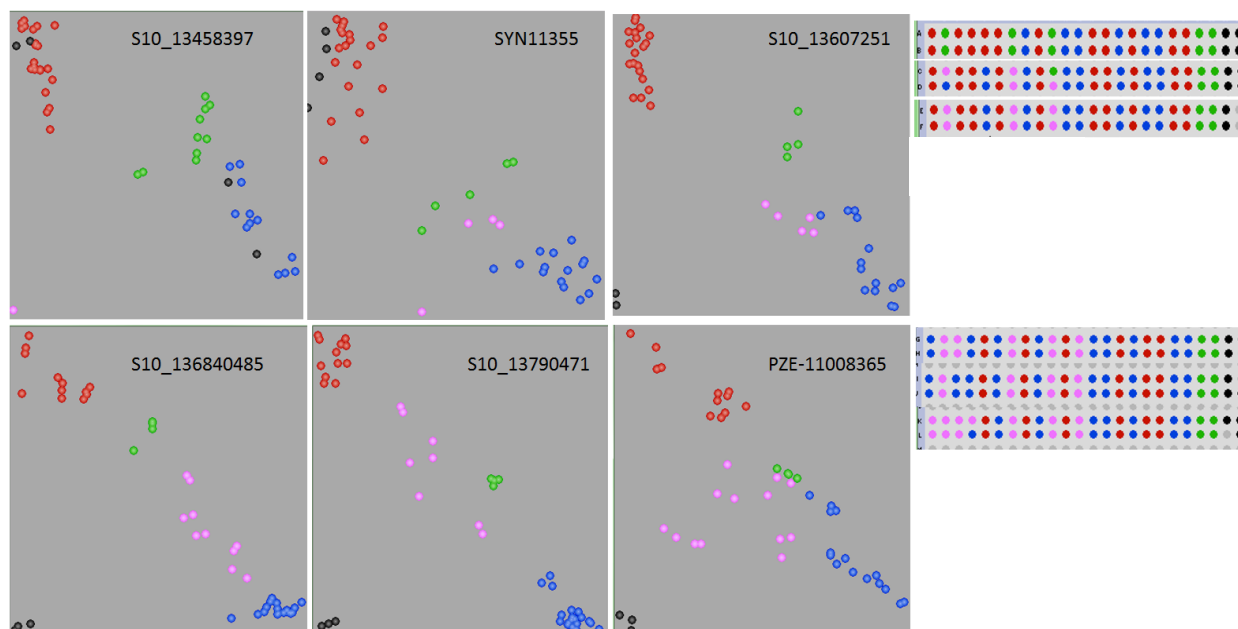


Figure 3: Compilation of the clustering and genotypes of tests 3-7

Table 1. Crude prep dilutions and volume per PCR reaction (check test number to match)

SNP	short name	Dilution	ul template per rxn
10_134583972	1345	1:1	4
S10_134655704	1346	1:1	4
SYN11355	1355	1:2	3
PZE-110083653	1100	1:2	3
S10_136072513	1360	1:2	3
S10_136840485	1368	1:2	3
S10_137904716	1379	1:2	3

The two markers, S10_134583972 and S10_134655704, were found not suitable for use with the Biomek because they could not be diluted to at least a 1:2 ratio and still provide enough quality DNA for PCR. Of the remaining five markers, three were inclined towards either the HEX or the FAM, meaning that the data points for the heterozygotes were located closer to either the negative or the positive controls. Because of this, in some cases it was difficult to distinguish between the positives and the heterozygotes or the negatives and the heterozygotes. The control, positive or negative, that corresponded to HEX and FAM depended entirely on the marker (see table 2). FAM corresponds with the blue data points, while HEX corresponds with the red data points.

Table 2. Attributes of KASP SNP markers for crtRB1 and their suitability for automatic pipetting.

SNP (short name)	NTC amplification	Heterozygous location	suitable for robot	Comments
1345	high	centered	No	Additional cycles increase the amplification of water.
1346	low	centered	No	The assay stock does not work after several repeated defrosts. It should be handled carefully. Small aliquots (20 ul) should have been prepared once it arrived to the lab from LGC.
1355	high	centered	Yes	The marker needs optimization, requires extra cycles to cluster, water amplifies, and the PCR products were dispersed.
1100	null	inclined FAM	to Yes	The maker needs optimization and correction of coordinates, controls were very close, and the PCR products were dispersed.
1360	null	inclined FAM	to Yes	The heterozygotes must be defined manually.
1368	low	inclined HEX	to Yes	The heterozygotes must be defined manually
1379	low	centered	Yes	heterozygotes are well defined if we increase the number of cycles, but the water amplifies

Tests with the Biomek FXP Liquid Handler

The DNA extraction protocol functioned very well with the Biomek Liquid Handler. Using the Biomek, we could extract the crude DNA in about half the time because the robot eliminated the need for repetitive pipetting, and the samples could also be vortexed or mixed by the machine. The process was only semi-automated because human supervision was required, and some actions, such as taking the samples to the water bath to incubate or to the centrifuge, required human action.

Setting up semi-automation using KASP marker 1379

In test one with the Biomek, we extracted DNA from a set of 96 randomly selected seeds and used SNP marker 1379 to genotype each sample. The assay 1379 amplified the samples very well and there is clear segregation and clustering of the favorable and unfavorable genotypes. In earlier tests, most of the samples had been identified as heterozygous, in this test there were far more samples identified as negative. We found it a little difficult to distinguish between the

heterozygous and negative samples because the heterozygous control was inclined towards the negative control.

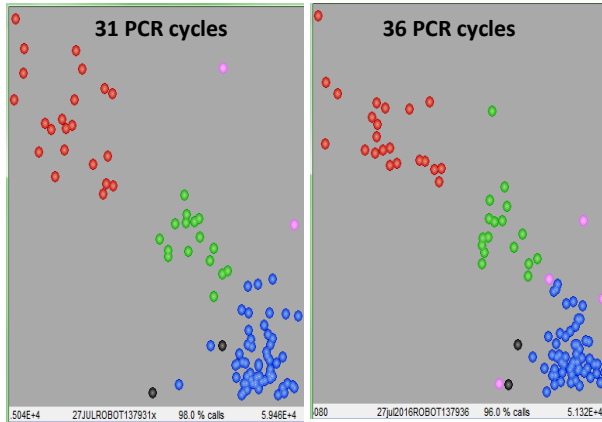


Figure 4: Clustering and genotypes of samples from test 1 with the Biomek

The NTC also amplified along with the DNA samples. However, very few of the samples were left unidentified by the KlusterCaller software. When we increased the number of PCR cycles from 31 to 36 cycles to see if we could get a better grouping of both the heterozygous and unfavorable genotypes, we could not see a significant change. Instead, we observed that the NTC, which was water, amplified even more during the PCR. In previous tests, marker 1379 had shown very

little dispersion of the data points, and little amplification of the NTC. We hypothesized that the greater dispersion was due to the contamination of the pipet tips used with the

Biomek. Supplies were very limited because the Biomek had not been utilized much in the lab, and new supplies did not arrive in time; we could secure only used pipet tips and sanitized them ourselves before each test. It is possible that the tips were not properly sanitized or became blocked, which would affect our results.

Comparison of four KASP markers 1346, 1360, 1368 and 1379

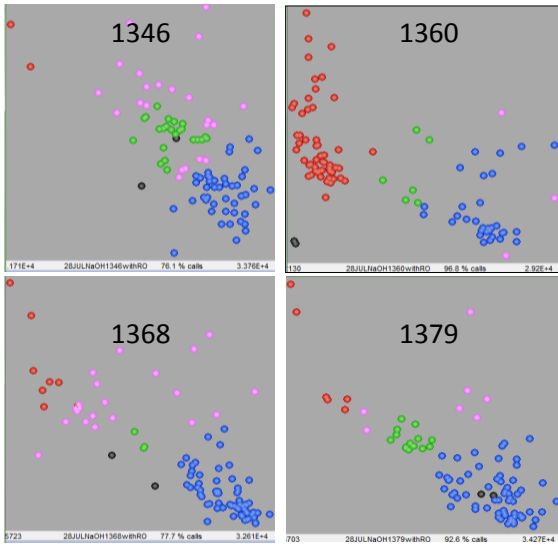


Figure 5: Comparison of clustering and genotypes of four SNP markers (Test 2)

In test two with the Biomek, we extracted DNA from a set of 96 seeds and amplified the DNA using 4 different markers. Two of the markers, 1346 and 1368, did not amplify the DNA well; the data points were much dispersed and many of the genotypes could not be determined. Marker 1346 did not amplify the controls well either. The other two markers amplified the DNA better, however, the genotypes determined using KlusterCaller were not consistent. The data points were also significantly dispersed, more than we would have liked them to be. With marker 1379, the NTC also amplified more than it did with marker 1360. All the markers showed some degree of over-amplification of certain samples, however, they were not always the same samples.

Effect of using defrosted DNA on KASP markers 1379 and 1360

In test 3, we used DNA extracted for test 1 and test 2 that had been kept at -20°C before being defrosted for this test. The samples were not diluted any further, and only two markers, 1360 and 1379, were used. The samples amplified very well with both markers. Using marker 1360, fewer samples amplified, however, the data points were less dispersed than in the amplification using marker 1379.

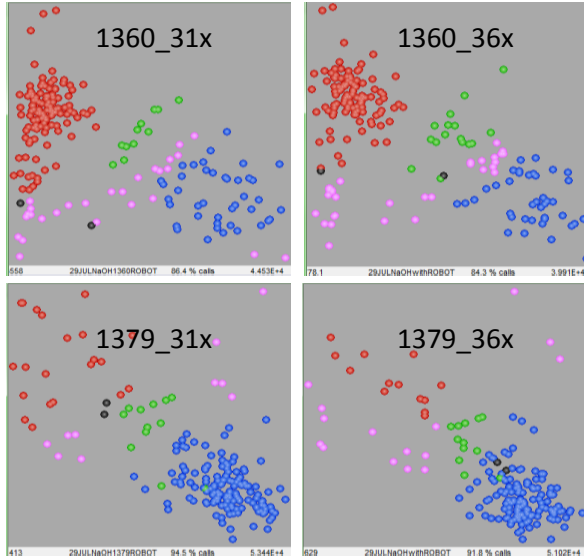


Figure 6: Clustering and genotypes of SNP markers 1379 and 1360 (Test 3)

Both markers also gave similar genotypes for the DNA, with most the samples being negative (the red data points for 1360 and the blue data points for 1379). More samples were identified as positive by marker 1360, while marker 1379 identified more heterozygous samples. As noticed in previous tests, the heterozygous and positive samples and controls were located quite close together when marker 1360 was used, making it difficult to be confident of the true genotype of many samples. This was not an issue using marker 1379. The unclear clustering of the data points does suggest that either more DNA should be used, or that the marker is not as suited for use with the crude DNA as we thought. When we increased the number of PCR cycles from 31 to 36, the data points became less dispersed, but both markers amplified the NTC.

Conclusions

In comparison to the controls extracted using the original CTAB method, the crude DNA extraction protocol functioned very well for most the markers. Some markers were sensitive to the NaOH used in the extraction, therefore the protocol had to be adjusted slightly for each marker. Some markers required more DNA to amplify properly, which meant that the preparation could not be diluted enough to use these markers with the Biomek FXP Liquid Handler. Most of the markers functioned with the same protocol and ratio of DNA preparation to water of 1:2, which suggests that this could be the standard protocol used with most markers. We determined that the best KASP marker for use with DNA extracted using the robot would be S10_137904716, abbreviated as marker 1397. When we tested this marker with crude DNA extracted by hand, it defined the heterozygotes well and showed low amplification of the NTC.

The modified semi-automated protocol with the Biomek FXP liquid handler worked well enough with two of the markers to be able to determine favorable homozygotes and heterozygotes. The amplification of this crude DNA was comparable to the control DNA extracted using the traditional CTAB preparation, however there was a greater chance of contamination using the Biomek. Using the Biomek decreased the time required for the crude DNA extraction by about an hour compared to manual crude DNA extraction. The semi-automation did not eliminate the need to pipet by hand because the Biomek often had issues transferring small volumes of about 2-3 ul. Therefore, many of the samples had to be pipetted in by hand, which took a significant amount of time. Any time that may have been saved during the DNA extraction by the machine

was spent transferring DNA to the PCR plate. We assumed that the Biomek was not transferring volume properly because they were being used repeatedly, not just within a trial, but also between trials. This may have caused them to warp slightly or become blocked between DNA transfers. We were unable to test this, however, because there were no new pipet tips to use and compare. Even with limitations, we could complete three tests with the Biomek and determine that the best KASP marker for use with DNA extracted using this robot was S10_136072513, although marker S10_137904716 was second best.

During the process, we also made an observation that may affect the quality of DNA, but it was not tested directly. Working with different sets of seeds in some of the tests, we noticed that the quality of the seed sample also affected the quality of the DNA extraction regardless of genotype. Although our seeds were randomly selected, we had 2 batches of seeds from different breeding seasons. One batch of seeds was generally dry and brittle while the other batch generally had a tough endosperm. We noticed that seeds from the second batch usually yielded higher quality DNA than seeds from the first batch. This means that more endosperm would have to be taken from these seeds to yield enough DNA, but these seeds would often be damaged during the seed chipping process. This may become an issue, so it is important to ensure the quality of the seeds being used.

Overall, we were able to achieve all three of our project objectives. We finalized a working protocol for crude DNA extraction using Sodium Hydroxide (NaOH) that will provide DNA of a high enough quality to be used with KASP SNP assays. We adjusted the protocol to function with the Biomek FXP Liquid Handler and determined that KASP markers S10_136072513, and S10_137904716 functioned the best with the DNA extracted using the robot. While the selected marker does not distinguish clearly between the heterozygotes and positive homozygotes, this method can still be used to eliminate negative homozygotes before planting. This semi-automated crude DNA protocol would allow for many samples of maize endosperm to be extracted and screened for predicted provitamin A content using KASP markers quickly and efficiently.

Personal Reflection

At the Borlaug-Ruan Intern orientation in May, Ambassador Quinn told our parents to take a good look at us for the next few weeks and remember who we were because when we came back after our two months as an Intern, we wouldn't be the same people that left. When I returned from Mexico, everything just felt a little off compared to what it had been before I left. It took me a while to realize that it wasn't everyone else who had changed, but me. My internship experience made me more confident and able to interact with people better, but at the same time it set me apart from my friends and family who would only know what I experienced through my stories and blog posts. They would never feel the way my heart melted when a little girl at the food kitchen in Texcoco ran up to us on our way out and asked us to take a picture of her drawing, or her shy smile when I handed her a packet of stickers. They would not know how nervous I felt at lunch every day during my first few weeks, surrounded by researchers and scientists with far more experience than I had, or the sense of relief when I accepted that it was okay to be inexperienced because I was there to learn and grow.

Before my internship, I hadn't considered that hunger comes in different forms. It is quite easy to assume that once you provide enough calories, that is enough. My internship reinforced the idea that food security is much more complex than it seems. The more I read about micronutrient deficiencies and biofortification for my research project, the more interested I became in this issue. My work as a Borlaug-Ruan Intern has solidified my determination and commitment to fighting food insecurity and given me hands-on experience that will guide me on my career path to agricultural research.

My Borlaug-Ruan internship was also my first experience working in a lab. Along with learning safety procedures and how to work lab equipment, I also discovered a lot about the process of conducting a research project. Although there were no major issues with my project, I quickly understood that everything won't always go your way. Often, a problem will arise, and the researcher or assistant needs to be flexible enough to resolve it. Working in the lab made me realize how much time and effort goes into many research projects. Sometimes the work can be tedious. One week I labeled over five thousand individual microtubes that would be used to collect leaf tissue from over ten thousand plants in a massive breeding project. It was not related to my research, but there was a deadline and the lab was shorthanded, and I was more than willing to help in any way that I could. In return, I got to go out to the fields and help in collecting leaf tissues. Again, the work was quite repetitive - filling tube after tube with little disks of leaf tissue. Each step was a necessary part of the larger project, therefore, each step was essential in increasing food security and global nutrition.

While I was in Mexico, I was a little surprised that I could connect with a lot of the culture and values. When I went with my supervisor, Martha, to Tlatizapan, we stopped by her parents' house so that she could bring them a few things. We stayed for lunch, which Martha's mom had prepared for all of us and included chicken. When she found out that I was a vegetarian, she began to fret about what I would eat. Everyone's meal was very simple, yet she insisted on preparing me something extra even after I assured her that I would be fine with what she had already prepared aside from the chicken. I was very touched by her concern, and even though the

meal was very simple, it was both surprising and refreshing how open and welcoming everyone was in Mexico. The culture was much more familiar and relaxed than it is here in the United States. Every morning in the biosciences building, the research assistants would come to work around 8:30 am and eat breakfast together in the lunch/breakroom. Although I was quite timid, everyone always gave me a cheerful greeting and warm smile. If I was ever sitting at the table while Martha and I waited for the PCR to finish up, they would offer me a part of their snack and express genuine interest in my project, even though we had never spoken before.

Being a Borlaug-Ruan intern in Mexico was a unique experience, and no less exciting than if I had travelled halfway across the world. While it would have been amazing to travel to Taiwan or Brazil, my internship allowed me to gain a deeper understanding of Mexico's situation and culture. It was one thing to study Mexico in Spanish class; it was an entirely different experience to see the history come to life before my eyes and experience some of the daily life myself. There were a lot of familiar sights in Mexico, from jeans and t-shirts to popular music to KFC. Sometimes, if it weren't for the fact that everything was in Spanish, it was possible to forget that I was in a different country. However, there was still a lot to set Mexico apart. Not everywhere was as developed as the center of Mexico City or the upscale neighborhoods we visited. When we made our way through Texcoco to the food kitchen or weaved our way through the markets, it was clear that we were still in a developing country.

Whenever we could, Michayla and I would take a bus with one of my mentors, Kate, into Texcoco to volunteer at a food kitchen held in a little room off to the side of a cathedral. Before we walked to the food kitchen though, we would stop by the markets to buy fruit for the breakfast we would be serving. Every week we went to the same fruit stall run by a small elderly woman. Kate told us that if there was ever a weekend that she wasn't there, the woman would be worried and ask her the next week if she had been sick or if everything was okay. Even though she didn't have much, she still did what she could, adding bananas or oranges to our bag for free or attempting to give Kate a discount on the fruit, although Kate always refused and paid the full price. After buying our fruit, we would walk over to the food kitchen to set up. People would begin to arrive at around 9, mostly elderly men and women, though there was one woman with two children. As I handed out fruit and yogurt, everyone welcomed me with a grateful smile and "gracias." One thing I noticed about everyone who came to the food kitchen was their positive energy and outlook on life, even though their situations were less than ideal. These people knew what true hunger felt like, yet they still came in with a smile on their face and said grace before breakfast with such hope and joy. While I was there, one of the women taught me how to play a traditional Mexican card game, and others proudly held their coloring pages up for me to admire at the end of the breakfast. Interacting with these people reminded me of the human impact of food insecurity. These were the people I was committed to helping. Their resilience and hope in the face of hunger reminded me to be grateful for all that I have and all that I can do to ensure that fewer people go hungry each day.

Some photos from the internship:



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Appendices

A1 through A8: Validation of corn crude DNA extraction with NaOH for KASP genotyping by hand

B1 through B3: Validation of corn crude DNA extraction for KASP genotyping using the Biomek FXP liquid handler

Appendix A1: 1st test with KASP Marker SNP-10_137904716

1st Test: SNP-10_137904716

date: 12.07.2016

Objective:

Demonstrate the corn crude extraction with NaOH for KASP SNP-10_137904716 genotyping.

Protocol:

1. Place ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in a water bath (incubation started at 65°C and finished at 60°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 116 ul of water to each sample to reach 1:1 dilution. Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 2 ul of dilution for PCR.

Controls: HP1037-35 (C:C or favorable, labeled with HEX, in red), HP1036-3 (T:T or unfavorable, labeled with FAM, in blue), HP1035-3 (T:C hetero, green). 100 ng each.

KASP Reaction Setup:

	1 rxn	30 rxn	
2x KASP Reaction	2.5	75.0	ul
Mix			
ddH ₂ O	0.73	21.9	ul
Assay mix	0.07	2.1	ul
DNA	2.0	---	ul
Volume per rxn		3.3	ul

Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 30 cycles of 95°C x 20 sec, 55°C x 1 min.

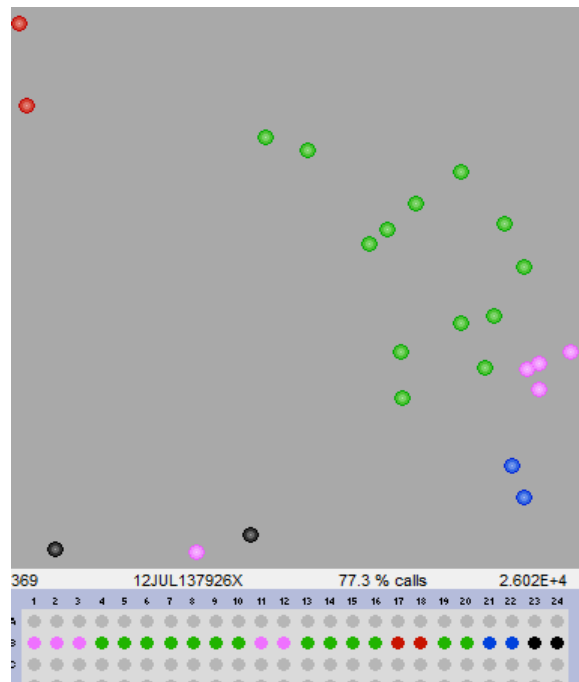
Results:

Negative and positive controls were well located, however the heterozygous control was inclined towards FAM.

Most of the samples amplified very well. Only one sample did not amplify.

One NTC (water) amplified.

Most of samples defined as heterozygous. The samples were a little dispersed, however.



Pherastar Test ID: 2592. Gain: 10%.

Appendix A2: 2nd test with KASP Marker SNP-10_137904716

2nd Test: SNP-10_137904716

date: 13.07.2016

Objective:

Demonstrate if a second dilution of the corn crude extraction with NaOH made one day before but kept at -20°C, is good to amplify the assay 10_137904716.

Protocol:

1. Defrost the corn crude extraction made on 12.07.2016 on ice.
2. Add 116 ul of water to each sample to reach 1:2 dilution. Mix by pipetting.
3. Centrifuge at 3750 rpm for 10 min at 24°C.
4. Take 4 ul of dilution for PCR.
5. Spin down the samples. Dry one hour at 65°C.
6. Aliquot 5.3 ul of KASP reaction.

Controls:

HP1037-35 (C:C or favorable, labeled with HEX, in red), HP1036-3 (T:T or unfavorable, labeled with FAM, in blue), HP1035-3 (T:C hetero, green). 100 ng each.

KASP Reaction Setup:

	1 rxn	30 rxn	
2x KASP Reaction	2.5	75.0	ul
Mix			
ddH ₂ O	2.73	81.9	ul
Assay mix	0.07	2.1	ul
DNA	4.0	----	ul
Volume per rxn		5.3	ul

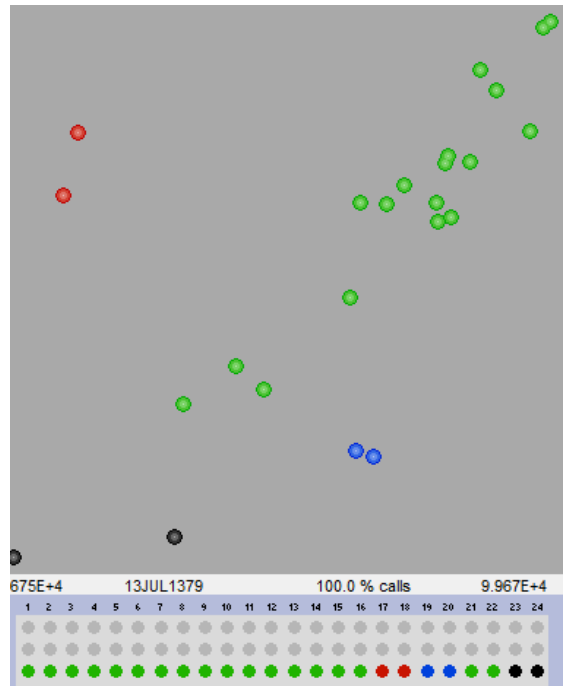
Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

Results:

The results were the same as the test carried out on 12.07.2016, but with all samples defined as heterozygous.

We also found that is possible to get a good amplification of crude preps made one day before and kept at -20°C. In this case, the samples over-amplified, indicating that is possible to dilute the preparation even more.

If we can dilute the DNA extraction even more, then it is possible to adjust the extraction volume so that the Biomek liquid handler can be used for pipetting.



Pherastar Test ID: 2596. Gain: 10%.

Appendix A3: 3rd test with KASP Marker SNP-10_137904716

3rd Test: S10_137904716

date: 14.07.2016

Objective:

Demonstrate the effect of several dilutions of the crude extracts on the amplification of the assay S10_137904716.

Protocol:

1. Place ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH* to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in a water bath.
4. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
5. Add 116 ul of water to each sample to reach 1:1 dilution (Vf=232 ul). Mix by pipetting.
6. Centrifuge at 3750 rpm for 10 min at 24°C.
7. Take 2 ul of 1X dilution for PCR.
8. Add 116 ul of water to each sample to reach 1:2 dilution (Vf=348 ul). Mix by pipetting.
9. Centrifuge at 3750 rpm for 10 min at 24°C.
10. Take 3 and 4 ul of 2X dilution for PCR.
11. Spin down the samples. Dry one hour at 65°C.
12. Aliquot 5.3 ul of KASP reaction.

Controls:

HP1037-35 (C:C or favorable, labeled with HEX, in red), HP1036-3 (T:T or unfavorable, labeled with FAM, in blue), HP1035-3 (T:C hetero, green). 100 ng each.

KASP Reaction Setup:

	1 rxn	90 rxn	
2x KASP Reaction	2.5	225.0	ul
Mix			
ddH ₂ O	2.73	245.7	ul
Assay mix	0.07	6.3	ul
Volume per rxn		5.3	ul

Cycling conditions:

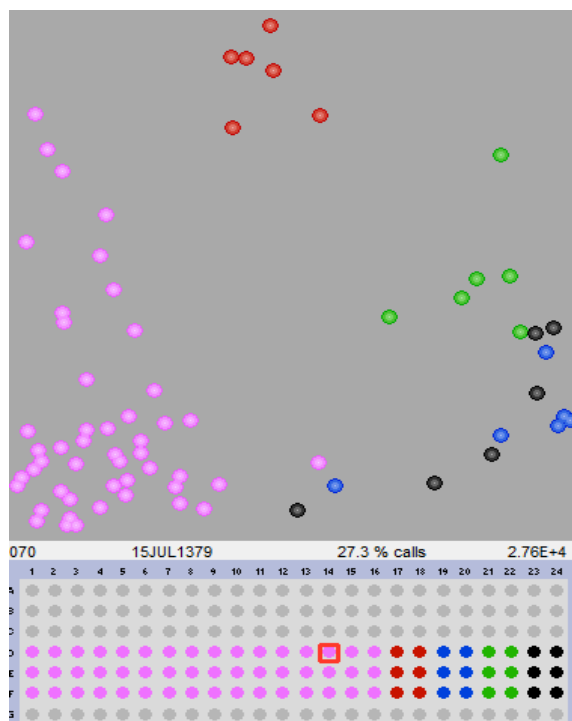
95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

Results:

The samples did not amplify due to excessive NaOH concentration.

The controls were very dispersed, indicating that the marker did not function very well or the PCR plate was contaminated. The heterozygous control was inclined towards FAM.

NTC (water) amplified as FAM control, also indicating that the marker did not function very well or the PCR plate was contaminated.



Pherastar Test ID: 2602. Gain: 10%.

*NaOH utilized was incorrect as its concentration was 2.5 M

Appendix A4: 4th test with KASP Marker SNP-10_134655704 and SNP-10_136840485

4th Test: S10_134655704 and S10_136840485

date: 19.07.2016

Objective:

Examine the effect of several dilutions of crude preparations in the amplification of the assays S10_134655704 and S10_136840485 and the possibility of an automatic pipetting.

Protocol:

1. Sample ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in water bath (incubation started at 68°C and finished at 65°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 116 ul of water to each sample to reach 1:1 dilution (Vf=232 ul). Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 2 ul of dilution for PCR and place in ROW A.
9. Add 116 ul of water to each sample to reach 1:2 dilution (Vf=348 ul). Mix by pipetting.
10. Centrifuge at 3750 rpm for 10 min at 24°C.
11. Take 2 and 3 ul of dilution for PCR and place in ROW B and ROW C, respectively.
12. Add 116 ul of water to each sample to reach 1:3 dilution (Vf=464 ul). Mix by pipetting.
13. Centrifuge at 3750 rpm for 10 min at 24°C.
14. Take 4 And 6 ul of dilution for PCR and place in ROW D and ROW E, respectively.
15. Spin down and dry samples, along with controls, at 65°C for 1 hour.

Controls: HP1037-35 (favorable, labeled with HEX, in red), HP1036-3 (unfavorable, labeled with FAM, in blue), HP1035-3 (hetero, green). 100 ng each (2 ul).

Seed sets: different for each marker.

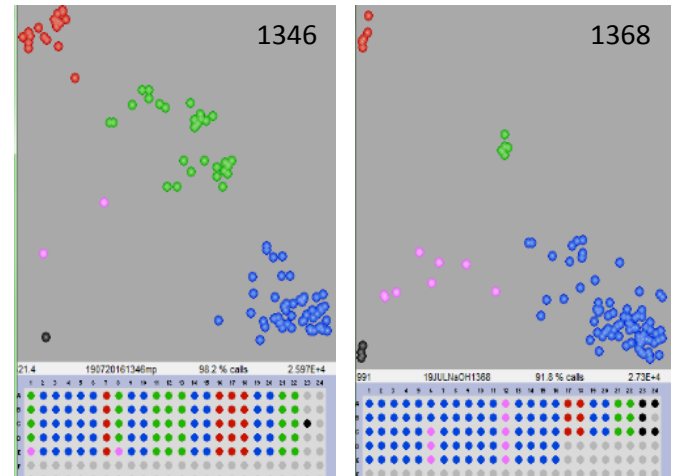
Pherastar Test ID: 2612 y 2613. **Gain:** 10%.

Cycling conditions: 95°C x 15 min, 10 cycles of 95°Cx20 sec, 61°Cx1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°Cx20 sec, 55°Cx1 min.

KASP Reaction Setup:

	1 rxn	150 rxn	
2x KASP Reaction	2.5	375.0	ul
Mix			
ddH ₂ O	0.73	409.5	ul
Assay mix	0.07	10.5	ul
Volume per rxn		5.3	ul

Results:



All DNA dilutions amplified and gave the same genotypes; therefore, it is possible to dilute the crude preparations more to reach a volume that can be used with the Biomek liquid handler for automatic pipetting. The clustering and amplification of the NTC (water) was dependent on the marker.

Appendix A5: 5th test with KASP Markers SNP-10_134583972 and SYN11355

5th Test: Validation of corn crude extraction with NaOH for KASP genotyping

date: 20.07.2016

Objective:

Examine the effect of several dilutions of crude DNA preparations on the amplification of the assays S10_134583972 and SYN11355 and the possibility of an automatic pipetting.

Protocol:

1. Sample ~20 mg of chips in 1.1 Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in water bath (incubation started at 68°C and finished at 67°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 232 ul of water to each sample to reach 1:2 dilution. Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 2 And 3 ul of dilution for PCR and place in ROW G and ROW H, respectively.
9. Add 116 ul of water to each sample to reach 1:3 dilution. Mix by pipetting.
10. Centrifuge at 3750 rpm for 10 min at 24°C.
11. Take 4 And 6 ul of dilution for PCR and place in ROW I and ROW J, respectively.
12. Spin down and dry samples, along with controls, at 65°C for 1 hour.

Controls: HP1037-35 (favorable, labeled with HEX, in red), HP1036-3 (unfavorable, labeled with FAM, in blue), HP1035-3 (hetero, green). 100 ng each (2 ul).

Seed sets: different for each marker.

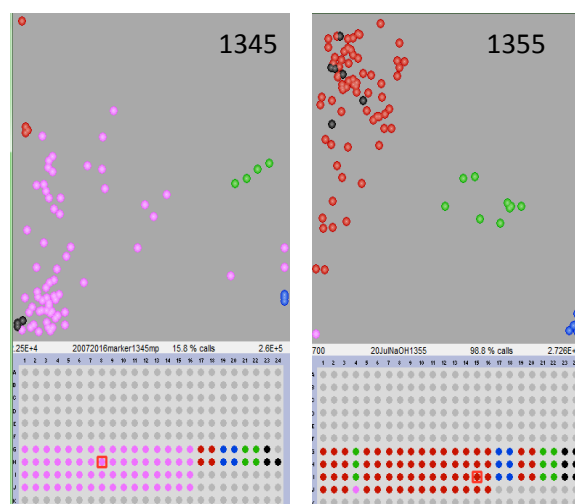
Pherastar Test ID: 261 y 2617. **Gain:** 10%.

Cycling conditions: 95°C x 15 min, 10 cycles of 95°Cx20 sec, 61°Cx1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°Cx20 sec, 55°Cx1min.

KASP Reaction Setup:

	1 rxn	150 rxn	
2x KASP Reaction Mix	2.5	300.0	ul
ddH ₂ O	0.73	327.6	ul
Assay mix	0.07	8.4	ul
Volume per rxn		5.3	ul

Results:



The assay S10_134583972 did not amplify any of the DNA samples, implying that it may require a higher concentration of DNA to amplify the DNA and group closely around the controls. All of the controls amplified very well, although the reading of the heterozygous control is inclined towards the negative control.

The PCR products for the assay SYN11355 amplified well, for the most part, and grouped with the controls. However, the PCR products were very dispersed, especially with the higher dilution (1:3). With this assay, the water amplified as if it were a sample.

Appendix A6: 6th test with multiple KASP markers and possible automatic pipetting

6th Test:

date: 21.07.2016

Objective:

Examine the effect of several dilutions of crude preparations on the amplification of the assays SYN11355 (1355), S10_136072513 (1360), S10_134583972 (1345) and S10_134655704 (1346) as well as the possibility of an automatic pipetting.

Protocol:

1. Sample ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in water bath (incubation started at 66°C and finished at 65°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 116 ul of water to each sample to reach 1:1 dilution ($V_f=232$ ul). Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 2 ul of dilution for PCR.
9. Add 113 ul of water to each sample to reach 1:2 dilution ($V_f=345$ ul). Mix by pipetting.
10. Centrifuge at 3750 rpm for 10 min at 24°C.
11. Take 2 and 3 ul of dilution for PCR.
12. Add 110 ul of water to each sample to reach 1:3 dilution ($V_f=455$ ul). Mix by pipetting.
13. Centrifuge at 3750 rpm for 10 min at 24°C.
14. Take 4 and 6 ul of dilution for PCR.
15. Spin down and dry samples and controls at 65°C for 1 hour.

Controls: HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each (2 ul).

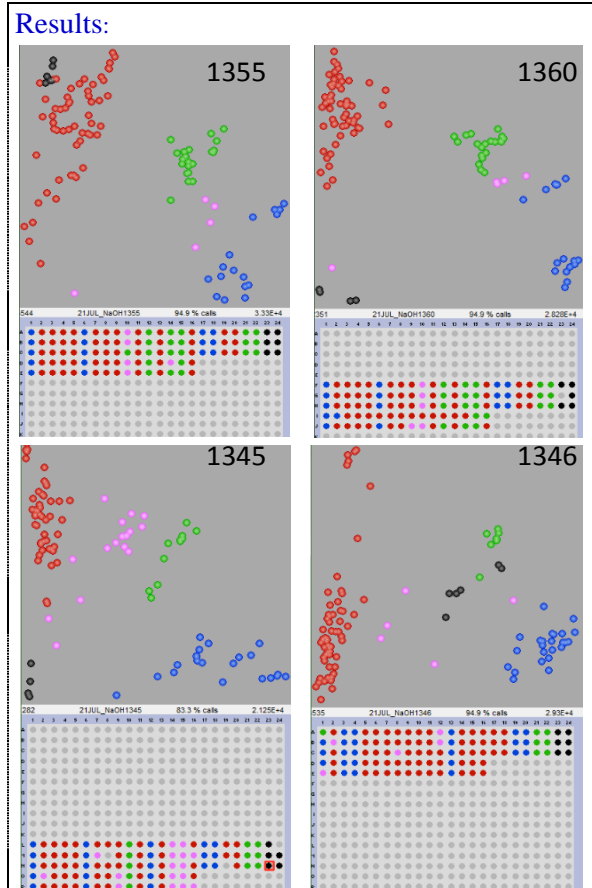
Seed sets: different set only for S10_134655704.

Pherastar Test IDs: 2633, 2634 y 2635. **Gain:** 10%.

Cycling conditions for SYN11355, S10_136072513, and S10_134655704: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

Cycling conditions for S10_134583972: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 65°C x 1 min decreasing -0.6°C per cycle until achieving 57°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

KASP reaction setup per rxn: 2.75 ul of 2X KASP Reaction mix, 2.73 ul of water and 0.07 ul of assay. Only for 1360, add 0.03 ul of 50 mM Mg₂Cl and adjust the water.



The amplification and clustering of the assays 1360 and 1345 were good. NTC for these markers had null or low amplification.

With respect to the assay 1355, we observed that the clustering of the PCR products improved with additional cycles of amplification; however, the NTC amplified as if it were a sample.

The assay 1346 efficiently amplified the samples with the unfavorable allele. The samples with the favorable allele showed a very weak amplification, indicating that they had a lower DNA concentration. The NTC also amplified as if it were a sample

Appendix A7: 7th test with multiple KASP markers to test reducing additional cycles of PCR

7th Test: Multiple SNP markers

date: 22.07.2016

Objective:

Demonstrate if a higher concentration of DNA improves the amplification of the assays SYN11355 (1355), S10_134583972 (1345) and S10_134655704 (1346), avoiding the need to apply additional cycles of PCR.

Protocol:

1. Place ~20 mg of chips in 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in a water bath.
4. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
5. Add 58 ul of water to each sample to reach 1:0.5 dilution (Vf=174 ul). Mix by pipetting.
6. Centrifuge at 3750 rpm for 10 min at 24°C.
7. Take 2 and 3 ul of dilution for PCR.
8. Add 58 ul of water to each sample to reach 1:1 dilution (Vf=232 ul). Mix by pipetting.
9. Centrifuge at 3750 rpm for 10 min at 24°C.
10. Take 4 and 6 ul of 1X dilution for PCR.
11. Spin down the samples. Dry one hour at 65°C.
12. Aliquot 5.3 ul of KASP reaction.

Controls: HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each (2 ul).

Seed sets: different set only for S10_134655704.

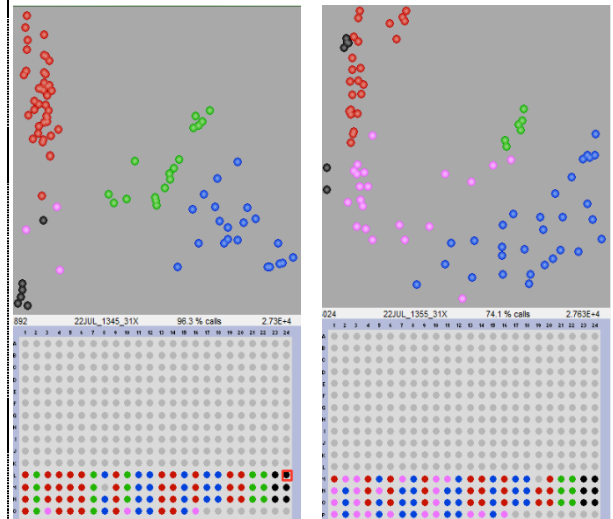
Pherastar Test ID's: 2638, 2642 y 2643. **Gain:** 10%.

Cycling conditions for SYN11355 and S10_134655704: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

Cycling conditions for S10_134583972: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 65°C x 1 min decreasing -0.6°C per cycle until achieving 57°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.

KASP reaction setup per rxn: 2.75 ul of 2X KASP Reaction mix, 2.73 ul of water and 0.07 ul of assay. Only for 1360, add 0.03 ul of 50 mM Mg₂Cl and adjust the water.

Results:



The samples did not amplify well until the PCR was run for additional cycles.

In this test, only the controls and the positive and heterozygous samples for the marker 1345 clustered well. The positives samples inclined towards the heterozygous control. Even so, they were genotyped as positives by the Kluster Caller program.

For the marker 1355, the amplification was dispersed and some samples did not amplify, indicating that NaOH concentration may affect amplification. Again, the NTC amplified excessively.

Another observation is that the genotypes with both markers corresponded in most cases, but in other cases they did not.

The amplification with assay 1346 was considered to have failed (image not shown), since the controls were not rightly located.

Appendix A8: Examine suitability of using Biomek station in all 7 KASP markers

8th Test

date: 25.07.2016

Objective: Examine the suitability of using the Biomek station with all 7 SNP markers.

Template: same crude preps for all 7 markers. The crude extracts were prepared with 100 mM NaOH, 65°C during 10 min, and neutralized with 0.2 mM Tris, pH 7.8. Volumes of template per rxn are indicated in Table 1. Templates were dried at 65°C for 1 hour, after kept at -20°C.

Controls: HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each.

KASP reaction setup per rxn: 2.75 ul of 2X KASP Reaction mix, 2.73 ul of water and 0.07 ul of assay. Only for 1360, add 0.03 ul of 50 mM Mg₂Cl and adjust the water.

Table 1. Crude prep dilutions and volume per PCR reaction:

SNP	short name	Dilution	ul template per rxn
10_134583972	1345	1:1	4
S10_134655704	1346	1:1	4
SYN11355	1355	1:2	3
PZE-110083653	1100	1:2	3
S10_136072513	1360	1:2	3
S10_136840485	1368	1:2	3
S10_137904716	1379	1:2	3

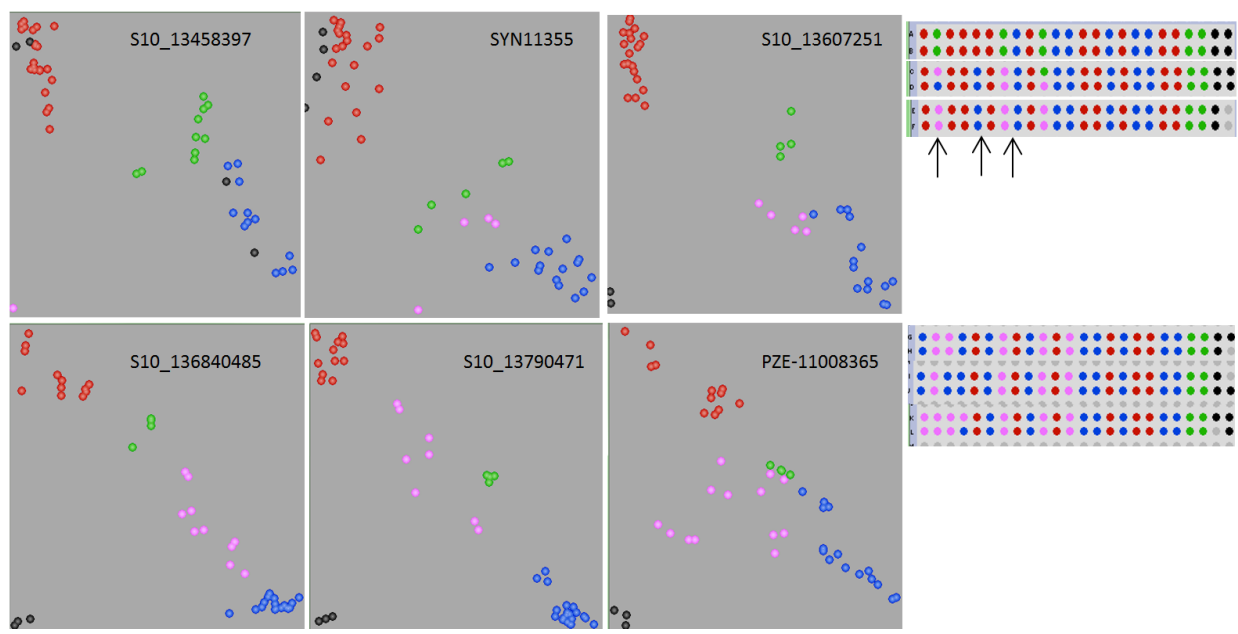
Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1min. Only for 1345, apply 65°C and 57°C instead of 61°C and 55°C, respectively.

Results:

Table 2 describes some features to be considered if we use the Biomek liquid handler. Figure 1 shows the cluster pattern of each marker. Marker 1346 failed to amplify the samples that we used for this test. Characteristics of this marker were described taking into account other previous results.

Table 2. Attributes of KASP SNP markers for crtRB1 and their suitability for an automatic pipetting.

SNP (short name)	NTC amplification	Heterozygous location	suitable for robot	Comments
1345	high	centered	No	Additional cycles increase the amplification of water.
1346	low	centered	No	The assay stock does not work after several repeated defrosts. It should be handled carefully. Small aliquots (20 ul) should have been prepared once it arrived to the lab from LGC.
1355	high	centered	Yes	The marker needs optimization, requires extra cycles to cluster, water amplifies, and the PCR products were dispersed.
1100	null	inclined to FAM	Yes	The marker needs optimization and correction of coordinates, controls were very close, and the PCR products were dispersed.
1360	null	inclined to FAM	Yes	The heterozygotes must be defined manually.
1368	low	inclined to HEX	Yes	The heterozygotes must be defined manually
1379	low	centered	Yes	heterozygotes are well defined if we increase the number of cycles, but the water amplifies



Cluster pattern and genotyping of 6 KASP SNP for the crtRB1 gene. Only two markers (1345 and 1355) were discrepant in defining the genotype of some of the same samples.

Appendix B1: Biomek FXP Liquid Handler test with marker 1379

1st Test: Marker 1379

Date: 27.07.2016

Objective:

Demonstrate that the NaOH-based DNA extraction and the transfer of DNA into the PCR plate can be done with the Biomek FXP liquid handler.

Protocol:

1. Sample ~20 mg of chips into 1.1 Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix by vortexing.
3. Incubate at 65°C for 10 min in water bath (incubation started at 68°C and finished at 68°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix by pipetting.
6. Add 232 ul of water to each sample to reach 1:2 dilution. Mix by pipetting.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 4 ul of dilution for PCR.
9. Spin down and dry samples, along with controls, at 65°C for 1 hour.

Controls:

HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each (2 ul).

Seed sets: one set, seeds from HP screening-2014.

Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1min.

Recycling conditions: 5 cycles of 94°C x 20 sec, 57°C x 1min.

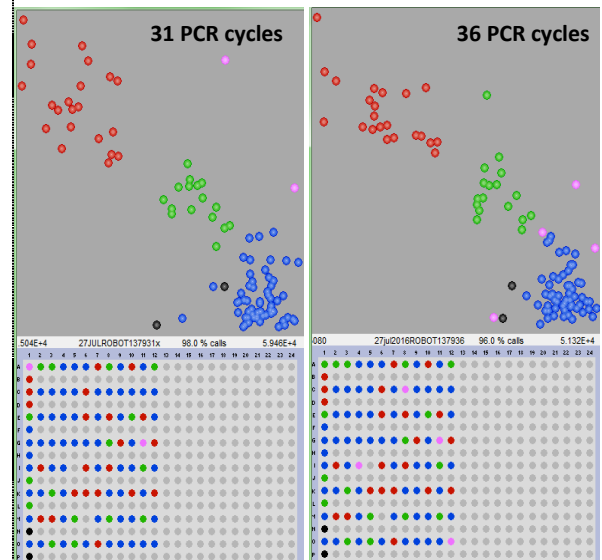
KASP Reaction Setup per 1 rxn: 2.75 ul of 2X KASP Reaction mix, 2.73 ul of water and 0.07 ul of assay 10_137904716 (1379).

Pherastar Test ID: 2665 and 2666. **Gain:** 10%

Time spent for chipping 96 seeds: 2 hours

Time elapsed from extraction to PCR: 1.5 hours

Results:



Shown here is a DNA extraction and PCR of a complete plate performed by the Biomek liquid handler (robot). The data points correspond to the marker 1379.

We made 2 modifications to original protocol in order to use the robot for the extraction and transfer of DNA to PCR plate. The first one was to prepare a 2X dilution to make it possible for the 20ul tips used by the robot to draw up liquid. The second modification was to aliquot 4 ul of DNA instead of 2 ul.

The results of amplification after making these modifications showed that the assay 1379 amplified the samples very well and there is clear segregation and clustering of the favorable and unfavorable genotypes. With this marker it was a little difficult to distinguish between the heterozygous and negative samples because the heterozygous control is inclined towards FAM.

When we increased the number of PCR cycles from 31 to 36 cycles trying to get a better grouping both heterozygous and unfavorable genotypes, we could not see a significant change. Instead, we observed that the water amplified during the PCR.

We can conclude that it is possible to use the robot to perform the DNA extraction and transfer the DNA to PCR plate.

Appendix B2: Biomek FXP Liquid Handler test with 4 KASP markers

2nd Test: Four KASP Markers (1346, 1360, 1368, 1379)

Date: 28.07.2016

Objective:

Determine the amplification of 4 KASP assays using the Biomek FXP liquid handler and the same set of tips.

Protocol:

1. Sample ~20 mg of chips into 1.1 ml Qiagen microtubes.
2. Add 64 ul of 100 mM NaOH to each sample. Mix.
3. Incubate at 65°C for 10 min in water bath (incubation started at 68°C and finished at 68°C).
4. Cool down 5 min at RT.
5. Add 52 ul of 0.2 M Tris pH 7.8 to each sample. Mix.
6. Add 232 ul of water to each sample to reach 1:2 dilution. Mix.
7. Centrifuge at 3750 rpm for 10 min at 24°C.
8. Take 4 ul of dilution for PCR. Program the Biomek to USE THE SAME TIPS to aliquot the same extract for all 4 markers.
9. Spin down and dry samples, along with controls, at 65°C for 1 hour.

Controls: HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each (2 ul).

Seed sets: 1-6 columns are seeds from HP screening-2014, 7-12 columns are seeds from HP screening-2015

KASP assays: S10_134655704 (1346), S10_136072513 (1360), S10_136840485 (1368) and S10_137904716 (1379).

Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1min.

Recycling conditions: 5 cycles of 94°C x 20 sec, 57°C x 1min.

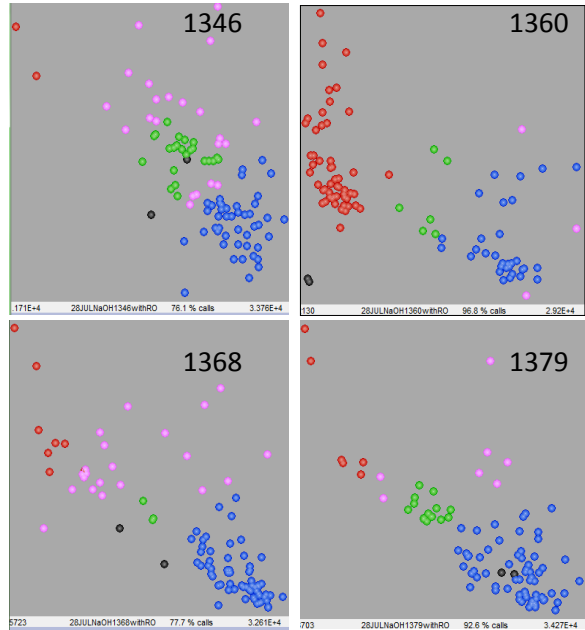
KASP Reaction Setup per 1 rxn: 2.75 ul of 2X KASP Reaction mix, 2.73 ul of water and 0.07 ul of assay. Only for 1360, add 0.03 ul of 50 mM Mg₂Cl and adjust the water.

Pherastar Test ID: 2667. Gain: 10%

TIME SPENT FOR CHIPPING 96 SEEDS: 2 hours.

TIME ELAPSED FROM EXTRACTION TO PCR: 2.5 h.

Results:



Shown in the top row of the Figure are the readings for markers 1346 and 1360. It is clear that there is more clustering and segregation using marker 1360. Marker 1346 also did not amplify the controls very well, which could be due to contamination or the degradation of the marker.

Shown below are the readings for markers 1368 and 1379. Both markers performed similarly, but they were different to 1360. With this markers, the data points are very dispersed and do not show the desired clustering, indicating that they may require more DNA for PCR or are more sensitive to NaOH.

Regarding the water control, while it did not amplify using marker 1360, other markers did amplify in the water. For DNA samples, there were also quite a few samples that did not amplify well using 31X cycles (not shown here) but others over amplified. Running the plate in the PCR machine for 36 cycles did cause more of the samples with poor amplification amplified enough to be read by the Pherastar and those over amplified samples amplified significantly.

One of the challenges that were faced when using the same tips with the Blomek, is that some of the pipet tips were not drawing up the proper volume of DNA, or they were not drawing any volume. Therefore, many of the samples had to be pipetted in by hand, which took a significant amount of time. Any time that may have been saved during the DNA extraction by the machine was spent transferring DNA to the PCR plate.

Appendix B3: Biomek FXP Liquid Handler test with 4 KASP markers

3th Test:

Date: 29.07.2016.

Objective:

Determine whether DNA from samples that was extracted one or two days before and stored at -20°C can be diluted to 3x concentration and function in a PCR with 4 KASP assays as well as to know whether the Biomek liquid handler functions more efficiently with a greater volume of diluted crude prep.

Protocol:

1. Defrost samples from 27/7/16 and 28/7/16 on ice.
2. Add 108 µl of water to make a 3x dilution
3. Centrifuge at 3750 rpm for 10 min at 24°C.
4. Take 6 µl of dilution for PCR.
5. Spin down and dry samples, along with controls, at 65°C for 1 hour.

Controls: HP1037-35 (favorable), HP1036-3 (unfavorable), HP1035-3 (hetero). 100 ng each (2 µl).

Seed sets: 2 sets, one plate of seeds all from HP screening 2014, and second plate columns 1-6 are seeds from HP screening 2014, 7-12 are seeds from 2015.

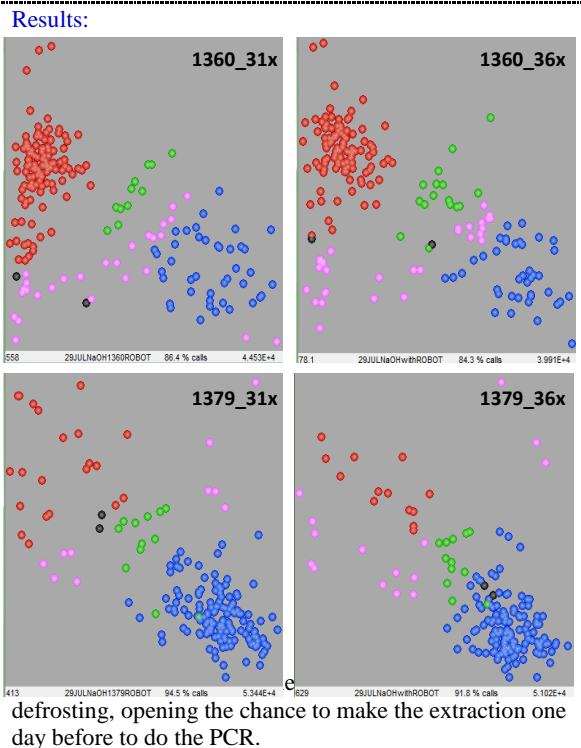
KASP assays: S10_134655704 (1346), S10_136072513 (1360), S10_136840485 (1368) and S10_137904716 (1379).

Cycling conditions: 95°C x 15 min, 10 cycles of 95°C x 20 sec, 61°C x 1 min decreasing -0.6°C per cycle until achieving 55°C; 31 cycles of 95°C x 20 sec, 55°C x 1 min.
Recycling conditions: 5 cycles of 94°C x 20 sec, 57°C x 1 min.

KASP Reaction Setup per 1 rxn: 2.75 µl of 2X KASP Reaction mix, 2.73 µl of water and 0.07 µl of assay. Only for 1360, add 0.03 µl of 50 mM Mg₂Cl and adjust the water.

Pherastar Test ID: 2687, 2688. **Gain:** 10%

TIME SPENT TO DO PCR: 1 h



With respect to amplification, with marker 1360, most of the samples amplified well. There is segregation and clustering of the three genotypes, although we need to consider two points. First, it is easy to distinguish between the negative (red dots) and heterozygous samples using this marker. Second, the distinction between the positive (blue dots) and heterozygous (green dots) samples is not as clear. When the number of cycles in the PCR machine was increased from 31 to 36, there was a slight increase in the clustering of the positive genotypes (blue dots), but there were not clear groups. With this marker, there is also little amplification of the water with 36 cycles.

With marker 1379, most of the samples amplified better with 36 cycles in the PCR machine instead of 31. With this marker, the distinction among the three genotypes was not very clear as on 27/Jul/2016 in spite of the samples were the same. The points for positive samples were dispersed and they were less compared to the same samples using marker 1360, indicating that marker 1379 is more depending on the amount of DNA and the number of PCR cycles. The water also amplified significantly.