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Assay Development for the Identification of Species and Bioactive compounds of Morinda citrifolia (Noni) in Dietary Supplements by rt-qPCR, DNA Sanger Sequencing and Spectroscopy (UV-Vis/FTIR)

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Comments

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ASSAY DEVELOPMENT FOR THE IDENTIFICATION OF SPECIES AND BIOACTIVE COMPOUNDS OF *MORINDA CITRIFOLIA* (NONI) IN DIETARY SUPPLEMENTS BY RT-qPCR, DNA SANGER SEQUENCING AND SPECTROSCOPY (UV-VIS/FTIR)

A Graduate Thesis Submitted to the Faculty of

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Abstract

Regulation standards for dietary supplements lack greatly. Due to this, fraud during the manufacturing of these products might be committed. Previous investigations on herbal dietary supplements have shown that the main ingredient was not present in most of the herbal supplements tested and that these products also contained other substances not stated in the label The purpose of this study was to develop a fluorescent qPCR assay for the identification of *Morinda citrifolia* herbal species in dietary supplements. Primers for genetic regions rbcL and matK of *M. citrifolia* were designed. The potential of novel matK primer showed discrimination potential of species during qPCR analysis. Results for matK amplified samples were verified through DNA Sanger sequencing using the 3730 XL Genetic Analyzer and searched using the BLAST search engine. Sequencing results showed *M. citrifolia* DNA, proving the discriminatory value of matK designed primer. Spectroscopy analysis for the identification of herbal species in dietary supplements was assessed. The details of the analysis and identification of DNA product brands is described within the study. The presence of bioactive compounds damnacanthal and kaempferol, present in *M. citrifolia,* were analyzed using FTIR and UV-VIS. Results showed that the extracted samples were not pure enough or were masked by the solvent used, which obstructed the use of spectroscopy for means of confirmation of those compounds.

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Introduction

"With increased trust there is the opportunity for growth." – Eric Pierce. Consumer trust enables the success or downfall of any business or entity. Herbal supplement products are trusted and consumed everyday by millions of people. A survey conducted by Ipsos Public Affairs determined that approximately 71% of United States adults consume dietary supplements, with 36% being herbal dietary supplements (2017 CRN Consumer Survey on Dietary Supplements). Even though these products are part of the everyday life of many citizens and according to the Council for Responsible Nutrition contribute up to 121.6 billion dollars to the United States economy, regulations and quality control for these supplements lack greatly (Center for Food Safety and Applied Nutrition). The Food and Drug Administration (FDA) is responsible for "taking action against any adulterated or misbranded dietary supplement product after it reaches the market", which by the time action is taken people may have already been exposed to contaminants, unapproved ingredients or a fraudulent product.

As previously mentioned, there are many types of dietary supplements which include vitamins, proteins, minerals or any other substance that provides a desired health "benefit" which might not be found in our regular diet. Herbal dietary supplements are one of the most common types of supplements which consist of an extract of a plant organism which are believed to have an effect on the individual's health (e.g. *Gingko biloba*, *Echinacea* species, *Hypericum perforatum* or St. John's Wort etc.). These supplements are mostly manufactured by vacuum freeze-drying (Figure 1). The herbal extract compound is frozen, and the water crystals formed

are removed from the frozen product by sublimation. Freezing temperatures reach to a low of approximately -20˚C which freezes the water present in the compound forming crystals that vary in size, depending on the amount of water present in the product and the freezing temperature used. These crystals are then removed by sublimation under low pressure using a vacuum pump and a source of heat energy. Depending on the size of the crystals formed, the product might lose some of the bioactive compounds present. Finally, this process results in a dehydrated form of the original product that contains most of the bioactive compounds present in the original plant material. The relative concentration of the bioactive compounds, however, may be unknown.

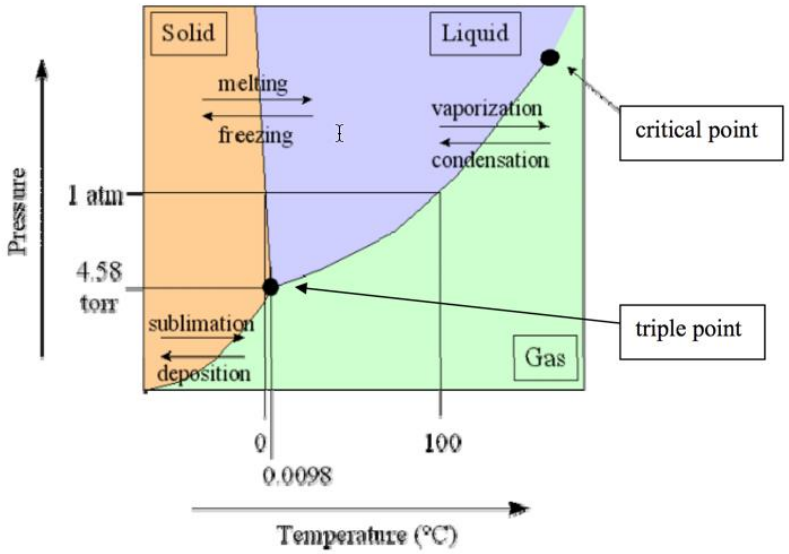


Figure 1: Table diagram which shows the point of interests of the freeze-drying process. (2012, Fuseideas (www.fuseideas.com))

Based on FDA standard regulations, any dietary supplement that has approved ingredients, does not state health claims in the label, and has been manufactured under Good Manufacturing/Distribution Practices (GMP/GDP), can be sold in market. These regulations are

purely superficial, in which a standardized proof of the contents of the final product is not required. Therefore, during manufacturing it is up to each entity to identify which tests to perform to ensure the quality of their product. Due to this, fraud during the manufacturing of these products might be committed, since a structured method for the identification of these products has not been established for herbal dietary supplements.

Previous investigations on the legitimacy of herbal dietary supplements have shown that the main ingredient stated in the label was not present (or was not able to be detected) in most of the herbal supplements analyzed and that these products also contained other substances not stated in the ingredients (Newmaster, Steven G, 2013). An investigation conducted by the Attorney General of New York State revealed "supplements that failed to contain their labelled ingredients". The method used in the investigation was DNA sequencing and it showed that not only the main ingredient (species) was not detected in most of the herbal supplements, but these products also contained other species (e.g. wheat) not stated in the label. The methodology of the investigation previously discussed was questioned since these products are highly processed during manufacturing (frozen and sublimed) which might cause the degradation of the herb's DNA, bringing up the argument that DNA sequencing alone may not be a suitable method for testing all supplements.

In this study we analyzed eight manufacturers of the herbal supplement Noni (*Morinda citrifolia*). Research investigations have exhibited the possible pharmacological benefits of Noni, which includes immunostimulatory, antitumor, anti-obesity, anti-inflammatory, leishhmanicidal, anti-oxidant, anti-allergic, anti-gastric, Esophagitis, anti-diabetic, anti-bacterial, anti-septic, anti-fungal, anti-viral, anti-nociceptive, wound healing, anti-helmintic, anti-mutagenic, anti-psychotic

anti-angiogenic, anxiolytic, photoprotective, tissue regeneration, and anti-wrinkle activities thus making Noni a great product for marketing (Torres, Mylena Andrea Oliveira, 2017). The purpose of this study was to verify the legitimacy of the *Morinda citrifolia* (Noni) herbal dietary supplement by analyzing the species detected as well as the bioactive compounds present in the final product, to determine if fraud during manufacturing or advertising was committed. The preliminary identification of the species present in the herbal supplements was identified using qPCR amplification of the matK genetic region of *M. citrifolia* of extracted DNA present in the supplements. Spectroscopic analysis was performed for supplement extracts to determine the presence of flavonoid kaempferol and anthraquinone damnacanthal, both of which are reportedly in the *M. citrifolia* species (Lv, Lishuang, 2011). The purpose of spectroscopic analysis was to verify potential bioactivity and determine for samples that did not show DNA amplification if they contained traces of these bioactive compounds common to *M. citrifolia* species.

Literature Review

Herbal supplement products are consumed everyday by millions of people. Approximately 80% of the world population, rely on HMPs for their primary health care (Abubakar, Bashir Mohammed, et al). Even though these products are part of the everyday life of many citizens, regulations and quality control for these supplements lack greatly. *Morinda citrifolia* contains a variety of phytochemical compounds that provide this herbal plant with numerous therapeutic effects (Ali, M., Kenganora, 2016) which has increased the interest of distributing this product as an herbal supplement. *In vitro, in vivo* and clinical trials for the verification of pharmacological functions of *Morinda citrifolia* are summarized in the review article by Assi, R. A., Darwis et al. (2017) which somewhat proves the functionality of this herbal plant.

For the authentication of herbal dietary supplements, a methodology for the identification of species and, bioactive compounds, and possible contaminants present in the product will assure consumers of the dietary supplement content. DNA sequencing is a method that allows us to identify the species present as well as the different DNA present within a mixed sample. DNA sequencing consists of using a short specific DNA genetic marker in an organism of interest to identify it as belonging to a species. DNA sequencing is already being employed in the food industry to detect market substitutions, trace pests through the forensic evaluation of trace "environmental DNA." , and to track parasitic infections in livestock which shows the potential of DNA sequencing to contribute to increased security and regulation methods (Littlefair, J. E., & Clare, E. L, 2016). DNA barcodes also provide accurate forensic tools for studying community

ecology and community evolution. A study carried out in the tropical forest in Puerto Rico which used up to three DNA barcoding gene specific locus combinations (rbcLa, trnH-psbA, and matK) allowed for the construction of a community phylogeny of trees in Puerto Rico (Kress, W. J., Erickson, D. L., Swenson. et al). The study demonstrates the effectiveness of using different gene locus combinations and how it impacts the DNA barcoding results.

According to Abubakar, Bashir Mohammed, et al. (2017), the identification of herbal supplements and its possible adulterants can be identified through DNA sequencing using primers for specific genetic regions that are distinct for each herbal species (rbcL, matK, psbA-trnH, and/or ITS regions). The matK gene is set in the group II intron between trnK exons of the chloroplast genome of most green plants. This gene encodes for proteins known as maturases which are enzymes that catalyze intron removal from premature RNAs. The structure of these proteins consists of three domains: a reverse-transcriptase (RT) domain, domain X (the proposed maturase functional domain), and a zinc-finger-like domain (Mohr, Georg, 1993). Insertions and deletions are frequent in matK, though these indels primarily occur in multiples of three, maintaining the reading frame. Some of the functions of the matK protein relate to regulation mechanisms of plant development and photosynthesis (Barthet, Michelle M, 2007).

The rbcL gene is located in the chloroplast genome of plant species and encodes for the Ribulose bisphosphate carboxylase large chain protein. This protein is responsible of acting as a catalyzing agent of two reactions: the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate in the photorespiration process (Houtz, R. L, 2012). The rbcL gene has been a phylogenetic

marker for studies of the evolution of land plants and a possible way of detecting the plant species based on sequence of this genetic region (Yamazaki, H, 1996).

In the case of *M. citrifolia* , the genetic region for the rbcL and matK gene with the partial trnK gene intron regions have been already coded and are available in the National Center for Biotechnology Information (NCBI) website. The use of DNA sequencing to identify species present in herbal supplements has been previously used, one example is the investigation executed by Newmaster, Steven G, et al. (2013). A wide set of herbal supplements (44) were tested with DNA sequencing using genetic regions rbcL and ITS2 with DNA extracted from herbal dietary supplements using the Nucleospin Plant II DNA Kit. Based on the results obtained from each DNA barcode, the dietary supplements were categorized as authentic (contains a DNA barcode for a species that is the main ingredient on the label), contaminated (contains barcode for main ingredient as well as DNA barcode belonging to a species other than what is labeled on the tested product), substitution (DNA barcode is only found for a species other than what is labeled on the tested product), and if fillers were present (DNA barcode is found for herbal product filler species including rice, soybean and wheat). Results demonstrated that only about 52% of the herbal products tested were authentic.

A similar investigation was carried out in China where they studied Chinese patents and medicines by developing a PCR method which incorporated the use of primers to the genetic region ITS of the respective plant species being studied (Chen, R., 2012). The study demonstrates the specificity of the ITS region and how it can be used to identify plant species present in medicinal products. Finally, the use of DNA sequencing with the ITS2 and rbcL gene specific regions was incorporated with Next-Generation Sequencing (NGS) to identify herbal

supplements (Ivanova, N. V, 2016). Their methodology incorporated the comparison of the two DNA barcoding methods, next generation sequencing and Sanger sequencing. Results demonstrated once again the specificity of the rbcL and ITS2 genetic regions for the identification of herbal species and that DNA sequencing through next-generation sequencing is preferred since DNA might be degraded during the manufacturing process and might not be detected through Sanger sequencing.

A polymerase chain reaction quantitative method (qPCR) followed by melting curve analysis was used by Castillo Cázares, A. (2016) for the detection of botanical composition of honey samples. The objective of this study was to develop and validate a system using quantitative PCR and SYBR® Green, as well as dissociation curve analysis, in order to characterize the plant species and to identify pollen sources in honey. Primers were developed for genetic regions Adh1, Hmg2, Brass lip, Plant 1, Plant nest, Act1, and Helli-all, and were used to amplify plant species and honey samples DNA. Comparisons of melting curves among plant and honey samples for each primer amplification were performed and taxonomic content of honey samples was determined.

The specificity of seven different genetic regions for plant DNA barcodes (psbA-trnH, matK, rbcL, rpoC1, ycf5, ITS2, and ITS) were compared based on PCR amplification efficiency, differential intra- and inter-specific divergences, and the DNA barcoding gap (Chen, S, 2010). Based on results obtained with the implementation of these barcodes for the identification of medicinal plant species, the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA was found to be the most suitable region for DNA barcoding applications for plants species.

As per Abubakar, Bashir Mohammed, et al. (2017), the combination of chromatography and DNA barcoding could prove for a comprehensive quality assessment of herbal medical products as these are necessary for quality control. The concentration of bioactive compounds of an herbal product, which determines the supplement functionality, can be determined through chromatography. A review article on the potential Noni effects in Obesity-Related Metabolic Dysfunction (Inada, Aline, 2017) listed the phytochemicals present in different parts of *Morinda citrifolia* (leaf, fruit, root). The article explains that flavonoids, which are present in Noni, have been used for the treatment of various metabolic disorders. Hence a determination of the presence of these bioactive compounds will determine the presence of Noni in the herbal supplement and its functionality. Of the six flavonoids present in Noni, one of the most common flavonoids is kaempferol. The study by Assi, R. A., et al. (2016) supports the presence of kaempferol in *Morinda citrifolia* by listing it as one of the pharmacological active ingredients present in the plant, making kaempferol a possible source for detection of bioactive compounds from *Morinda citrifolia* dietary supplements.

Regarding the determination of flavonoid content, Mitra, Sankar. (2014) realized an investigation on the level of catechin/poly–phenolics in commercially available green and black teas. In this article, the use of High-Pressure Liquid Chromatography with UV detector (HPLC-UV) was employed to detect the level of total phenolic content of a product which determined the catechin content on green and black teas. The extraction of the bioactive compounds was carried out with ethanol with continuous shaking at room temperature repeating for five days. The mobile phase consisted of methanol-ethanol with trifluoroacetic acid. The results showed the determination of the amount of total phenolic compounds present in black and green teas. Based

on Stefova, M. (2003) a reverse phase HPLC is the best method for the calculation of amount and for separation of flavonoids such as catechin. Also, it recommends the use of an acidic compound during the mobile phase since it improves peak quality. We can see that these two suggestions were implemented by Mitra, Sankar (2014) in the research article previously discussed which assures the methodology implemented to be the most efficient in calculating the levels of catechin content.

Kaempferol is a natural flavonoid found in a variety of fruits, vegetables and herbs including *M. citrifolia* . Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings and are classified according to substitutions (Jia, L., & Liu, F. 2013). This biologically active compound exhibits many pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and anticancer activities (Inada, Aline, 2017). There have been studies determining the anticancer effect of this flavonoid which are thought to be mediated through different modes of action, including anti-proliferation, apoptosis induction, cell-cycle arrest, generation of reactive oxygen species (ROS), and anti-metastasis/anti-angiogenesis activities (Rajendran, 2014).

Damnacanthal is an anthraquinone (3-hydroxy-1-methoxy-anthraquinone-2-aldehyde) that was initially isolated from the phenolic phase of noni roots, although it is also present in other parts of the plant (Lv, Lishuang, et al. 2014). Damnacanthal has been known to be a selective inhibitor of $p56^{lck}$ tyrosine kinase activity, a protein activity involved in the chemotactic response of T cells to CXCL12 and targets several tyrosine kinases and has antitumoral effects (García-Vilas, J., 2015). Studies have shown that damnacanthal also enhances transcription factor CCAAT/enhancer binding protein β (C/EBPβ), which controls NAG-

1 transcriptional activity, and therefore increases antitumorigenic activity in human colorectal cancer cells (Nualsanit, T., 2012).

Extraction and identification methods of phenolic compounds from plant material are reviewed in the article by Sasidharan, S. (2017). Different extraction methods reviewed yielded different concentrations of phenolic compounds. Some of the possible extraction methods include using solvents based on the polarity of the bioactive compound of interest such as ethanol and methanol. Microwave assisted extraction (MAE) with the most effective temperature for extracting phenolic compounds from herbal products being 170 ◦C and ultrasonic assisted extraction (UAE) with levels greater than 20 kHz used to disrupt plant cell walls, which improve the solvent's ability to penetrate the cells and obtain a higher extraction yield as reviewed by Altemimi (2017).

A review article by Yahya, N. A. (2018) discussed the different extraction techniques for the extracting of bioactive compounds from plants. One of the techniques mentioned was solid phase extraction (SPE) which consists of the exhaustive elimination of chemical constituents from a flowing liquid sample, which is carried out via retention of the chemicals on a contained solid sorbent (C-18 column) and the later recovery of selected constituents by elution from the sorbent. Some of the advantages of SPE explained in the article state that this extraction procedure uses less organic solvent, is faster, has lower costs and is straightforward in practice, but the analyst needs to keep in consideration the processing of the compound of interest and its related restriction in capacity of sorbent (adsorption to column) and dislocation of the analyte (elution from column).

As a method of identification of bioactive compounds, UV-visible spectroscopy can be used for quantitative analysis since aromatic molecules are powerful chromophores in the UV range. A validated method for the identification of kaempferol using UV-Vis spectroscopy was performed by Telange, Darshan R., (2014). Kaempferol UV spectra was found to have a wavelength absorbance at 265nm and 365 nm when dissolved in methanol with a detection limit of 0.015ug/ml. Analysis of FTIR spectra was able to indicate the presence of phenol, carboxylic acid, alkanes, nitro and aliphatic amines functional groups when analyzing crude ethyl acetate extract from *Streptomyces cavouresis* (Duraikannu, D., 2015). All of which shows the potential of spectroscopy analysis to determine the presence of bioactive compounds in herbal products.

Finally, the determination of the main ingredient in an herbal supplement, as well as contaminants and certain bioactive compounds will indicate if the supplement is legitimate or fraud. The methodologies previously discussed are able to achieve each and every one of these goals which in the end will determine if they are of quality standards and most important improve the consumers overall trust in the consumption of herbal dietary supplements.

Material and Methods

Biology (DNA Analysis)

- **DNA Extraction**

Samples for eight manufacturers of *Morinda citrifolia* herbal dietary supplements were ordered online and randomly labeled MCS-001 to MCS-008 (questioned samples). Each sample collected stated *Morinda citrifolia* as the main ingredient present. Specimen of *Morinda citrifolia* fruit was obtained from Miami, Florida (Miami Fruit, https://miamifruit.org/) and testing was carried out as a positive control measure. Organic wheat powder (*Triticum aestivum*, obtained from Just Jaivik - India) was used as a negative control (see Appendix 1 (Materials: Sample Set) for list of samples obtained).

The samples were treated with the Nucleospin Plant II DNA Kit (column-based extraction) to extract DNA present. The protocol from the kit allowed for two possible methods of cell lysis. Either CTAB (cetyl trimethylammonium bromide) or SDS (Sodium Dodecyl (lauryl) Sulfate). Both methods were performed. DNA extraction consisted of taking 200mg of dried product (homogenous fruit sample for positive control) and following one of the two lysis procedures. For CTAB lysis, 400ul of buffer PL1 was added to the 200mg sample with 10ul of RNase A and mixed well. Samples were then incubated for 1 hour at 65˚C. For SDS lysis, 300ul of buffer PL2 and 10ul of RNase were added to the 200mg sample and mixed well. Samples were then incubated for 1 hour at 65˚C, then 75ul was added of buffer PL3, mixed well and incubated in ice for 5 minutes.

Clarification of lysate and removal of residue was the same for both extraction procedures. The product was transferred to a column filter tube (Nucleospin Violet Ring Filter tube) with a new collection tube. The column was centrifuged at 11,000g for 5 minutes. The filter

product was then treated with 450ul of buffer PC (DNA binding buffer), mixed, and transferred to the Nucleospin Plant II Column for DNA binding. After transfer, sample was centrifuged at 11,000g for one minute. Washing of column consisted of adding 400ul of washing buffer PW1 and centrifuging for one minute at 11,000g. Flow through was discarded and 700ul of buffer PW2 was added followed by centrifugation at 11,000g for one minute. The final washing step consisted of "drying' the column with 200ul of buffer PW2 and centrifuging for two minutes at 11,000g. DNA was finally eluted with 100ul of buffer PE (elution buffer) previously warmed to 65˚C. Samples were incubated for 5 minutes at 65 ˚C and centrifuged at 11,000g for one minute. The quantity and purity of the extracted DNA was determined using the Nanodrop UV Microspectrophotometer by calculating the absorbance of UV light of the extracted DNA. The instrument was blanked with 2ul of deionized water, and 2ul of each unknown sample were analyzed in the instrument.

- **Real Time PCR (qPCR)**

The identification of species present in the *Morinda citrifolia* herbal supplements and its possible adulterants were identified through a qPCR assay for presence/absence of *M. citrifolia* species with the Applied Biosystems 7500 Real-Time PCR System. Genetic regions for the matK and rbcL loci of *Morinda citrifolia* were previously sequenced by Wikstrom, N., 2014 (matK) and Razafimandimbison, S. G., 2017 (rbcL) and obtained from the NCBI (The National Center for Biotechnology Information) website for primer design (https://www.ncbi.nlm.nih.gov/). Wheat genetic regions for rbcL as well as matK were also obtained to serve as reference control during the qPCR process, if necessary. Primer design was carried out using the Primer 3 software for suitable parameters of primer design (Thornton, Brenda, 2011). After design based on primer

length, primer stability (mispriming, self-complementarity), and stringency (GC content), primers were ordered from Invitrogen (see Appendix 2 for primer information). The expected amplicon size of the primers are shown in Table 1. The real time amplification (qPCR) process was carried out using the QuantiFast SYBR Green PCR Kit (referencing the Schnable Lab Plant Genomics protocol for amplification of plant genomic material using SYBR Green I, manufacturers instruction (QUANTITATIVE RT-PCR PROTOCOL Plant Sciences Institute Iowa State University, Table 2). Sample parameters for qPCR runs are stated in Table 3. As a standard, known concentrations with *Triticum aestivum* (wheat) and *Morinda citrifolia* fruit sample were performed with each primer pair (rbcL and matK) to compare amplification results of each. Non-template controls (NTC) were performed for each primer with the addition of water. As a control measure, template DNA of wheat and *M. citrifolia* were run with primer designed for the other to determine specificity of the primers designed.

Table 1: Designed genetic primers and expected amplicon size.

Primer	**Species**	**Expected Amplicon Size (bp)**
rbcL	*Morinda citrifolia*	138
matK		109
rbcL	*Triticum aestivum*	88
matK		86

Table 2: qPCR well content and final concentration.

Component	Volume	Final Concentration
2X SYBR Green Master Mix	10ul	1X
Forward Primer	0.8ul*	1uM, 2uM, 5uM
Reverse Primer	0.8ul*	1uM, 2uM, 5uM
Water	1.6	N/A
Template DNA	6.8ul	≈3-5 ng/ul
Total Volume:	20uL	

*Multiple primer concentrations were used to establish optimal concentration needed.

Table 3: ABI instrument parameters for qPCR run.

Cycle Step	Temp. (˚C)	Time	Cycles
Stage 1	50	2 min	1
Stage 2	95	10 min	1
Stage 3	95	15 sec	40
	60	1 min,	
Diss. Stage	60	1 min	1

- **PCR**

In order to observe fragment size and perform sequencing, conventional PCR was performed using the Applied Biosystems GeneAmp PCR -Thermal Cycler. Two PCR reaction procedures were followed. The previously mentioned control parameters (NTC, Positive and Negative controls) in qPCR process, aside for primer specificity, were performed for each PCR run. The first PCR process was carried out with the 2.5 U *Pfu* DNA Polymerase following standard procedures recommended by kit and Schnable Lab protocol (see Tables 4-5). Second PCR procedure was performed using the Terra PCR kit (Taq polymerase) with recommended parameters from kit as well as parameters used in qPCR procedure (see Tables 6-8).

Table 4: PCR well content and final concentration using Pfu DNA polymerase.

Component	Volume (ul)	Final Concentration
5X Phusion Buffer	2.5	1X
10mM dNTPs	0.5	200uM each
50 mM MgCl2	0.5	100uM
Forward Primer	2.0	5uM
Reverse Primer	2.0	5uM
Water	11.5	N/A
Template DNA	5.0	≈3-5 ng/ul
Phusion DNA Polymerase	1.0	X
Total Volume:	25uL	

Table 5: PCR cycling conditions with Pfu DNA polymerase-treated samples.

Cycle Step	Temp. (˚C)	Time	Cycles
Initial Denaturation	94	3 min	1
Denaturation	94	30 sec	32
Annealing	60	30 sec	
Extension	72	1 min, 30 sec	
Final Extension	72	10 min	1
Hold	4	infinity	1

Table 6: PCR well content and final concentration using Terra DNA Polymerase.

Component	Volume (ul)		Final Concentration
2X Terra PCR Direct Buffer	25.0	12.5	1X
Forward Primer	2.0	1.0	0.3uM, 2uM, 5uM
Reverse Primer	2.0	1.0	0.3uM, 2uM ,5uM
Water	15.0	2.5	N/A
Template DNA	5.0	7.0	≈1-5 ng/ul
Terra DNA Polymerase Mix	1.0	1.0	X
Total Volume:	50uL	25ul	

Table 7: PCR cycling conditions with Terra DNA polymerase treated samples (protocol recommended).

Cycle Step	Temp. (˚C)	Time	Cycles
Initial Denaturation	98	2 min	1
Denaturation	98	10 sec	40
Annealing	60	15 sec	
Extension	68	30 sec	
Hold	4	infinity	1

Table 8: PCR cycling conditions with Terra DNA polymerase treated samples (qPCR process).

Cycle Step	Temp. (˚C)	Time	Cycles
Pre-Heat	50	2 min	1
Initial Denaturation	95	10 min	1
Annealing	95	15 sec	40
Extension	60	1 min,	
Diss. Stage	60	1 min	1
Hold	4	infinity	1

- **Gel Electrophoresis/ Agilent Bioanalyzer Procedure**

Amplified product was analyzed using 1% and 2% agarose gel electrophoresis with ethidium bromide to determine the length/size of the DNA fragments amplified. Gel was prepared using 1 or 2 grams of agarose powder and dissolved in 100ul of 1X TBE buffer. The mixture was heated for 1 to 2 minutes in the microwave, until the agarose powder was fully dissolved. An amount of 10ul of ethidium bromide was added, the solution was well mixed, and a gel was cast and cooled to room temperature. A 1Kb DNA ladder (15,000 – 100bp) was used for reference in sizing. Based on protocol recommendations, 2 ul of 1kb DNA ladder was mixed with 2 ul of loading dye 10X BlueJuice (provided by kit) and 16 ul of deionized water. For amplified samples, 20ul of sampled product was mixed with 2ul of loading dye BlueJuice 10X. The prepared solution was well mixed and centrifuged for 10 seconds. Solidified gel was placed in the electrophoresis chamber and submerged with 800 – 900ul of 1X TBE buffer. The samples were added (20ul) to each well and electrophoresis was run at 109V for 2 – 3 hours (until dye traveled to ¾ of the gel). Separated DNA fragments were cut out of the gel and placed in 0.5ml gel breaker tube inside a 2ml collection tube for DNA extraction for DNA sequencing. Gel breaker/collection tubes were

centrifuged for 2 minutes at 20,000 x g. Each gel breaker tube was discarded and 200ul of water were added to the flow through. Samples were placed in a shaker overnight and transferred to a 5um filter tube. Lastly, samples were centrifuged for 10 seconds at 600 x g and the DNA was analyzed in the UV Nanodrop instrument.

The amplified product for the *M. citrifolia* matK region was analyzed using the Agilent 2100 Bioanalyzer with the Agilent DNA Kit. The DNA chip was placed in a priming station, a total of 9ul of previously prepared gel/dye mix was added to the G well circled in black of the DNA chip. The priming station was closed, and the DNA chip was pressurized for 60 seconds. The plunger syringe was gently pulled to the 1 mL position, the priming station was opened and 9ul of gel/dye mix were added to the remaining three G wells on the DNA chip (not circled in black). An amount of 5ul of Marker were added to the ladder and sample wells. A size reference ladder (1ul) was added to the L well and sample well 1 for control purposes. Finally, 1ul of previously amplified sample using the Terra PCR kit was added to sample wells 3 – 12 which consisted of Non template control, positive control and unknown samples (MCS-001 to MCS-008). The DNA chip was vortexed for 60 seconds at 2,400 rpm and analyzed (inserted) in the instrument.

- **3730XL DNA Sequencing**

DNA bands extracted from gel electrophoresis were then submitted to Yale Keck for sequencing (https://medicine.yale.edu/keck/dna/). Based on Yale Keck sequencing requirements, 40 – 50 ng (fill with water) gel purified DNA were premixed with 2ul of specific forward or reverse primer at a concentration of 4uM. Sequencing was carried out with fluorescently-labelled dideoxynucleotides (Big Dye Terminators) and Taq FS DNA polymerase in a thermal cycling

protocol in order to optimize chemistry to increase cluster density and read length and improve sequencing quality scores. The separation matrix used for the run was POP-7 Polymer on a 36 cm capillary array. Following completion of sequencing, a zip file with the actual base by base sequence of extracted DNA was provided. Sequence results were searched on the BLAST website against the NCBI reference DNA database for candidate species matches (www.ncbi.nlm.nih.gov).

Chemistry (Spectroscopy)

The bioactive compounds present in *M. citrifolia* that were studied were kaempferol and damnacanthal. Standards were obtained from Tocris Bioscience (damnacanthal) and Sigma Aldrich (kaempferol) and used as control references for comparison to extracts from samples.

- **Extraction of Bioactive Compounds**

A total amount of 5g of product was collected from the herbal dietary supplements (without capsules), wheat extract, and fruit control. Product was macerated and was placed in 45mL polypropylene tubes. An amount of 20 ml of methanol were added to each tube, samples were well mixed and left sitting overnight. A solid phase extraction was carried out for the extraction of kaempferol and damnacanthal. Hypersep C-18 cartridge columns were obtained from Thermo Scientific and extraction protocol was followed (McDonald, Patrick D., 1995). The C18 column was pretreated with 1mL of methanol. The flow through of methanol through the column was performed with a syringe pump by pressure. After pretreatment, 1 mL of sample mixture was added to the column. Before addition, powder and methanol mixture was

centrifuged for 1 minute at 2500 rpm to move solid particles to the bottom of the tube. The methanol-sample mixture was pushed through the column slowly with pressure. A wash of the C18 column was performed with 1 mL of deionized water and pushed through by pressure. The kaempferol bioactive compound was extracted using hot ethanol and by applying pressure. For damnacanthal, the same procedure was followed, but instead the final washing method was performed with dimethylsulfoxide (DMSO) and not hot ethanol. As a control measure, the procedure was repeated without the addition of sample extract.

- **UV-VIS and Fourier Transform Infrared Spectroscopy (ATR-FTIR) Procedure**

The extracted sample was transferred (700ul) to a Hellma Microglass cuvette with a 10mm pathway. Samples were run in the UV-Vis instrument (kaempferol) and UV-NanoDrop Spectrophotometer (kaempferol and damnacanthal). Sample parameters for the UV-Vis instrument (Shimadzu 1700 series) included a wavelength read length of 190 to 700nm at medium scan speed with light source from W1 lamp (visible) and D2 lamp (UV). UV- Nanodrop One Spectrophotometer (Thermo Fisher Scientific) wavelength read was of 190 to 850nm at fast speed. Dilutions of the extracts were performed when necessary. Spectra of the standard for kaempherol were analyzed with external spectra for reference (Telange, Darshan R., 2014). FTIR analysis was performed with the ThermoFisher ATR-FTIR instrument. Sample extracts were mixed and 8ul of sample were placed in the ATR built in detector (DTGS KBr). Dried herbal dietary supplement samples were also analyzed, as well as fresh fruit samples, wheat samples, damnacanthal and kaempferol control standards. A small amount of product was placed in the built in ATR detector, enough to cover the aperture. Thirty-two scans were performed per

sample. The resolution value was 4.0, the sample gain of 2.0 and optical velocity was 0.4747 on an 80.0 aperture.

Results

The analyzed extracted DNA samples showed a consistent amount of DNA between extracted samples from the same source of herbal dietary supplements and fruit control of *M. citrifolia* species. DNA concentrations ranged between 3.8 – 15.8 ng/ul of extracted samples from the *M.citrifolia* control and unknown samples. A260/A280 absorbance values for unknowns and fruit control were consistent (between 1.40 – 1.71) as well as A260/A230 ratios (0.49 – 0.60). Wheat control DNA was extracted using the same procedure and high yield results were obtained. DNA wheat control extracted samples showed a concentration of 100.3ng/ul with absorbance values of 1.80 in the A260/280 ratio and 2.16 in the A260/230 ratio. Results using the CTAB and SDS extraction method showed consistent results with no major difference in the two species.

Table 9: Summarized results of UV-Nanodrop Spectrophotometer assessing DNA quantity and quality of extracted DNA.

Sample	Concentration (ng/ul)	A260/A280	A260/A230
***M. citrifolia* (Fruit)**	**5.5**	**1.40**	**0.59**
MCS-001	**15.8**	**1.50**	**0.49**
MCS-002	**5.5**	**1.50**	**0.49**
MCS-003	**9.0**	**1.58**	**0.57**
MCS-004	**4.1**	**1.79**	**0.56**
MCS-005	**3.8**	**1.57**	**0.38**
MCS-006	**4.2**	**1.74**	**0.56**

MCS-007	**6.0**	**1.52**	**0.60**
MCS-008	**6.0**	**1.71**	**0.51**
Organic Wheat	**100.3**	**1.80**	**2.16**

- **qPCR Amplification Results:**

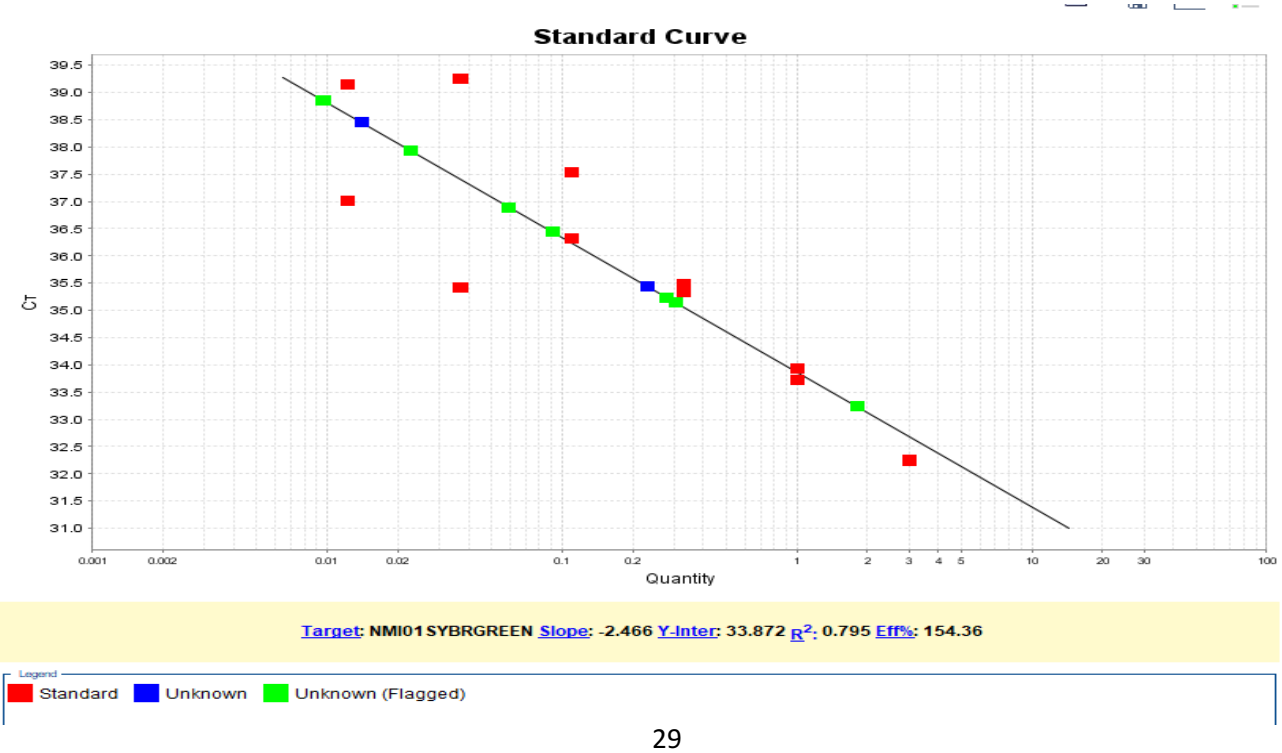
Duplicate analysis of unknown samples using the 5uM primer concentrations of both rbcL and matK genetic regions for *M. citrifolia* was performed. A qPCR run was performed using *M. citrifolia* DNA as a reference standard with both the rbcL and matK regions. Triplicate testing performed for non-template of rbcL and matK for *M. citrifolia* showed Ct values of 35.57 – 39.31. The positive controls for matK *M. citrifolia* showed consistent Ct results with standard concentration 1 (same sample used) with values ranging between 31.94 – 32.49. Dilution standard of *M. citrifolia* rbcL primer showed no results. Duplicate testing was performed for each primer standard and unknown samples. Standard curve results showed a slope of -2.466 and a R^2 value of 0.795. These values are less than optimal for quality check of standard curve but were included for the development of the plant custom assay; see criteria for the human Quantiblot, see table 9 for results and Figure 2 for standard curve. All unknown samples showed amplification with the matK primer (32.21 – 39.16 Ct values). Unknown sample MCS-004 and MCS-007 showed Ct values of 33.34 and 37.43 respectively in one of the test duplicates using rbcL primer (*M. citrifolia*). MCS-004 and 005 showed Ct values (33.34 – 39.26) with the rbcL primer in both repetitions.

Table 9: Summarized for 5uM primer concentration using *M. citrifolia* as DNA standard reference.

Sample Name	Task	Ct Value			DNA Qty (ng/ul)		
		T1	T2	T3	T1	T2	T3
NTC MC MATK	NTC	35.57	39.31	Und.	/	/	/
POS CTR MATK	Unknown	31.94	32.31	32.49	14.5	4.28	3.64
ST1 MATK	Standard	32.25	32.27		3.0	3.0	
ST2 MATK	Standard	33.73	33.93		1.0	1.0	
ST3 MATK	Standard	35.49	35.34		0.330	0.330	
ST4 MATK	Standard	37.53	36.33		0.110	0.110	
ST5 MATK	Standard	39.26	32.43		0.0370	0.0370	
ST6 MATK	Standard	39.16	37.01		0.0123	0.0123	
MCS-001 MATK	Unknown	35.46	Und.		0.232	Und.	
MCS-002 MATK	Unknown	36.96	39.28		0.056	0.006	
MCS-003 MATK	Unknown	39.13	36.86		0.007	0.062	
MCS-004 MATK	Unknown	36.44	37.93		0.091	0.023	
MCS-005 MATK	Unknown	38.85	38.45		0.01	0.014	
MCS-006 MATK	Unknown	36.89	35.15		0.06	0.303	
MCS-007 MATK	Unknown	32.82	32.21		2.678	4.729	
MCS-008 MATK	Unknown	35.24	33.23		0.279	1.816	

Legend: NTC: Non-Template Control, ST: Standard

Figure 2: Standard curve generated using 5uM primer concentration of rbcL and matK *M. citrifolia* as standard.



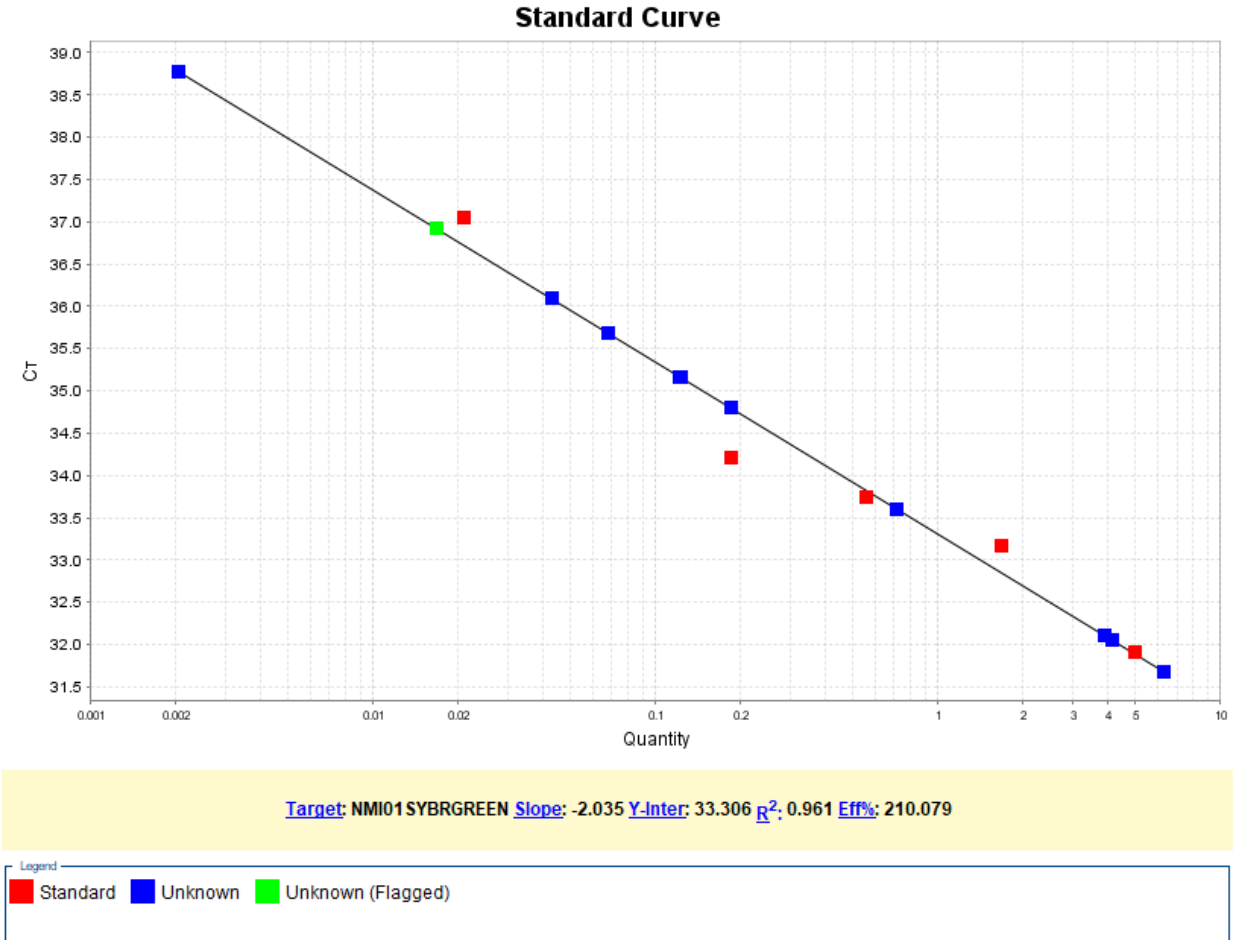
Standard curve using 2uM primer concentrations of both rbcL and matK genetic regions for *M. citrifolia* and wheat standard dilutions showed an R^2 value of 0.39 and a slope of -1.64 which is not optimal for reporting data. A second standard curve was performed using only matK (*M. citrifolia*) standard results improved to a R^2 value of 0.96 and a slope of -2.04. Testing for specificity of primers to species by adding primers designed for *M. citrifolia* to wheat and primers designed for wheat to *M. citrifolia* and unknown samples was performed. Wheat DNA showed no amplification with matK and rbcL *M. citrifolia* designed primers. Unknown samples MCS-005 and 006 showed Ct values over 36 with wheat specific primers (matK and rbcL respectively, both samples do not have wheat listed in the ingredients).

Table 10: Summarized results using for 2uM primer concentration using *M. citrifolia* and *T. aestivum* as DNA standard reference.

Sample Name	Task	Ct Value			DNA Qty (ng/ul)		
		T1	T2	T3	T1	T2	T3
NTC MATK MC	NTC	Und.	Und	Und.	Und.	Und.	Und.
POS CTR MATK MC	Unknown	31.82	31.88	31.5	4.121	3.888	6.274
ST1 MATK MC	Standard	31.77			5.0		
ST2 MATK MC	Standard	32.97			1.67		
ST3 MATK MC	Standard	33.57			0.556		
ST4 MATK MC	Standard	34.08			0.185		
ST5 MATK MC	Standard	Und.			Und.		
ST6 MATK MC	Standard	36.91			0.0210		
MCS-005 MATK (W)	Unknown	36.36			0.130		
MCS-006 RBCL (W)	Unknown	37.03			0.0509		
MCS-001 MATK (MC)	Unknown	35.56			0.396		
MCS-002 MATK (MC)	Unknown	Und.			Und.		
MCS-003 MATK (MC)	Unknown	38.67			0.00507		
MCS-004 MATK (MC)	Unknown	36.8			0.0699		
MCS-005 MATK (MC)	Unknown	35.89			0.252		
MCS-006 MATK (MC)	Unknown	34.69			1.34		
MCS-007 MATK (MC)	Unknown	35.08			0.779		
MCS-008 MATK (MC)	Unknown	33.46			7.61		

Legend: NTC: Non-Template Control, W: Wheat, M.C: *M. citrifolia* , ST: Standard (): Species primer used

Figure 3: Standard curve generated using 2uM primer concentration matK *M. citrifolia* standard dilutions.



Analysis with 2uM primer concentration using only matK genetic region for *M. citrifolia* generated a standard curve with an R^2 value of 0.98 and a slope of -3.84. Three out of the six non template controls for matK showed positive results with Tm values that were not consistent with positive and standard controls. All test repeats of unknown samples MCS-006, 007 and 008 showed positive results. Positive controls for matK *M. citrifolia* showed consistent CT results with standard concentration 1 (same sample used).

Table 11: Summarized results using for 2uM primer concentration using *M. citrifolia* matK as standard reference.

Sample Name	Task	Ct Value			DNA Qty (ng/ul)		
		T1			T1		
NTC MC MATK	NTC	35.92	Und	32.56	/	/	/
		Und.	36.35	Und.	/	/	/
POS CTR MC MATK	Unknown	26.08	25.78	26.17	3.92	4.67	3.71
		25.87	25.95	25.56	4.44	4.24	5.36
ST1 MC MATK	Standard	25.72	25.71	25.64	5.97		
		26.10					
ST2 MC MATK	Standard	26.84	27.16	27.40	1.99		
		26.94					
ST3 MC MATK	Standard	28.91	28.74	28.53	6.63E-01		
		28.69					
ST4 MC MATK	Standard	30.88	30.92	30.63	2.21E-01		
		30.90					
ST5 MC MATK	Standard	32.00	31.86	32.75	7.37E-02		
		32.05					
ST6 MC MATK	Standard	34.88	35.66	35.23	2.46E-02		
		34.79					
MCS-001	Unknown	36.93	37.47	39.50	0.006	0.004	0.001
		36.62	36.69	36.40	0.007	0.007	0.008
		36.45			0.008		
MCS-002	Unknown	35.75	Und.	Und.	0.012	Und.	Und
		38.93	37.00	36.89	0.002	0.006	0.006
		38.50			0.002		
MCS-003	Unknown	34.95	36.86	34.70	0.019	0.006	0.022
		38.47	37.64	37.30	0.002	0.004	0.005
		36.21			0.009		
MCS-004	Unknown	Und.	35.70	33.71	Und.	0.012	0.04
		38.29	38.03	36.68	0.003	0.003	0.007
		Und.			Und.		
MCS-005	Unknown	Und.	38.41	35.32	Und.	0.002	0.015
		36.51	35.09	35.60	0.007	0.018	0.013
		35.33			0.015		
MCS-006	Unknown	31.57	31.58	31.37	0.145	0.144	0.163
		32.19	33.92	33.65	0.100	0.035	0.042
MCS-007	Unknown	36.92	34.65	35.17	0.006	0.023	0.017
		37.34	36.40	33.91	0.004	0.008	0.036

		35.73			0.012		
MCS-008	Unknown	29.49	29.66	29.81	0.505	0.457	0.417
		30.38	33.41	33.52	0.297	0.048	0.045
		32.10			0.106		

Figure 4A: Standard curve generated using multiple replicates 2uM primer concentration matK *M. citrifolia* standard dilutions.

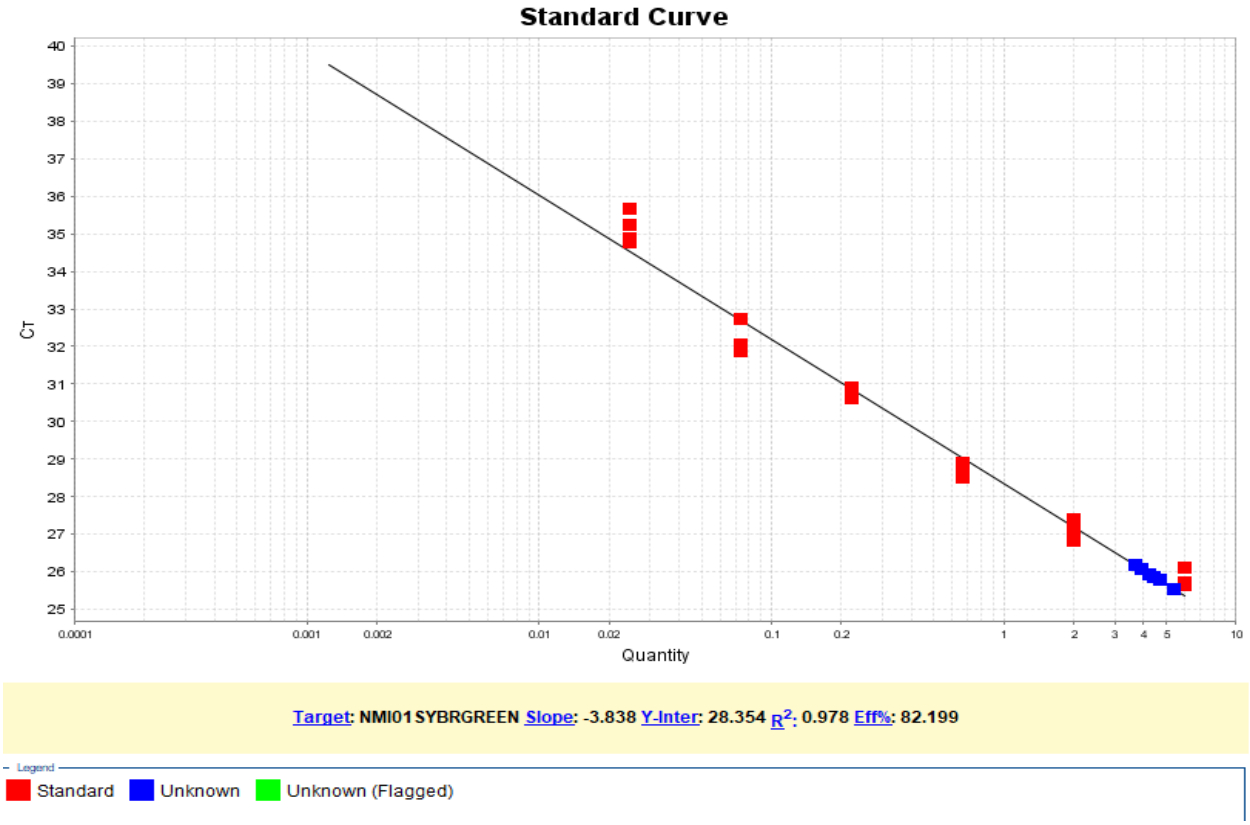
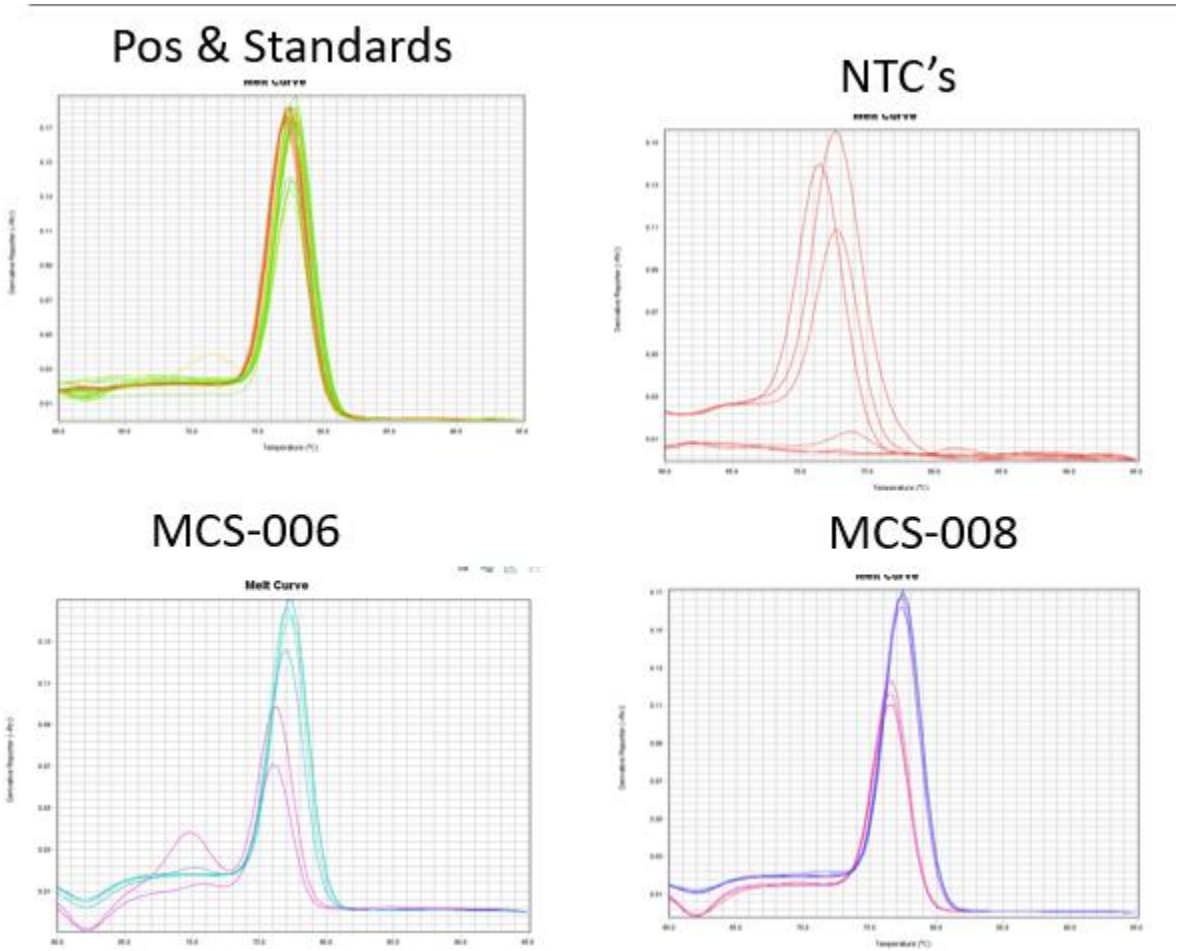


Figure 4B: Melting curve graph of standard dilutions, positive controls, amplified NTC and Unknown samples MCS-006 and MCS-008 at 2uM primer concentration.



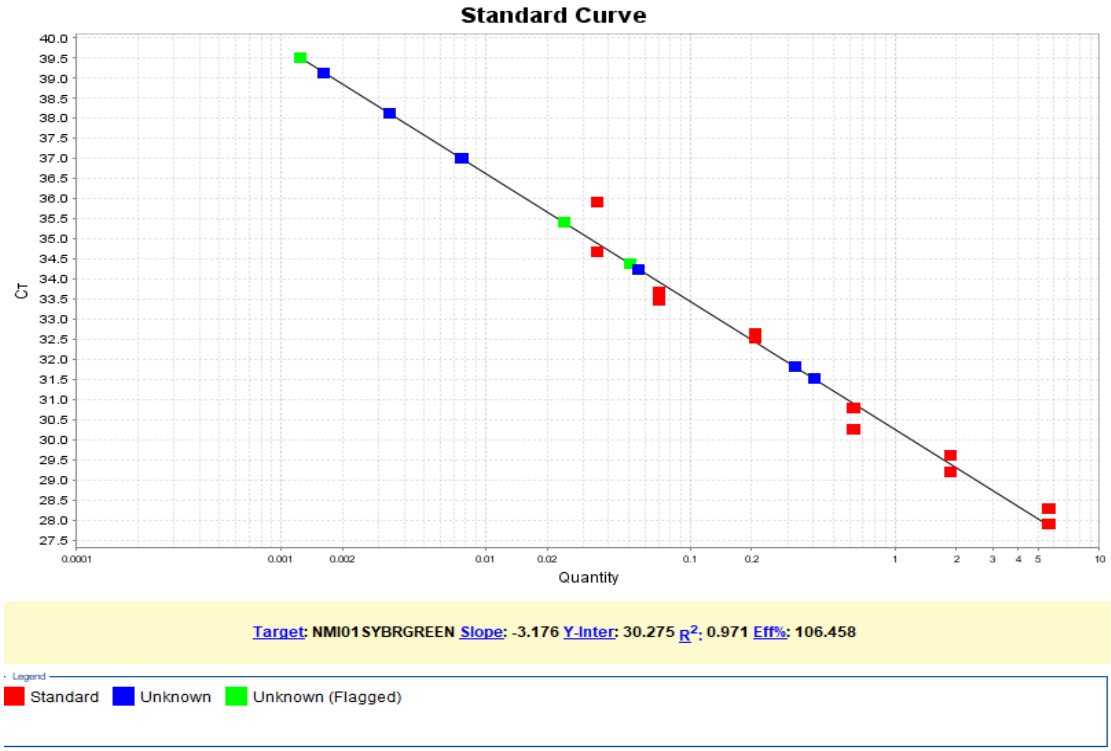
Analysis with 2uM primer concentration using only rbcL genetic region for *M. citrifolia* showed Ct results in MCS-001 (36.57) and MCS-004 (39.6). Standard dilutions, non-template controls and positive control results were negative. A standard curve was not able to be determined since standards for genetic regions rbcL did not show results. Standard curve using 5uM primer concentrations matK genetic regions for *M. citrifolia* showed an R^2 value of 0.97 and a slope of -3.15. Positive controls with rbcL primer for *M. citrifolia* did not show results. Specificity

testing of *M. citrifolia* matK primer with wheat DNA showed a Ct value of 32.59. Unknown samples did not show results below 38 Ct.

Table 12: Summarized results using for 5uM primer concentration using *M. citrifolia* matK as DNA standard reference.

Sample Name	**Task**	**Ct**	**Qty**
RBCL NTC	NTC	Undetermined	/
RBCL NEG CTR (Wheat)	Unknown	Undetermined	/
RBCL POS CTR (M.C)	Unknown	Undetermined	/
MATK NEG CTR (WHEAT)	Unknown	32.59	6.45E-02
MATK POS CTR (MC)	Unknown	26.61	5.07
MATK NTC	NTC	Undetermined	Und
MCS-001 (RBCL)	Unknown	38.1	1.16E-03
MCS-002 - MCS-004 (RBCL)	Unknown	Undetermined	Und
MCS-005 (RBCL)	Unknown	38.68	7.54E-04
MCS-006 (RBCL)	Unknown	Undetermined	Und
MCS-007 (RBCL)	Unknown	39.81	3.30E-04
MCS-008 (RBCL)	Unknown	Undetermined	Und
MCS-001 (MATK)	Unknown	36.69	3.22E-03
MCS-002 (MATK)	Unknown	Undetermined	Und
MCS-003 (MATK)	Unknown	35.67	6.79E-03
MCS-004 (MATK)	Unknown	34.03	2.26E-02
MCS-005 (MATK)	Unknown	32.93	5.02E-02
MCS-006 (MATK)	Unknown	30.27	3.50E-01
MCS-007 (MATK)	Unknown	37.91	1.32E-03
MCS-008 (MATK)	Unknown	30.04	4.16E-01

Figure 5: Standard curve generated using multiple replicates 2uM primer concentration matK *M. citrifolia* standard dilutions.



- **Gel Electrophoresis and Bioanalyzer**

Extracted but not amplified DNA from the control samples *M. citrifolia* (positive) and wheat (negative) showed a band size of approximately 15,000bp (likely extracted genomic DNA). The intensity of the wheat samples was higher compared to the *Morinda* fruit.

RbcL gel electrophoresis of amplified rbcL *M. citrifolia* primer showed multiple bands in positive and negative control. Non template control showed no bands. A mixture was observed in samples MCS-006 – 008. No results were observed in samples MCS-001 – 005.

Mat K amplified MCS-008 sample using matK primer showed a slight band near the lower 100bp region (expected 108bp). An amplified region using 5uM of matK primer showed

consistent results in all samples (including NTC and negative controls) near the 130bp region. A duplicate gel confirmed the results. A slight band was observed with the 2uM matK primer concentration for *M. citrifolia* on unknown samples MCS-006 and MCS-008 near the 130 bp region. See images below for results.

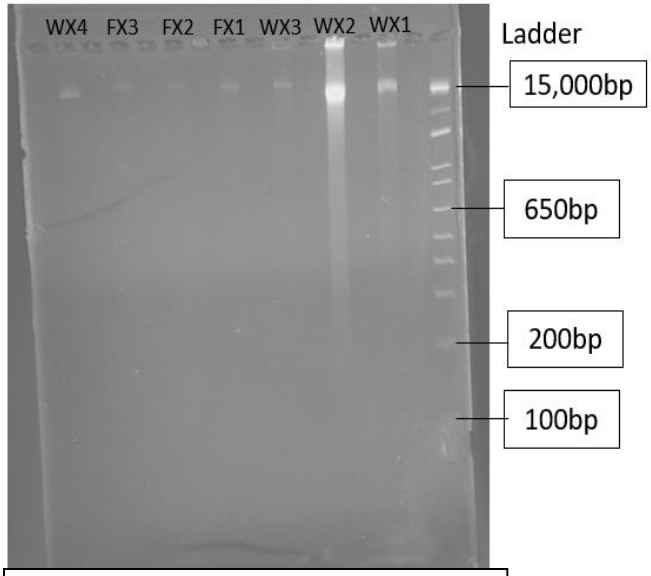


Image 1: Extracted control samples (no amp) WX: Wheat, FX: Fruit

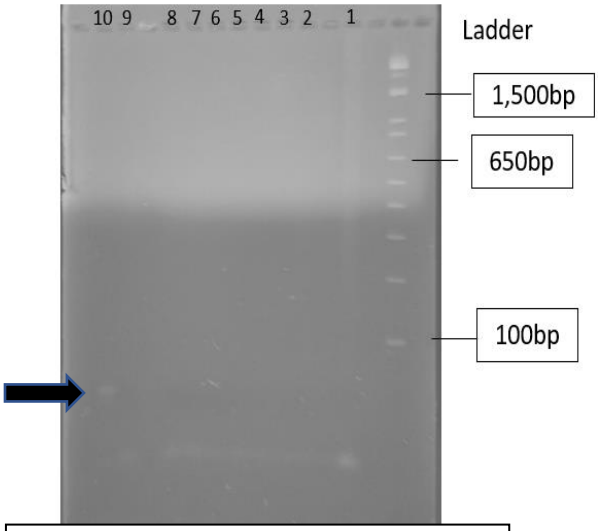


Image 2: Amplified samples using matK (2uM) primer with Pfu polymerase

1: Pos Ctr 2: Neg. Ctr. 3-10: MCS-001-008

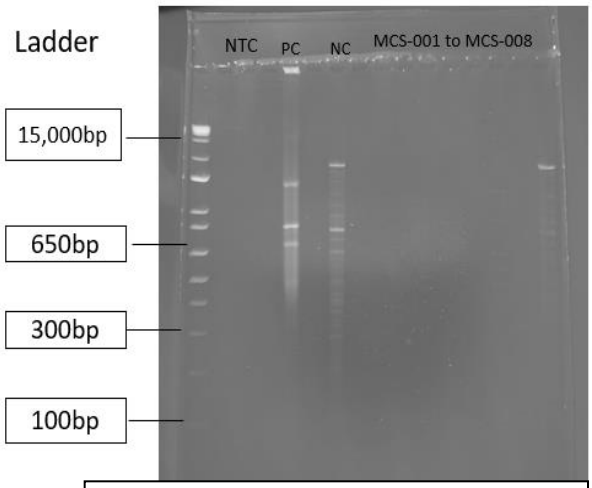


Image 3: Gel electrophoresis of *M.citrifolia* rbcL (5uM) primers

(Terra primer PCR Kit)

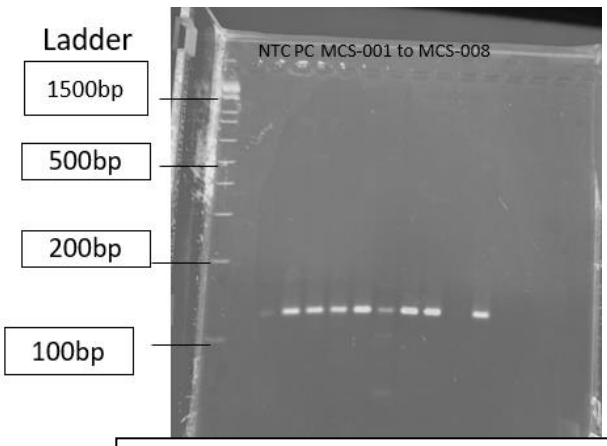


Image 4: Gel electrophoresis of *M.citrifolia* matK (5uM) primers

(Terra 5uM primer PCR Kit)

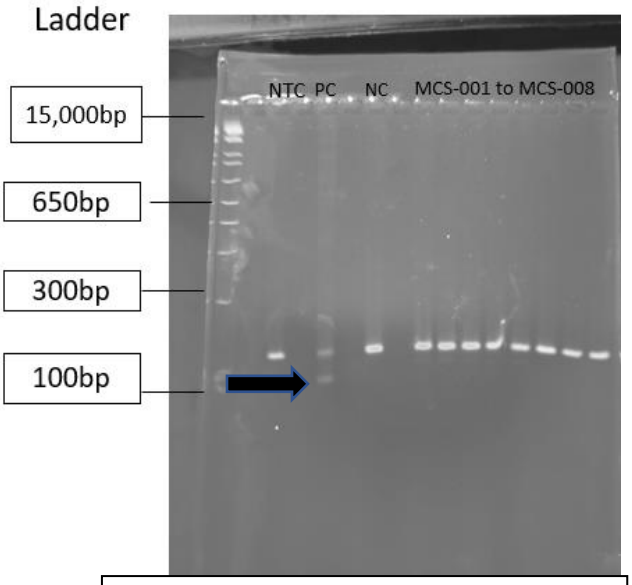


Image 5: Gel electrophoresis of *M.citrifolia* matK (5uM) primers (dup.)

(Terra primer PCR Kit)

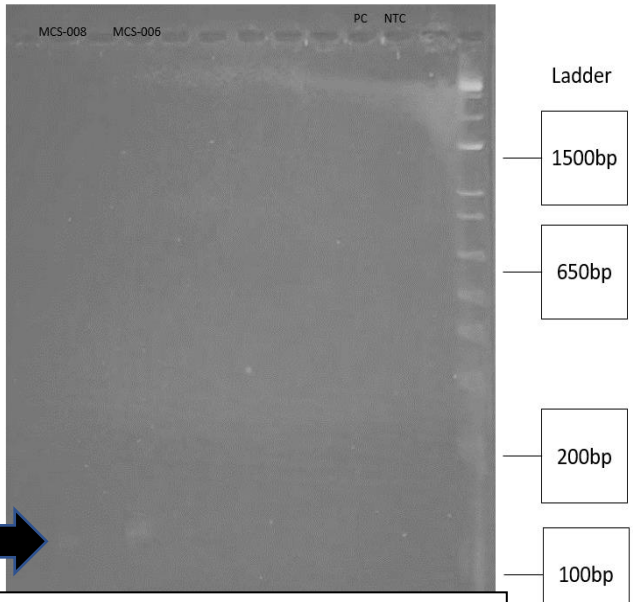


Image 6: Gel electrophoresis of *M.citrifolia* matK (2uM) primers

(Terra Kit Polymerase)

Analysis of amplified product with the matK *M. citrifolia* primer at 5uM showed results in all samples except unknown MCS-001 and MCS-004. Negative control wheat was not analyzed. Non-template control, positive control, and unknown samples MCS-002 & 003 showed consistent peak that showed a DNA fragment of approximately 362 -380bp. Unknown sample MCS-002 showed a mixture of amplified DNA with a total of 7 peaks detected. Unknown samples MCS-005, 006, and 008 showed consistent results that showed the presence of two DNA fragments which ranged between 75 – 83bp and 122-141bp respectively. Results for unknown sample MCS-007 show two DNA fragments of 24bp and 39bp. A comparison between the results obtained from the Bioanalyzer and Gel electrophoresis are stated in table 12. Bioanalyzer gel image is shown in Figure 6.

Figure 6: Bioanalyzer Gel Image of matK amplified samples using Terra PCR Kit

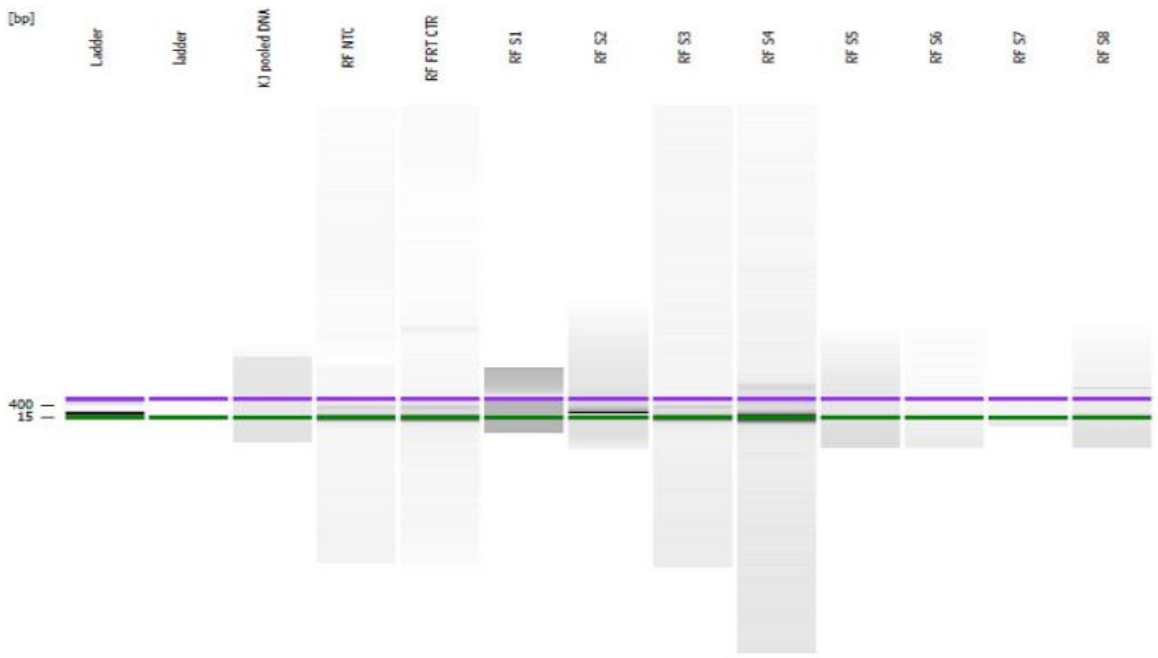


Table 12: Comparison between Bioanalyzer and Gel Electrophoresis data of amplified matK (5uM) samples with Terra Kit protocol.

Bioanalyzer Data				Gel Electrophoresis	
Sample	**Peak(s)**	**Size ((bp)**	**Conc. (ng/ul)**	**Band Observed Gel Electrophoresis**	**Size (ladder bp)**
NTC	1	366	4.81	1	Near 130
Fruit Positive Control	1	380	4.35	2	100, Near 130
MCS-001	None	N/A	N/A	1	Near 130
MCS-002	7	80; 201; 294; 362; 489; 803; 863	10.70, 58.49, 14.20, 10.92, 3.61, 1.98, 2.06	1	Near 130
MCS-003	1	373	5.22	1	Near 130
MCS-004	None	N/A	N/A	1	Near 130
MCS-005	2	77; 122	20.03, 8.60	4	>100 (2x), 100, Near 130
MCS-006	2	75; 125	1.80, 0.57	1	Near 130
MCS-007	2	24; 39	104.06, 43.76	1	Near 130
MCS-008	2	83; 141	81.45, 32.90	1	Near 120
*Detection limit for DNA using the Bioanalyzer is 50pg/ul, for Gel electrophoresis is 10ng/ul.					

- **Sequencing Results**

Since the RT-PCR assay fluorescence was evaluated as being a result of DNA fragment mixtures, a band purification method was used to isolate and sequence bands independently to establish what the matK primer set was binding to and the resultant sequence of the amplification products. The separated bands in gel electrophoresis were submitted for sequencing to Yale School of Medicine W. M. Keck Foundation for sequencing (https://medicine.yale.edu/keck/dna/). Data obtained showed a nonspecific sequence in the not amplified NTC control for matK primer, and positive control for matK. Forward primer sequence for amplified matK NTC (gel extracted) match *M. citrifolia* with an identity of 97% having one mismatch base and amplicon gaps. Non-specific sequences with the rbcL forward primer were obtained for the non-amplified NTC and positive control (*M. citrifolia* DNA, and unknown sample MCS-008 first band. For the rbcL reverse primer, non- specific sequences were obtained in the non-amplified NTC and unknown sample MCS-007 (last band), *Mus musculus* lacZ tagged mutant allele was sequenced in the three bands for unknown sample MCS-008 as well as unknown sample MCS-006 last band with the rbcL reverse primer and the not amplified matK NTC. (See table 13 for summarized results). A comparison between the sequence obtained that matched with *M. citrifolia* was performed to see match possibility with other Morinda species (see Image 8).

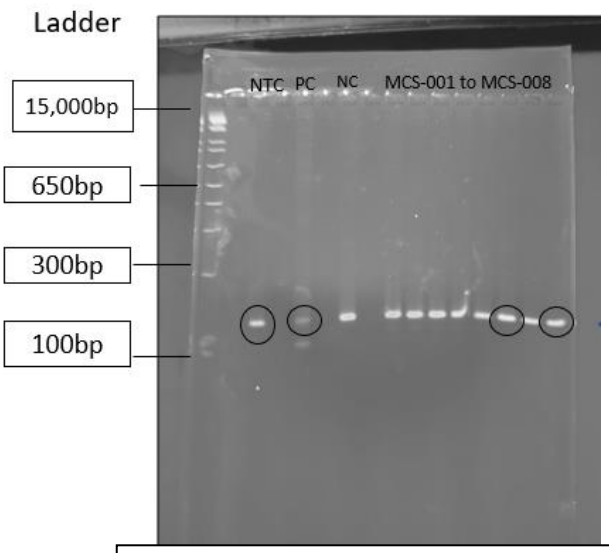


Image 7: Circled, bands that showed results in sequencing (matK).

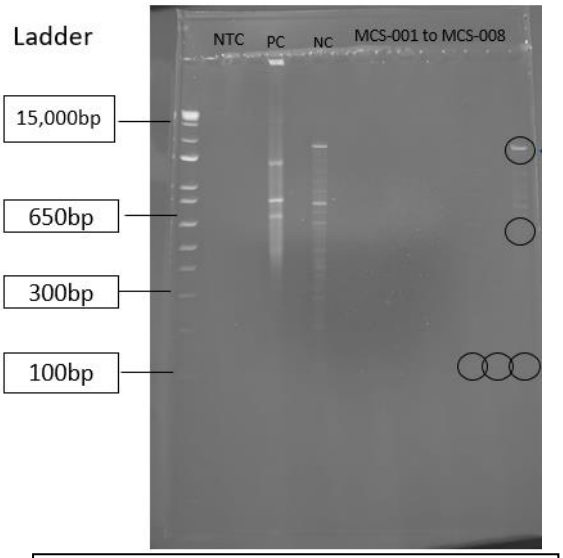


Image 8: Circled, bands that showed results in sequencing (rbcL).

Table 13: Summary of sequencing results with Blast search results.

Sample	Primer	Size Sequenced (bp)	Blast Search	%ID
NTC (No amp.)	matK Forward	191	No match	N/A
PC	matK Forward	189	No match	N/A
NTC (Amped)	matK Forward	152	*M. citrifolia* matK and partial trnK gene intron	96
NTC (No amp)	rbcL Forward	155	No match	N/A
MCS-008 (1st band)	rbcL Forward	279	No match	N/A
NTC (No amp)	rbcL Reverse	176	No match	N/A
MCS-006 (Last band)	rbcL Reverse	206	*Mus musculus* lacZ tagged mutant allele	92.6
MCS-007 (Next to last band)	rbcL Reverse	406	Synthetic construct modified HIV-1 subtype C backbone	79.9
MCS-007 (Last band)	rbcL Reverse	319	No match	N/A
MCS-008 (1st band)	rbcL Reverse	400	*Mus musculus* lacZ tagged mutant allele	86.7
MCS-008 (2nd Band)	rbcL Reverse	206	*Mus musculus* lacZ tagged mutant allele	96.6

MCS-008 (Last Band)	rbcL Reverse	242	*Mus musculus* lacZ tagged mutant allele	92
Pos. Ctr. (No amp)	rbcL Forward	159	No match	N/A
NTC (No amp.)	matK Forward	281	*Mus musculus* lacZ tagged mutant allele	87.8

Image 8: Sequence search comparing possible Morinda species of sequenced result matK

Morinda citrifolia chloroplast matK gene for maturase K and partial trnK gene intron

Range 1: 571 to 649 GenBank Graphics Next Match Previous M

Score	Expect	Identities	Gaps	Strand
128 bits(69)	5e-26	76/79(96%)	2/79(2%)	Plus/Plus

```
Query  5    GTTGA-AAGTTTCT-CCCGTCCCCGAGGTATCTATTCTTATGAGAATACTTTGTTTTTGA  62
            ||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  571  GTTGATAAGTTTCTACCCGTCCCCGAGGTATCTATTCTTATGAGAATACTTTGTTTTTGA  630

Query  63   CTGTATCGCACTACGTATC  81
            ||||||||||||| |||||
Sbjct  631  CTGTATCGCACTATGTATC  649
```

Morinda officinalis chloroplast, complete genome

Score	Expect	Identities	Gaps	Strand
111 bits(60)	5e-21	73/79(92%)	2/79(2%)	Plus/Minus

```
Query  5     GTTGA-AAGTTTC-TCCCGTCCCCGAGGTATCTATTCTTATGAGAATACTTTGTTTTTGA  62
             ||||| |||||||  |||| ||||||||||||||||||| ||||||||||||||||||||
Sbjct  3669  GTTGATAAGTTTCGACCCGCCCCCGAGGTATCTATTCTTACGAGAATACTTTGTTTTTGA  3610

Query  63    CTGTATCGCACTACGTATC  81
             ||||||||||||| |||||
Sbjct  3609  CTGTATCGCACTATGTATC  3591
```

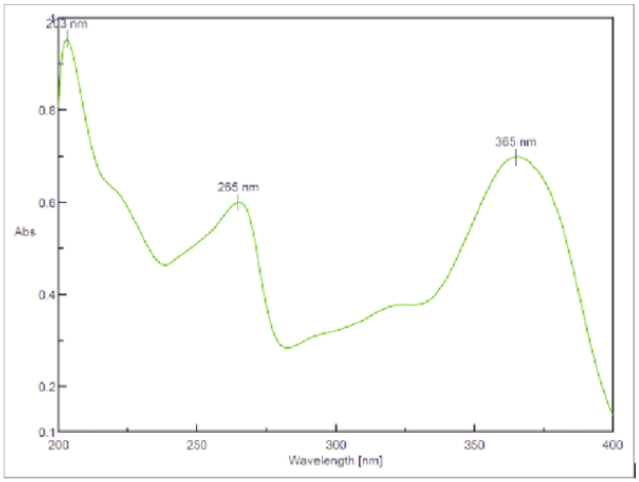
- **UV-Vis and FTIR Results**

UV-Vis spectra was obtained for bioactive compound kaempferol. Standard control of kaempferol show consistent peaks with reference literature (Telange, Darshan R., 2014 See Image 9). Data shows one consistent peak between unknown samples and positive control (*M. citrifolia*) near the 272 – 299 wavelength regions. Unknown samples MCS-003,004, and 006 showed consistent UV spectra showing a peak near the 665 region. Unknown samples MCS-001 and 008 also showed consistence spectra with an absorbance at 3 peak region. Positive and unknown samples did not show peak near the 368 wavelength region, present in kaempferol

standard control. Negative control (wheat) showed peaks that were not consistent with standard, positive, and unknown samples (see summarized data below).

Image 9: Standard control for kaempferol from literature search (panel A) and performed in experiment (panel B)

Panel A



Panel B

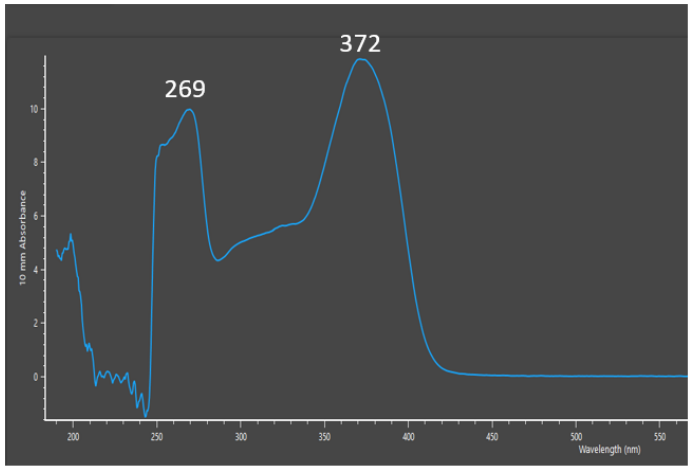
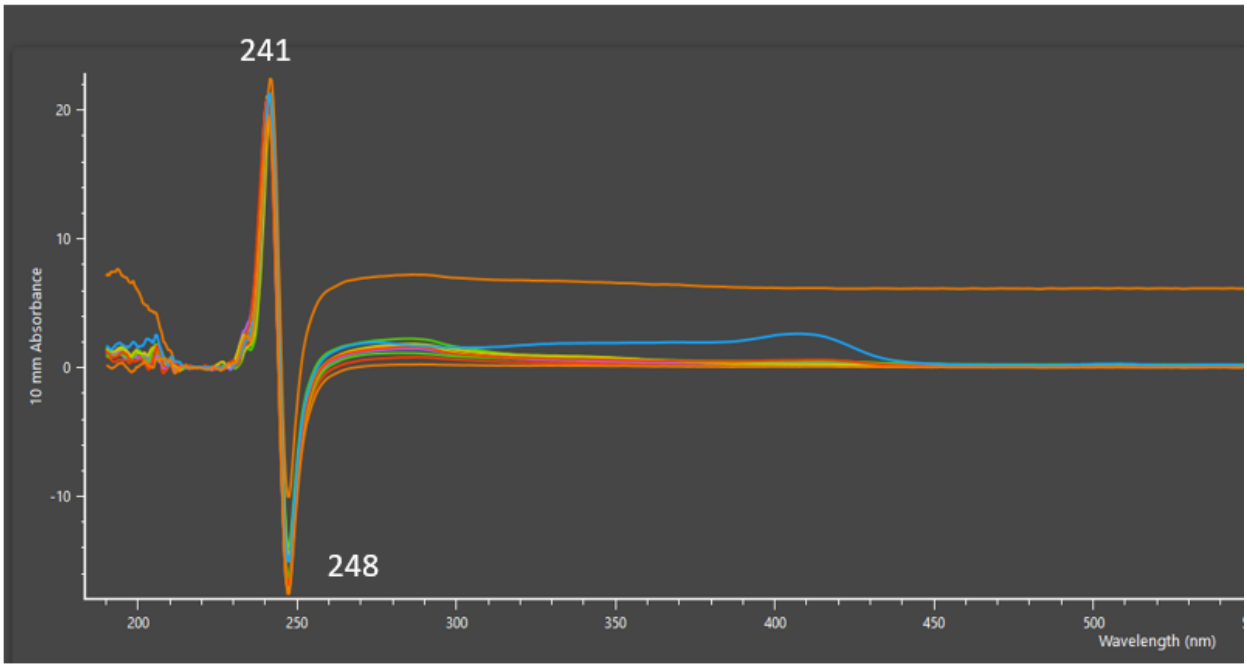


Table 14: Summary of UV-Vis results peaks for kaempferol extraction.

Sample:	**KC**	**NC**	**PC**	**MCS -001**	**MCS -002**	**MCS -003**	**MCS -004**	**MCS -005**	**MCS -006**	**MCS -007**	**MCS -008**
Peak (s) Observed	269	252	272	273	283	299	275	283	286	274	281
	368	291		412		665	665		664		411
		301		663							666
		414									
		426									
		505									
		538									
		610									
		664									

Analysis results for damnacanthal bioactive compound using the UV-Vis showed consistent results between all samples (NTC, standard, positive and negative controls, and unknown samples) Same peaks were observed (positive absorbance at 241nm and negative absorbance at 248nm) at different absorbance ratios (See image 10).

Image 9: Overlay spectra of samples (DMSO blank).



FTIR data results for dried product showed that wheat negative control had similar peaks as in fresh fruit. Different parts of the fresh fruit were analyzed in the FTIR instrument (see table below). Kaempferol control show peaks not present in powder supplements or fruit sample controls. Wheat negative control shared highly similar results to fruit control. For methanol extracts, peaks of herbal supplements and wheat negative control are highly consistent with methanol blanks. The same occurrence happened when analyzing DMSO extracts, results of

herbal dietary supplements and wheat extracts are consistently similar with DMSO blank controls.

Table 15: Summary of FTIR data results peaks for fresh fruit, kaempferol, and dried product.

Sample	MC Fruit (Inside Center)	MC Fruit (Seed)	MC Fruit (Skin)	MC Fruit (Skin Dot)	Kaempferol	MCS-001	MCS-002	MCS-003	MCS-004	MCS-005	MCS-006	MCS-007	MCS-008	Wheat
Peak	3334.06	3334.82	3334.77	3331.90	3306.41	3332.40	3286.15	3331.39	3328.86	3328.80	3286.46	3320.85	3293.65	3286.44
		2943.18	2922.22	2921.48	2916.29	3009.20	2954.62	2921.81	2921.82	2923.37	2918.51	2919.14	2923.25	2917.77
		1732.63	1732.55	1732.60	2848.85	2923.15	2919.09	2852.33	2852.36	2853.85	2850.67	2850.75	2853.55	2849.65
	1633.80	1634.39	1639.48	1633.88	1655.16	2853.56	2850.98	1742.70	1742.66	1738.04	1741.93	1741.66	1741.14	1633.88
	1422.39	1441.02	1441.98	1422.08	1608.55	1741.14	1741.20	1614.28	1614.50	1710.72	1633.48	1614.30	1710.48	1548.28
	1371.20	1369.94	1370.55	1370.23	1593.41	1712.56	1711.49	1538.88	1539.31	1614.62	1539.13	1511.00	1620.92	1377.91
	1314.37	1314.90	1316.68	1316.65	1566.46	1608.44	1633.39	1509.86	1509.94	1510.57	1463.63	1417.14	1510.67	1316.62
	1257.66	1239.77	1240.76	1241.86	1506.09	1509.23	1538.90	1454.99	1455.01	1417.26	1426.83	1370.84	1416.56	1238.92
	1157.68	1150.53	1202.97	1146.74	1440.96	1420.66	1464.35	1416.77	1416.62	1371.18	1414.24	1315.96	1371.27	1202.86
	1103.26	1101.59	1156.49	1100.70	1375.00	1371.54	1426.79	1371.29	1371.28	1316.29	1370.92	1227.80	1316.84	1035.89
	1053.75	1051.49	1101.87	1050.48	1299.68	1316.87	1414.43	1316.83	1316.72	1236.26	1317.06	1050.33	1236.43	898.78
	1033.00	1017.99	1053.30	1020.09	1275.88	1236.35	1371.59	1232.92	1233.93	1154.39	1235.12	1031.56	1027.60	666.68
	661.19	664.90	1019.51	664.57	1248.27	1030.95	1317.47	1155.03	1155.21	1027.20	1147.54	896.11	895.17	630.03
	599.34	633.63	664.56	645.43	1221.55	895.17	1231.76	1030.97	1030.69	896.34	1020.42	780.36	814.84	599.71
	568.36	602.91	599.62	599.17	1191.66	818.36	1050.84	896.28	895.77	811.59	815.21	667.05	667.48	561.16
	564.09	599.14	595.50	568.31	1163.83	758.23	1033.03	812.10	665.76	780.44	758.74	630.56	633.37	553.31
	560.65	564.63	568.51	564.13	1129.56	665.34	896.53	666.94	599.65	667.06	715.33	603.47	599.06	534.41
		560.92	564.75	560.21	1087.26	630.80	807.40	630.53	561.30	630.79	667.16	561.18	567.93	
		553.37	560.83		1007.49	603.45	780.53	595.56		599.67	626.34	557.42	557.50	
					974.91	595.87	715.74	568.65		595.55	599.27	553.75	548.07	
					881.54	572.99	666.34	561.29		572.72	568.56	526.99	533.43	
					864.30	564.65	626.58	557.53		561.43	561.01		526.41	
					845.14	557.43	599.60	553.54		553.62	553.63			
	Legend:				817.12	553.50	568.69	549.55		542.01	534.33			
	Yellow : Peak not found in fruit control				794.51	542.26	561.10	534.09		534.35	527.02			
	Blue: Peak within 10 from fruit control				721.34	533.93	557.37			526.64				
	Green: Peak within 5 from fruit control				701.99	526.65	553.38							
					667.74		526.73							
					637.12									
					621.37									
					599.16									
					583.95									
					564.78									

Legend: Yellow: peak not found in fruit control, Green: peak within 5 values of peak in fruit control, Blue: peak within 10 values of peak in fruit control.

Discussion

DNA extraction results from unknown samples and fruit control showed consistent concentrations of DNA but showed impurity. A desired value of 1.8 for A260/280 and 2.0 for A260/230 is recommended since it might indicate the presence of contaminants that might prevent amplification. Since we are dealing with plant material, it is most likely that phenols and carbohydrates are present in the extracted DNA samples which is affecting this value. An extraction of wheat DNA was performed with the same procedure and purity values were within recommendations. This shows that the extraction procedure was carried out correctly and the low purity values are isolated to *M. citrifolia* when following the protocol extraction. The low A260/230 values had no negative impact since amplification was later carried out successfully.

In order to accurately determine the presence of *M. citrifolia* in our unknown samples using the SYBR green dye in qPCR, a fluorescence assay development needed to be carried out. Analysis in qPCR showed that the designed primer for the matK region of *M. citrifolia* had better potential to serve as a possible primer when using the SYBR green fluorescent dye. Serial dilutions of DNA with different concentrations of primer showed that the matK and rbcL primers for wheat had some potential as serving as a reference standard when amplified at 2uM primer concentration. Wheat serial dilution Ct values were mostly consistent with their respective DNA concentration, but when combined with the standard dilutions for the matK primer for *M. citrifolia* with *M.citrifolia* DNA, it affected the results of the standard curve and therefore the ability of the assay to determine the accurate amplification of the unknown samples. Since we are trying to find *M. citrifolia* DNA in unknown samples and our designed *M. citrifolia* primers worked, using primer designed for *T. aestivum* with wheat DNA as a size standard was stopped.

When using only *M. citrifolia* amplified with matK product, a consistent standard curve was generated which helped us determine the concentration of our samples.

Since the qPCR process was carried out with the SYBR green dye, which attaches to any double stranded DNA that amplifies, we need to determine the specificity of our designed primers to the *Morinda* species and check the nature of our designed primer sets to create primer dimers. Specificity testing showed that matK primer has some cross-hybridization to wheat DNA when amplifying with 5uM primer concentration. This showed the potential of the matK *M. citrifolia* primer to be universal and not *Morinda* specific, which might pose a problem when using it for the identification of a plant species. The possibility of primer dimers was tested with our non-template controls (NTC's). Results for NTCs of matK showed some results when amplifying with 5uM and 2uM concentrations, but their Ct values were on the higher end. This is most probably due to background amplification since NTC for matK primer was carried out multiple times during qPCR analysis and most results were negative. Melting curve analysis showed a consistent Tm value of approximately 77 in all positive control samples and standard concentrations using *M. citrifolia DNA*. Melting curves of amplified DNA in NTC samples showed a Tm value that ranged from 71 – 73, which is not consistent with our positive controls. DNA analysis in qPCR showed that unknown samples MCS-006 and MCS-008 had consistent amplification results with Tm values consistent with our positive controls, which could indicate the presence of *M. citrifolia* in the herbal dietary supplements. Based on data, amplified of matK primer for M. citrifolia product needs to be sequenced in order to determine if the bands that are showing fluorescence in the qPCR assay are *M. citrifolia* specific and correlate to the desired target sequence of matK.

Gel electrophoresis of extracted DNA from controls showed that our extraction procedure performed successfully and extracted genomic DNA. Gel electrophoresis with Pfu polymerase PCR product showed amplified product for the matK genetic region that was not consistent in size with our expected matK primer amplicon size. Since gel electrophoresis was performed with a DNA 1kb ladder and our desired product size for matK (108 bp) it is on the lower end of that ladder, it is possible that a misread of size on the ladder was performed and also ladder bands present were not taken into account because they are not visible in the gel. With gel electrophoresis, a minimum concentration of 10ng/ul is needed to visualize genomic DNA material. It can also be possible that our amplified DNA does not reach that detectable concentration.

Gel electrophoresis with Terra PCR kit amplified product at 2uM primer concentration showed band results that were consistent with our desired primer size (108bp). These results correlated with qPCR results obtained for unknown samples MCS-006 and MCS-008. Positive control results for same gel did not show any results, it is possible since unknown samples showed a slight band, that the amplified product concentration of the positive control was not enough to create fluorescence. Gel electrophoresis of matk 5um primer concentration showed amplification in all samples. The procedure was repeated since negative results were expected in the non-template control samples. Second gel electrophoresis confirmed the results. Bioanalyzer data showed that a mixture of DNA was present in unknown sample MCS-002 that was not observed in MCS-002 in the gel electrophoresis. Also, results for the non-template control and fruit positive control showed product that was consistent. Since none of the observed peaks matched our designed amplicon size (108bp), gel bands were cut, purified, and DNA

extracted for sequencing. Gel electrophoresis of amplified product with rbcL primer at 5uM concentration was performed and results were not consistent with expected results. Therefore, gel bands were sequenced to determine if the amplified DNA belonged to *M. citrifolia.*

Sequencing results for the matK primer in NTC (not amplified) sample showed an amplicon sequence that could not be determined. This result shows the possibility of amplification of the NTC matK primer at 5uM which was consistent with qPCR testing with NTC showing high CT values in three out of the six NTC performed with this primer. Sequence of amplified band of matK NTC at 5uM (extracted from gel) belonged to *M. citrifolia.* Since non amplified NTC showed a sequence that did not belong to *M. citrifolia* , this result is most probably due to a mis labeling error since it shows that *M. citrifolia* DNA was present in this sample, was amplified and finally sequenced. Sequencing length does not match our desired amplicon size by 47bp (108bp matK) but is within expected amplicon size. This may due to loss of the sequencing region near where the primers attach to the template. Based on blast search, results for the sequencing of rbcL primer at 5uM showed that the DNA sequenced from the gel electrophoresis did not belong to *M. citrifolia* Unknown sample MCS-008 showed consistent sequencing results for mouse DNA, which might indicate contamination of either the sample or the reagents used in the analysis or during the manufacturing and packaging of the herbal supplement.

Spectroscopy data showed no reliable results for the identification of the herbal species by identifying bioactive compounds present using known standard for kaempferol and damnacanthal. UV-Vis data of extracted kaempferol was consistent between unknown samples, known standard and positive control, but not enough to make an identification. Only one peak

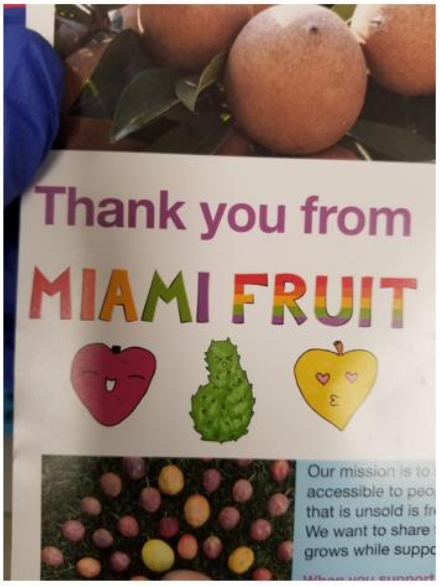
was consistent with the samples and it could not serve as a good discriminatory procedure. Extracted damnacanthal product showed the same results for all samples which indicates that the solvent used is masking the bioactive compound present in the samples or the compound is not extracting properly per procedure. Analysis of dried samples showed that powder extracts (as sold) cannot be identified using FTIR using standard reference of bioactive compound kaempferol alone. FTIR data of methanol and DMSO extracts showed high similarities between blank controls and unknown samples, which indicates that the solvent used for the extraction procedure is masking the bioactive compounds present making them undetectable by means of FTIR spectroscopy.

Conclusion

In order to detect fraud in manufacturing of herbal dietary supplement *M. citrifolia*, a fluorescence assay development was established. Results showed that designed genetic region of matK primer of *M. citrifolia* species has a degree of discriminatory potential in amplifying DNA for this species by melt curve analysis and PCR. In this study, we tested the primer dimer occurrence, specificity and discriminatory power of a novel matk primer for *M. citrifolia.* Mixtures of PCR product were obtained but at least one sample confirmed the presence of *Morinda citrifolia* by DNA sequencing and a high homology search of the NCBI database using the blast feature. Spectroscopy data showed that the purity of the bioactive compound used to possibly identify an herbal supplement plays a great role, therefore, multiple extraction procedures should be analyzed to ensure that spectroscopic results belong to the bioactive compound of interest. Nonetheless, the methodology implemented could be used for the fluorescent assay development of Morinda plant species with additional band purification and sequencing steps. Future optimization of PCR primer selectivity could be addressed to achieve greater specificity. Since herbal supplements are used by millions, this study provides an approach in developing a suitable method for the identification and regulation of herbal dietary supplement products and contents, which serves to protect consumer safety and wellbeing.

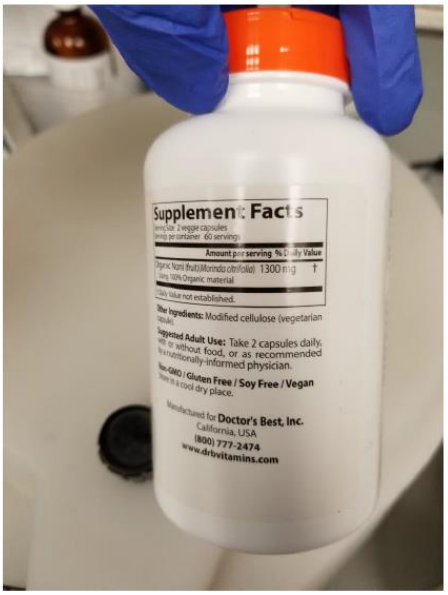
Appendix 1: Material (Samples)

Positive Control (*Morinda citrifolia* fruit)

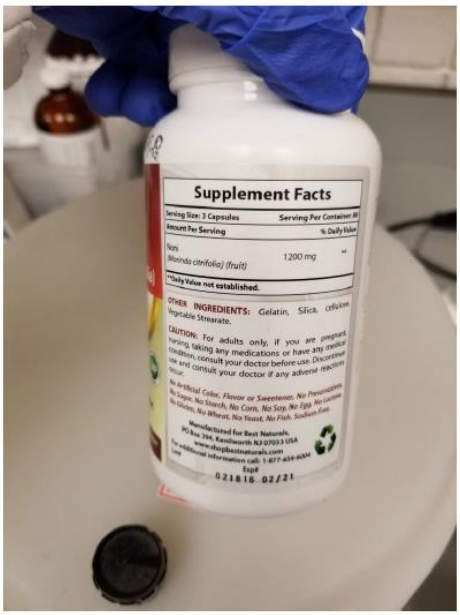


Wheat Negative Control

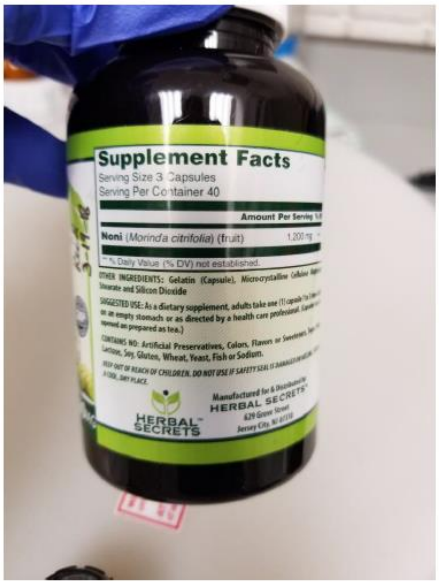
MCS-001



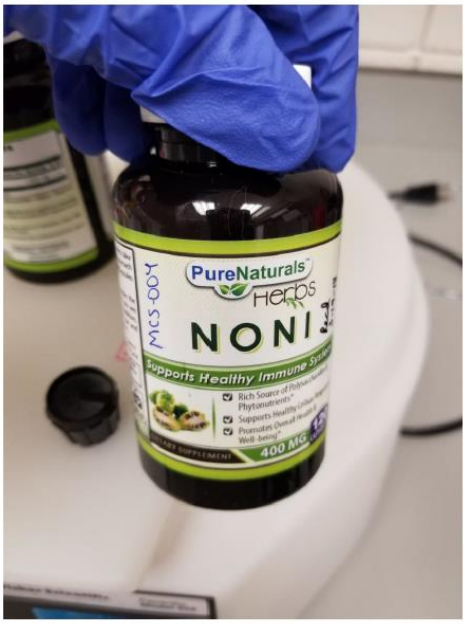
MCS-002



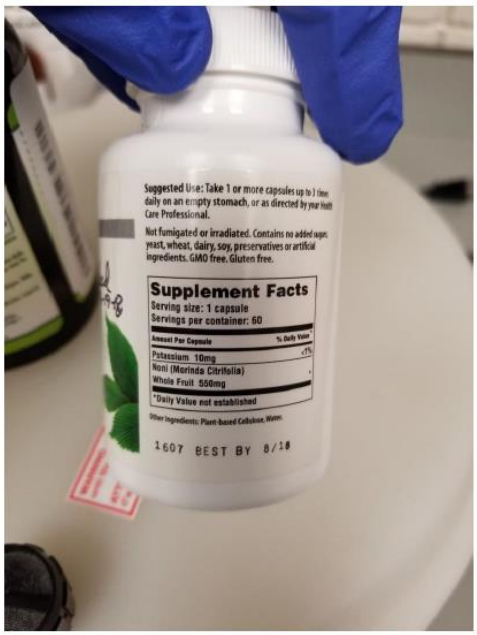
MCS-003



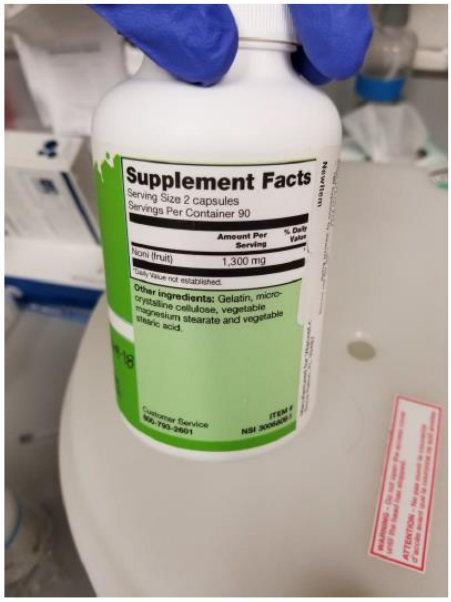
MCS-004



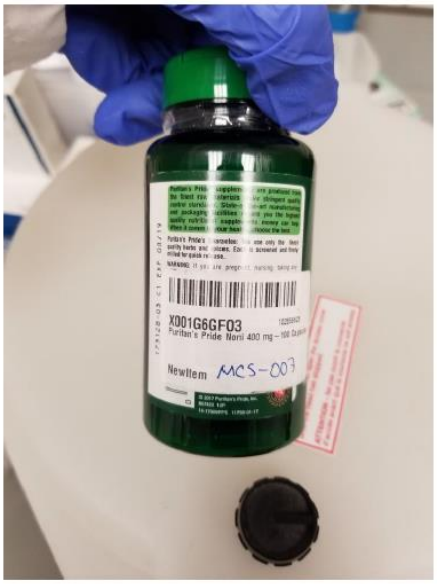
MCS-005



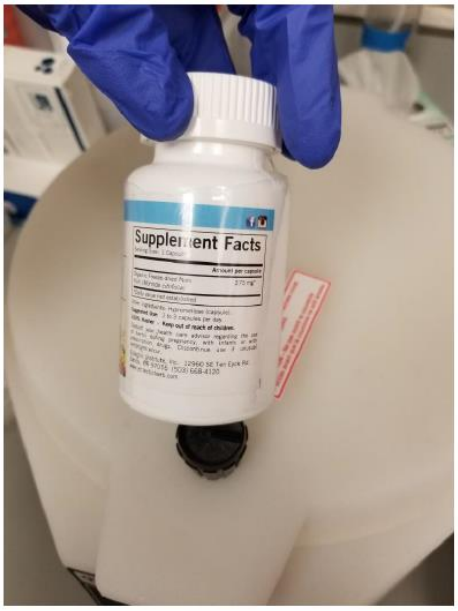
MCS-006



MCS-007



MCS-008



Appendix II: Primer Design Information

invitrogen™
by life technologies™

Order Number	Order Date	Page No.
41298900	7/10/2018	Page 1 of 1

Please refer to this No. on all inquiries

Made in U.S.A.

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DODDS HALL RM 402
300 BOSTON POST RD
Sandra Hartman-Neumann
WEST HAVEN, CT 06516 US
Ship to Account No.: 309489

CUSTOMER REP.	CUSTOMER P.O. NO.	CUSTOMER CONTRACT NO./RELEASE NO.	PLACED BY:
GES	VISA7528	/	Sandra Hartman-Neuma

Line No.	Cat No./Lot No.	Type	Description/Sequence	Quantity	Amount(OD)
1	A15609 - UP2YHJF 362176 C06	DNA	rbcL F M. Citrifolia for Ricardo L Fernandez Walker TGGCATTCAAGTCGAGAGAGATAAA	1 ea @ 25 bases	12.97
2	A15609 - UP34C4D 362176 C07	DNA	rbcL R M. citrifolia for Ricardo L Fernandez Walker ACAGAAGCACCACCTGAACTAAA	1 ea @ 23 bases	12.16
3	A15609 - UP496PA 362176 C08	DNA	mat K F M. citrifolia for Ricardo L Fernandez Walker AAAGGCGAGGATAGAGAAATAGAGA	1 ea @ 25 bases	13.69
4	A15609 - UP7EY97 362176 C09	DNA	mat K R M. citrifolia for Ricardo L Fernandez Walker AAACTGACATAGCGTGATACATAGT	1 ea @ 25 bases	11.53
5	A15609 - UP9JUU4 362176 C10	DNA	rbcL F T. aestivum for Ricardo L Fernandez Walker TGGTCGTCCTTTATTGGGATGTACT	1 ea @ 25 bases	10.93
6	A15609 - UPACJ7V 362176 C11	DNA	rbcL R T. aestivum for Ricardo L Fernandez Walker TCTCGCACAATACTCACAGATGCA	1 ea @ 24 bases	12.37
7	A15609 - UPCGETT 362176 C12	DNA	matK FT. aestivum for Ricardo L Fernandez Walker TTACCATTAGCATCTTCTGGAACT	1 ea @ 24 bases	12.91
8	A15609 - UPDK9DP 362176 D01	DNA	matK R T. aestivum for Ricardo L Fernandez Walker AAACCCTATTACATGGGACCAAA	1 ea @ 23 bases	11.62

UNIV OF NEW HAVEN	
Order Number:	41298900
PO:	VISA7528
Order Date:	10-Jul-2018

Invitrogen Custom Primers

Certificate of Analysis

Line 1 - Cat No. A15609 - UP2YHJF - Manufactured: 7/10/2018

Primer Number: **362176C06**

Primer Name:	rbcL F M. Citrifolia	Primer Length:	25
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - TGG CAT TCA AGT CGA GAG AGA TAA A		
Molecular Weight (µg/µmole):	7,764.1	µg per OD:	25.51
Millimolar Extinction Coeff.: (OD/µmol):	304.3	nmoles per OD:	3.29
Purity	**Desalt**	**OD's**	**12.97**
% GC Content:	**40**	**µg's**	**330.88**
Tm (1M Na+)	**85**	**nmoles**	**42.7**
Tm (50 mM Na+)	**63**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Line 2 - Cat No. A15609 - UP34C4D - Manufactured: 7/10/2018

Primer Number: **362176C07**

Primer Name:	rbcL R M. citrifolia	Primer Length:	23
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - ACA GAA GCA CCA CCT GAA CTA AA		
Molecular Weight (µg/µmole):	7,004.6	µg per OD:	25.55
Millimolar Extinction Coeff.: (OD/µmol):	274.1	nmoles per OD:	3.65
Purity	**Desalt**	**OD's**	**12.16**
% GC Content:	**44**	**µg's**	**310.64**
Tm (1M Na+)	**82**	**nmoles**	**44.4**
Tm (50 mM Na+)	**60**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Line 3 - Cat No. A15609 - UP496PA - Manufactured: 7/10/2018

Primer Number: **362176C08**

Primer Name:	mat K F M. citrifolia	Primer Length:	25
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - AAA GGC GAG GAT AGA GAA ATA GAG A		
Molecular Weight (µg/µmole):	7,871.2	µg per OD:	23.77
Millimolar Extinction Coeff.: (OD/µmol):	331.1	nmoles per OD:	3.02
Purity	**Desalt**	**OD's**	**13.69**
% GC Content:	**40**	**µg's**	**325.38**
Tm (1M Na+)	**82**	**nmoles**	**41.3**
Tm (50 mM Na+)	**60**	**Format**	**Liquid (100 uM in Water)**
Notes:			

UNIV OF NEW HAVEN	
Order Number:	41298900
PO:	VISA7528
Order Date:	10-Jul-2018

Invitrogen Custom Primers

Certificate of Analysis

Line 4 - Cat No. A15609 - UP7EY97 - Manufactured: 7/10/2018

Primer Number: **362176C09**

Primer Name:	mat K R M. citrifolia	Primer Length:	25
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - AAA CTG ACA TAG CGT GAT ACA TAG T		
Molecular Weight (µg/µmole):	7,699.1	µg per OD:	25.89
Millimolar Extinction Coeff.: (OD/µmol):	297.4	nmoles per OD:	3.36
Purity	**Desalt**	**OD's**	**11.53**
% GC Content:	**36**	**µg's**	**298.63**
Tm (1M Na+)	**78**	**nmoles**	**38.8**
Tm (50 mM Na+)	**56**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Line 5 - Cat No. A15609 - UP9JUU4 - Manufactured: 7/10/2018

Primer Number: **362176C10**

Primer Name:	rbcL F T. aestivum	Primer Length:	25
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - TGG TCG TCC TTT ATT GGG ATG TAC T		
Molecular Weight (µg/µmole):	7,686.0	µg per OD:	29.52
Millimolar Extinction Coeff.: (OD/µmol):	260.4	nmoles per OD:	3.84
Purity	**Desalt**	**OD's**	**10.93**
% GC Content:	**44**	**µg's**	**322.77**
Tm (1M Na+)	**85**	**nmoles**	**42.0**
Tm (50 mM Na+)	**64**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Line 6 - Cat No. A15609 - UPACJ7V - Manufactured: 7/10/2018

Primer Number: **362176C11**

Primer Name:	rbcL R T. aestivum	Primer Length:	24
Researcher	Ricardo L Fernandez Walker	Scale of Synthesis:	50 N
Sequence (5' to 3'):	(DNA) - TCT CGC ACA ATA CTC ACA GAT GCA		
Molecular Weight (µg/µmole):	7,266.8	µg per OD:	27.58
Millimolar Extinction Coeff.: (OD/µmol):	263.5	nmoles per OD:	3.80
Purity	**Desalt**	**OD's**	**12.37**
% GC Content:	**46**	**µg's**	**341.08**
Tm (1M Na+)	**87**	**nmoles**	**47.0**
Tm (50 mM Na+)	**65**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Invitrogen Custom Primers

Certificate of Analysis

UNIV OF NEW HAVEN	
Order Number:	41298900
PO:	VISA7528
Order Date:	10-Jul-2018

Line 7 - Cat No. A15609 - UPCGETT - Manufactured: 7/10/2018

Primer Name:	matK FT. aestivum	Primer Number:	**362176C12**
Researcher	Ricardo L Fernandez Walker	Primer Length:	24
Sequence (5' to 3'):	(DNA) - TTA CCA TTA GCA TCT TCT GGA ACT	Scale of Synthesis:	50 N
Molecular Weight (µg/µmole):	7,278.8	µg per OD:	28.51
Millimolar Extinction Coeff.: (OD/µmol):	255.3	nmoles per OD:	3.92
Purity	**Desalt**	**OD's**	**12.91**
% GC Content:	**38**	**µg's**	**367.93**
Tm (1M Na+)	**80**	**nmoles**	**50.6**
Tm (50 mM Na+)	**58**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Line 8 - Cat No. A15609 - UPDK9DP - Manufactured: 7/10/2018

Primer Name:	matK R T. aestivum	Primer Number:	**362176D01**
Researcher	Ricardo L Fernandez Walker	Primer Length:	23
Sequence (5' to 3'):	(DNA) - AAA CCC TAT TAC ATG GGA CCA AA	Scale of Synthesis:	50 N
Molecular Weight (µg/µmole):	7,010.6	µg per OD:	25.97
Millimolar Extinction Coeff.: (OD/µmol):	270.0	nmoles per OD:	3.70
Purity	**Desalt**	**OD's**	**11.62**
% GC Content:	**39**	**µg's**	**301.67**
Tm (1M Na+)	**82**	**nmoles**	**43.0**
Tm (50 mM Na+)	**61**	**Format**	**Liquid (100 uM in Water)**
Notes:			

Mat K M. citrifolia

Primer3 Output

WARNING: Numbers in input sequence were deleted.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        542   25   61.49   40.00  2.00  0.00
aaaggcgaggatagagaaatagaga
RIGHT PRIMER       650   25   60.71   36.00  3.00  0.00
tgatacatagtgcgatacagtcaaa
```

SEQUENCE SIZE: 2217
INCLUDED REGION SIZE: 2217

PRODUCT SIZE: 109, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

```
    1 gtgcgactagcctcttttacacatttagatgaagcaagggattcgtccataccatcggta

   61 aagtttggaagaccccgactgatcttaaaagggaatgactggaaaaaatagcatgtcggg

  121 ttgagggagagtgtttcattctttctggatcagtacaaaacaaaaacctggtcgggattc

  181 ttggaacgtaagaaaagaaagttggttcaatgaataaatggaaagggccctgcggcttca

  241 attaattataggaagaggcaaagcaacgagcttctgttttgaatttgaattatttcctga

  301 tctaattagatgttaaaaatatattagtacctaatgcaggaggggtttttcccatgagcg

  361 gagtcgctgttttttgaattaatcctaactattctaattctccattatgaaacggagata

  421 tgtgtgtcaaagaagcagtatattgataacgaaagtttttccgaactcaaaagagcgatt

  481 cggttgaaaaaataaaagattgctaatcatcttgctatcccaggcatagcctaaaaatgg

  541 aaaaggcgaggatagagaaatagagaatctgttgataagtttctacccgtccccgaggta
       >>>>>>>>>>>>>>>>>>>>>>>>

  601 tctattcttatgagaatactttgtttttgactgtatcgcactatgtatcattcgaaaatt
                                <<<<<<<<<<<<<<<<<<<<<<<<

  661 ccccaatcttctaccttcgtttcaaatatactattaaatcaaatatactattaaatggag

  721 gaaatcaaaagatatttacagcttgatagatctcaacagcccggctttctatatccactt

  781 atctttcaggagtatatttacggacttgctcatgattatagtttaaatcgatctcgcttg

  841 ttggagaatccgggttatgacaataaatacagtttactacttgtgaaacgtttaattact

  901 cgaatgtatggacaaaatcattttattatttttgctaatgattctaataaaaatcgattt

  961 tttggtcgcaacaagaatttctaccctcaaacgatatcagaaggatttgcatttattgtg

 1021 gaaattccatttgatatacgattaataccttctcaagacgggaaaaaaatattaaaatct
```

1081 cataatttacgatcaattcattcactatttcctttcttagagaacaatttttcacatttt

1141 aattctgtgttagatatactaataccccgcgccgtccatctggaaattctggttcaaacc

1201 cttcattattgggtaaaagatgcctccgccttgcatttattacgattatttttccacgaa

1261 tattggactcttagtgctacaaagaaatcccatttttcaccaaaaagaaataaaagattt

1321 tttttcttattatataattctcatgtatatgaatacgaatccattttggcctttctccgt

1381 aaccaatcttcgcatttgcaatcaacatcttttgtattatttctcgaacgactttatttt

1441 tatggaaaaaaagaacgtcttgtagaagttgttgctaaggattttcgggttagtctatgg

1501 ctgttcacagacccttttcatgcagtatgttaggtatcaaggaaaatcaattctgctttca

1561 aagggtacacctcttttgatgaataaatggaaatcttatcttgtcaatttttggcaatat

1621 cactttgatctgtggtttcgctcgggaagggtttatataaatcaattttctaatcattca

1681 cttgactttgtgggctatcgttcaagtgtgcgactaaatccggcaatggtacggggtcaa

1741 atgctagaaaattcatttctaattaataatgctattaagaaattggatatgcttgttcca

1801 attattcctcttattagatcattggctaaagcgaaattttgtaacctattaggacatccc

1861 cttagtaagccggtttggactgatttatcagattctgatatgattgaacgctttgggtat

1921 atatgcagaaacctttctcattatcatagcgggtcttcaaaaaaaaagattttgtatcga

1981 ataaagtatatacttcgactttcttgtgctaaaactttggcggggaaacacaagagtact

2041 gtacatatttttttgaaaagattaggttcggaatttttggaagaattcttcgcgtcggga

2101 gaagacgacctttctttgatcttctcaagagcttcttcgacttttcgggggtatataga

2161 agtcgaatttggtatttggatattacttatatcaacgatttgagcaaccttcaatga

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS

	start	len	tm	gc%	any	3'	seq
1 LEFT PRIMER	543	25	61.49	40.00	2.00	0.00	aaggcgaggatagagaaatagagaa
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 108, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

2 LEFT PRIMER	543	24	60.92	41.67	2.00	0.00	aaggcgaggatagagaaatagaga
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 108, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

3 LEFT PRIMER	544	24	60.92	41.67	2.00	0.00	aggcgaggatagagaaatagagaa
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 107, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

4 LEFT PRIMER	542	26	62.02	38.46	2.00	0.00	aaaggcgaggatagagaaatagagaa
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 109, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

5 LEFT PRIMER	542	24	60.18	41.67	2.00	0.00	aaaggcgaggatagagaaatagag
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 109, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

6 LEFT PRIMER	544	23	60.30	43.48	2.00	0.00	aggcgaggatagagaaatagaga
RIGHT PRIMER	650	25	60.71	36.00	3.00	0.00	tgatacatagtgcgatacagtcaaa

PRODUCT SIZE: 107, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

7 LEFT PRIMER	542	25	61.49	40.00	2.00	0.00	aaaggcgaggatagagaaatagaga
RIGHT PRIMER	684	23	60.93	43.48	2.00	0.00	tgaaacgaaggtagaagattggg

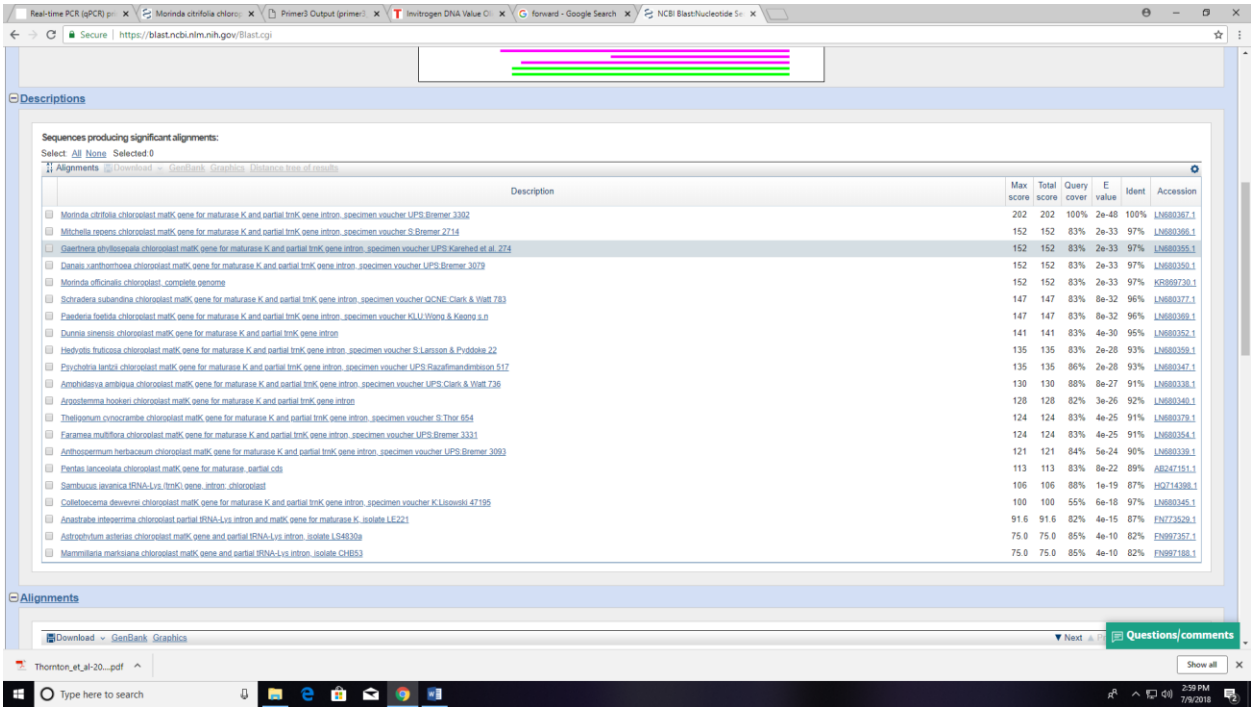
PRODUCT SIZE: 143, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

8 LEFT PRIMER	542	25	61.49	40.00	2.00	0.00	aaaggcgaggatagagaaatagaga
RIGHT PRIMER	650	24	60.12	37.50	3.00	0.00	tgatacatagtgcgatacagtcaa

PRODUCT SIZE: 109, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

```
 9 LEFT PRIMER        543   25   61.49   40.00  2.00  0.00
aaggcgaggatagagaaatagagaa
   RIGHT PRIMER       684   23   60.93   43.48  2.00  0.00
tgaaacgaaggtagaagattggg
   PRODUCT SIZE: 142, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

Statistics
         con   too    in    in          no    tm    tm  high  high
high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly
end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X
stab    ok
Left   13160     0     0     0  9091     0  1715    78   532   163   565
0  1016
Right  13268     0     0     0  9367     0  1678    58   501   141   568
0   955
Pair Stats:
considered 970280, unacceptable product size 934238, tm diff too large 22189,
high any compl 7422, high end compl 126, ok 6305
primer3 release 1.1.4
```

Blast Search



RBCL M. citrifolia

Primer3 Output

WARNING: Numbers in input sequence were deleted.

```
No mispriming library specified
Using 1-based sequence positions
WARNING: Unrecognized base in input sequence

OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        407   25   62.65   40.00  4.00  0.00
tggcattcaagtcgagagagataaa
RIGHT PRIMER       544   23   62.96   43.48  3.00  0.00
aaatcaagtccaccacgaagaca
SEQUENCE SIZE: 1343
INCLUDED REGION SIZE: 1343

PRODUCT SIZE: 138, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

    1 aagagtacaaattgacttattatactcctgaatacgaaaccaaagatactgatatcttgg

   61 cagcattccgagtaactgcccaacctggagttccaccggaagaagcgggggccgcggtag

  121 ctgccgagtcttctactggtacatggacaactgtatggacggatggacttaccagtcttg

  181 atcgttacaaagggcgatgctaccacatcgagccagttcctggagaagaagatcaattta

  241 ttgcttatgtagcttacccgttagacctttttgaagaaggttctgttactaacatgttta

  301 cttccattgtaggtaatgtatttgggttcaaagccctgcgcgctctgcgtctggaagatt

  361 tgcgaattcccatttcttatgttaaaaccttccaaggcccgcctcatggcattcaagtcg
                                                    >>>>>>>>>>>>>>

  421 agagagataaattgaacaagtatggtcgtcccctgttgggatgtactattaaacctaaat
      >>>>>>>>>>>

  481 taggtttatctgctaaaaactacggtagagcagtttatgaatgtcttcgtggtggacttg
                                                  <<<<<<<<<<<<<<<<<<

  541 attttaccaaagatgatgaaaacgtgaactcccaaccatttatgcgttggagagatcgtt
      <<<<

  601 tcttattttgtgccgaagcactttataaagcgcaggctgaaacaggtgaaatcaaagggc
```

661 attacttgaatgctactgcaggtacatgcgaagaaatgatcaaaagagctgtatttgcta

721 gagaattgggagttcctatcgtaatgcatgattacttaacaaggggattcactgcaaata

781 ctagcttggctcattattgccgagataatggcctacttcttcacattcaccgtgcaatgc

841 atgcagttattgataggcagaagaatcatggtatgcacttccgcgtactagctaaagcgt

901 tacgtatgtctggcggagatcatattcacgccggtactgtcgtcgggaaacttgaaggcg

961 aaagagatatcactttgggctttgttgatttactgcgcgatgattatattgaaaaagatc

1021 gatcccgcggtatttatttcactcaagattgggtctctttaccgggtgttatacccgtgg

1081 cttcaggaggtattcacgtttggcatatgcctgctctgacggagatctttggagatgatg

1141 cggtactacagttcggtggaggaactttgggacacccttggggtaatgcgccaggtgctg

1201 tagcgaatcgagtagctctagaagcatgygtaaaagctcgaaacgaggggcgtgatcttg

1261 ctgctgagggtaatgaaattatccgcaaggctagtaaatggagtcctgaactagcgkctg

1321 cttctgagatatggaaggagatc

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

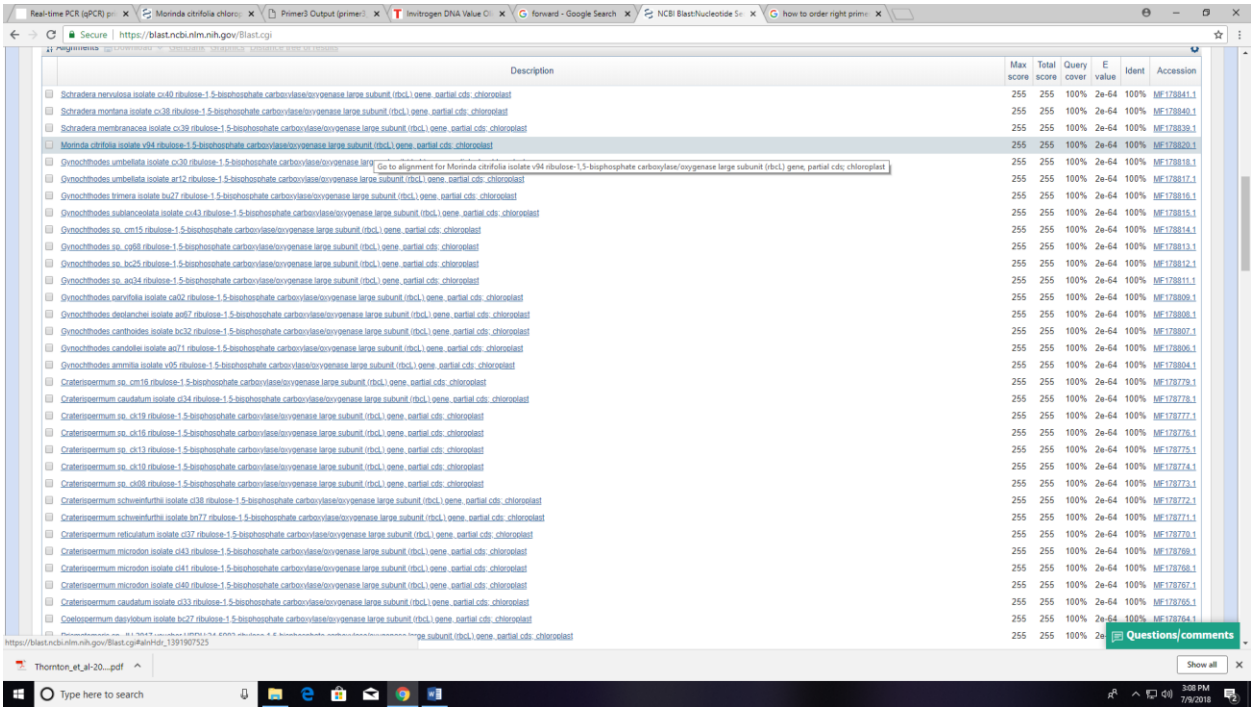
ADDITIONAL OLIGOS

	start	len	tm	gc%	any	3'	seq
1 LEFT PRIMER	520	25	63.76	40.00	3.00	0.00	aatgtcttcgtggtggacttgattt
RIGHT PRIMER	663	25	63.28	40.00	4.00	0.00	aatgccctttgatttcacctgtttc
PRODUCT SIZE: 144, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00							
2 LEFT PRIMER	407	25	62.65	40.00	4.00	0.00	tggcattcaagtcgagagagataaa
RIGHT PRIMER	543	22	62.41	45.45	3.00	0.00	aatcaagtccaccacgaagaca
PRODUCT SIZE: 137, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00							

```
 3 LEFT PRIMER        521   24   63.29   41.67  3.00  0.00
atgtcttcgtggtggacttgattt
   RIGHT PRIMER       663   25   63.28   40.00  4.00  0.00
aatgccctttgatttcacctgtttc
   PRODUCT SIZE: 143, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 4 LEFT PRIMER        516   25   63.60   44.00  3.00  0.00
tatgaatgtcttcgtggtggacttg
   RIGHT PRIMER       665   26   63.29   38.46  4.00  0.00
gtaatgccctttgatttcacctgttt
   PRODUCT SIZE: 150, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 5 LEFT PRIMER        520   25   63.76   40.00  3.00  0.00
aatgtcttcgtggtggacttgattt
   RIGHT PRIMER       665   27   64.01   40.74  4.00  0.00
gtaatgccctttgatttcacctgtttc
   PRODUCT SIZE: 146, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 6 LEFT PRIMER        520   25   63.76   40.00  3.00  0.00
aatgtcttcgtggtggacttgattt
   RIGHT PRIMER       665   26   63.29   38.46  4.00  0.00
gtaatgccctttgatttcacctgttt
   PRODUCT SIZE: 146, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 7 LEFT PRIMER        520   25   63.76   40.00  3.00  0.00
aatgtcttcgtggtggacttgattt
   RIGHT PRIMER       664   26   63.07   38.46  4.00  0.00
taatgccctttgatttcacctgtttc
   PRODUCT SIZE: 145, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 8 LEFT PRIMER        521   24   63.29   41.67  3.00  0.00
atgtcttcgtggtggacttgattt
   RIGHT PRIMER       665   26   63.29   38.46  4.00  0.00
gtaatgccctttgatttcacctgttt
   PRODUCT SIZE: 145, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 9 LEFT PRIMER        516   25   63.60   44.00  3.00  0.00
tatgaatgtcttcgtggtggacttg
   RIGHT PRIMER       607   25   63.38   40.00  4.00  0.00
aataagaaacgatctctccaacgca
   PRODUCT SIZE: 92, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

Statistics
         con   too    in    in          no    tm    tm  high  high
high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly
end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X
stab    ok
Left    6150    20     0     0  1788     0  1435   210   621   325   254
0  1497
Right   6002    44     0     0  1579     0  1462   212   614   288   258
0  1541
Pair Stats:
considered 2307635, unacceptable product size 2191232, tm diff too large
67031, high any compl 23976, high end compl 416, ok 24980
primer3 release 1.1.4
```

(primer3_results.cgi release 0.4.0)

Blast Search



Primer3 Output Wheat rbcL

WARNING: Numbers in input sequence were deleted.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len       tm      gc%   any   3' seq
LEFT PRIMER        440   25    64.16    44.00  4.00  2.00
tggtcgtcctttattgggatgtact
RIGHT PRIMER       527   24    64.14    45.83  4.00  0.00
acgtagacactcataacacgctct
SEQUENCE SIZE: 545
INCLUDED REGION SIZE: 545

PRODUCT SIZE: 88, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

    1 attataaattgacttactacaccccagagtatgaaactaaggatactgatatcttggcag
```

```
   61 cattccgagtaagtcctcagcctggggttccgcccgaagaagcaggggctgcagtagctg

  121 ccgaatcttctactggtacatggacaactgtttggactgatggacttaccagtcttgatc

  181 gttacaaaggacgatgctatcacatcgagcctgttgctggggaagacagccaatggatct

  241 gttatgtagcttatccattagacctatttgaagagggttccgttactaacatgtttactt

  301 ccattgtaggtaacgtatttggtttcaaagccctacgtgctctacgtttggaggatctac

  361 gaattccccctacttattcaaaaactttccaaggcccgcctcatggtatccaagttgaaa

  421 gagataagttgaacaagtatggtcgtcctttattgggatgtactattaaaccaaaattgg
                        >>>>>>>>>>>>>>>>>>>>>>>>

  481 gattatccgcaaaaaattatggtagagcgtgttatgagtgtctacgtggtggacttgatt
                             <<<<<<<<<<<<<<<<<<<<<<<

  541 ttaca
```

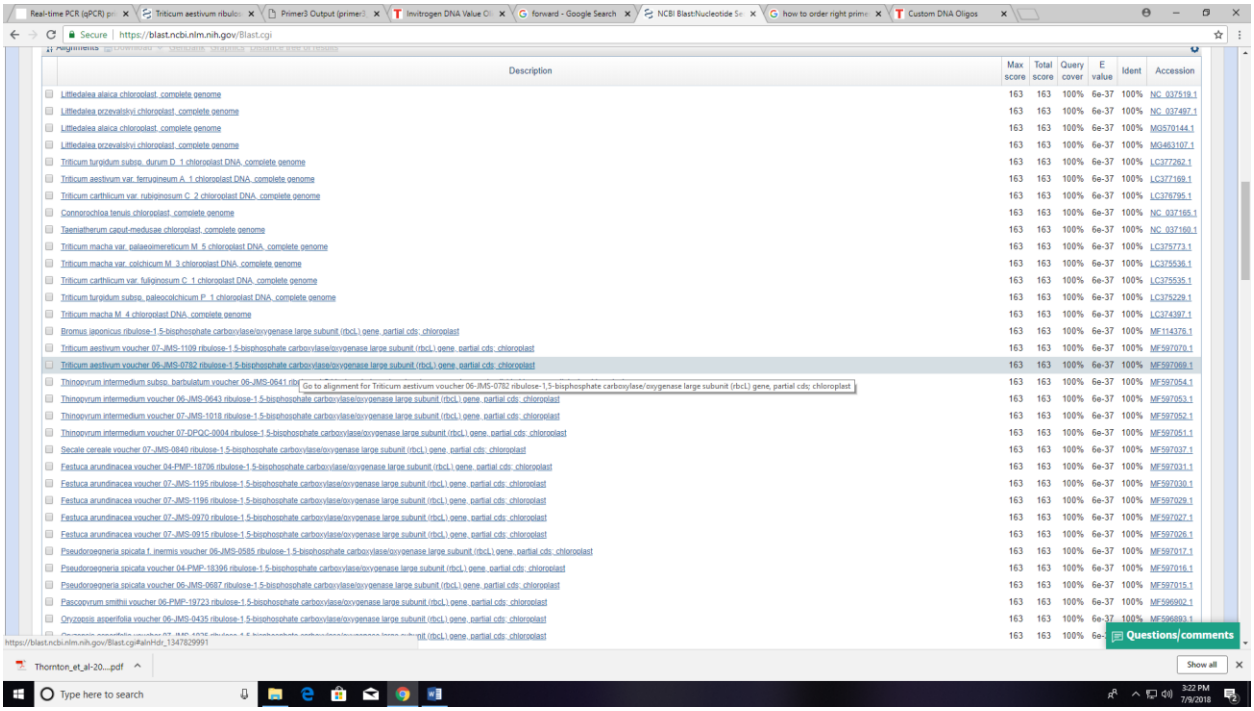
KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS

	start	len	tm	gc%	any	3'	seq
1 LEFT PRIMER	439	23	62.36	43.48	4.00	0.00	atggtcgtcctttattgggatgt
RIGHT PRIMER	526	23	62.64	47.83	3.00	0.00	cgtagacactcataacacgctct
PRODUCT SIZE: 88, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00							
2 LEFT PRIMER	441	24	62.68	45.83	4.00	2.00	ggtcgtcctttattgggatgtact
RIGHT PRIMER	522	23	62.60	47.83	2.00	0.00	gacactcataacacgctctacca
PRODUCT SIZE: 82, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00							
3 LEFT PRIMER	439	26	64.43	42.31	4.00	2.00	atggtcgtcctttattgggatgtact
RIGHT PRIMER	527	24	64.14	45.83	4.00	0.00	acgtagacactcataacacgctct
PRODUCT SIZE: 89, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00							
4 LEFT PRIMER	328	22	64.48	50.00	4.00	0.00	aagccctacgtgctctacgttt
RIGHT PRIMER	415	21	64.46	57.14	4.00	0.00	acttggataccatgaggcggg
PRODUCT SIZE: 88, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00							

```
 5 LEFT PRIMER        327   23   64.94   47.83  4.00  0.00
aaagccctacgtgctctacgttt
   RIGHT PRIMER       415   21   64.46   57.14  4.00  0.00
acttggataccatgaggcggg
   PRODUCT SIZE: 89, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

 6 LEFT PRIMER        440   25   64.16   44.00  4.00  2.00
tggtcgtcctttattgggatgtact
   RIGHT PRIMER       522   26   63.20   42.31  2.00  1.00
gacactcataacacgctctaccataa
   PRODUCT SIZE: 83, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

 7 LEFT PRIMER        440   25   64.16   44.00  4.00  2.00
tggtcgtcctttattgggatgtact
   RIGHT PRIMER       528   25   65.08   48.00  4.00  0.00
cacgtagacactcataacacgctct
   PRODUCT SIZE: 89, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 8 LEFT PRIMER        442   26   61.33   38.46  4.00  2.00
gtcgtcctttattgggatgtactatt
   RIGHT PRIMER       522   22   61.00   50.00  2.00  0.00
gacactcataacacgctctacc
   PRODUCT SIZE: 81, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

 9 LEFT PRIMER        440   25   64.16   44.00  4.00  2.00
tggtcgtcctttattgggatgtact
   RIGHT PRIMER       526   23   62.64   47.83  3.00  0.00
cgtagacactcataacacgctct
   PRODUCT SIZE: 87, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

Statistics
         con   too    in    in          no    tm    tm  high  high
high
         sid  many   tar  excl   bad    GC   too   too   any    3'  poly
end
        ered    Ns   get   reg   GC% clamp   low  high compl compl     X
stab    ok
Left    2346     0     0     0   778     0   658    30   247   114   108
0   411
Right   2319     0     0     0   745     0   642    24   237   106   128
0   437
Pair Stats:
considered 179607, unacceptable product size 157231, tm diff too large 13192,
high any compl 4355, high end compl 95, ok 4734
primer3 release 1.1.4
```

Blast Search



(primer3_results.cgi release 0.4.0)

Primer Wheat matK

```
1 gattaaatgg ttctgaacct gtggaaatag ttgttagttg taataacaag aaatttagtt

       61 cactacttgt gaaacgttta attattcgaa tgtatcagca gaatttttg gataactcgg

      121 ttaatcatcc taaccaagat cgattattgg attacaaaaa ttatttttat tctgagtttt

      181 attctcagat tctatctgaa gggtttgcga tcgttgtgga aatcccattc tcgctacggg

      241 aattattttg tccgaaagaa aaagaaatac caaagtttca gaatttacgc tctattcatt

      301 caatatttcc ctttttttgaa gacaaatttt tgcatttgga ttatctatca catatagaaa

      361 taccctatcc tatccatttg gaaatcctgg ttcaactcct tcaataccgt atccaagatg

      421 ttccatcttt gcatttattg cgattctttc tcaactacta ttcgaattgg aatagtttta

      481 ttacttcaat gaaatccatt ttttttttc aaaaagaaaa taaaagacta tttcgattcc

      541 tatataactc ttatgtatca gaatatgaat ttttttgtt gtttcttcgt aaacaatctt
```

601 cttgcttacc attagcatct tctggaactt ttctggaacg aatccacttt tctaggaaga

661 tggaacattt tgggataatg taccctggtt tttctcggaa aaccctatgg ttctttatgg

721 atcctcttat gcattatgtt cgatatcaag gaaaggcaat tcttgcatca aaaggcactt

781 ttttttgaa gaagaaatgg aaatgctacc ttatcaattt atggcaatat tatttctgtt

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