Journal of Visualized Experiments

Field identification of Matricaria chamomilla using a portable qPCR system

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE60940R3
Full Title:	Field identification of Matricaria chamomilla using a portable qPCR system
Corresponding Author:	Zhengfei Lu Herbalife International of America Inc Torrance Torrance, CA UNITED STATES
Corresponding Author's Institution:	Herbalife International of America Inc Torrance
Corresponding Author E-Mail:	zhengfeil@herbalife.com
Order of Authors:	Zhengxiu Yang
	Zheng Quan
	Tiffany Chua
	Leo Li
	Yanjun Zhang
	Silva Babajanian
	Francesco Buongiorno
	Isabella Della Noce
	Lorenzo Colombo
	Steven Newmaster
	Tricia Chua
	Peter Chang
	Gary Swanson
	Zhengfei Lu
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please specify the section of the submitted manuscript.	Genetics
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the UK Author License Agreement (for UK authors only)
Please provide any comments to the journal here.	full size video was uploaded to dropbox link

TITLE:

Field Identification of *Matricaria chamomilla* Using a Portable qPCR System

AUTHORS AND AFFILIATIONS:

Zhengxiu Yang[1], Zheng Quan[2], Tiffany Chua[3], Leo Li[1], Yanjun Zhang[3], Silva Babajanian[2], Francesco Buongiorno[4], Isabella Della Noce[4], Lorenzo Colombo[4], Steven Newmaster[5,6], Tricia Chua[3], Peter Chang[3], Gary Swanson[3], Zhengfei Lu[2]

[1]Herbalife NatSource (Hunan) Natural Products Co., Ltd, 1318 Kaiyuan East Road, Changsha City, Hunan Province, China

[2]Herbalife International of America, Inc., Corporate Quality Laboratory, 950 W 190th Street, Torrance, CA, USA

[3]Herbalife International of America, Inc., Corporate Quality, 990 W 190th Street, Torrance, CA, USA

[4]Hyris Ltd, Lower Ground Floor, One George Yard, London, UK

[5]College of Biological Sciences, University of Guelph, Guelph, Ontario, Canada

[6]Natural Health Product Research Alliance, University of Guelph, Guelph, Ontario, Canada

Email addresses of co-authors:

Zhengxiu Yang	(shawnya@herbalife.com)
Zheng Quan	(zhengq@herbalife.com)
Tiffany Chua	(tiffanyc.consultant@herbalife.com)
Leo Li	(leoli@herbalife.com)
Yanjun Zhang	(yanjunzh@herbalife.com)
Silva Babajanian	(silvaba@herbalife.com)
Francesco Buongiorno	(francesco.buongiorno@hyris.net)
Isabella Della Noce	(isabella.dellanoce@hyris.net)
Lorenzo Colombo	(lorenzo.colombo@hyris.net)
Steven Newmaster	(snewmast@uoguelph.ca)
Tricia Chua	(triciac@herbalife.com)
Peter Chang	(peterch@herbalife.com)
Gary Swanson	(garysw@herbalife.com)

Corresponding author:

Zhengfei Lu	(zhengfeil@herbalife.com)

KEYWORDS:

field testing, qPCR, botanical identification, chamomile, dietary supplements, quality control, DNA, food

SUMMARY:

Presented here is a protocol for field identification of *Matricaria chamomilla* using a portable qPCR system. This easy-to-perform protocol is ideal as a method to confirm the identity of a botanical species at locations where access to laboratory equipment and expertise is limited,

such as farms and warehouses.

ABSTRACT:
Quality control in botanical products begins with the raw material supply. Traditionally, botanical identification is performed through morphological assessment and chemical analytical methods. However, the lack of availability of botanists, especially in recent years, coupled with the need to enhance quality control to combat the stresses on the supply chain brought by increasing consumer demand and climate change, necessitates alternative approaches. The goal of this protocol is to facilitate botanical species identification using a portable qPCR system on the field or in any setting, where access to laboratory equipment and expertise is limited. Target DNA is amplified using dye-based qPCR, with DNA extracted from botanical reference materials serving as a positive control. The target DNA is identified by its specific amplification and matching its melting peak against the positive control. A detailed description of the steps and parameters, from hands-on field sample collection, to DNA extraction, PCR amplification, followed by data interpretation, has been included to ensure that readers can replicate this protocol. The results produced align with traditional laboratory botanical identification methods. The protocol is easy to perform and cost-effective, enabling quality testing on raw materials as close to the point of origin of the supply chain as possible.

INTRODUCTION:
The practice of using botanicals to maintain and improve health dates back to thousands of years. Due to stresses on the supply chain brought by increased consumer demand[1], unsustainable harvesting practices and climate change[2], botanical adulteration is becoming a growing concern in the food and dietary supplement industry[3]. The presence of undeclared or misidentified botanical species may lead to reduced efficacy, or even safety issues. For example, black cohosh (*Actaea racemosa*), used for treating premenstrual discomfort, may be substituted with a low-priced Asian species with limited clinical data support for its efficacy[4]. In a more serious case, substitution of *Aristolochia fangchi* for *Stephania terandra* in a clinical study for weight loss using Chinese herbs led to severe nephrotoxicity and renal failure in some participants[5,6]. The two different species shared a Chinese common name “Fang Ji”. These cases highlight the need for more stringent quality control, starting with the identification of raw materials[7], preferably as close to the point of origin of the supply chain as possible, so resources can be efficiently allocated to the material of correct identity.

A number of orthogonal approaches can be used for botanical identification. Traditionally, botanical identification is performed through morphological assessment[8,9] and chemical analytical methods[10–13]. Morphological identification is based on differences in macroscopic and microscopic features of plant materials if differences exist (**Figure 1**). However, the lack of training programs on classical botany in the recent years has resulted in a shortage of experts[14], making this approach impractical for routine quality control. Its application in powdered botanical materials is also limited. Chemical analytical methods are widely used in pharmacopoeias and laboratories, but are not ideal for field testing due to the size of instruments such as High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), and Nuclear Magnetic Resonance Spectroscopy (NMR) (**Figure**

2), and environment requirements. Recently, genomic methods have emerged as an alternative technique for botanical species' authentication and substitution detection and has proven to be efficient and precise. Genomic methods exploit the high fidelity and specificity of genetic information in plant materials[15–19]. Molecular diagnostic tools are available in the form of portable devices, and often include automated data interpretation tools that lower the barrier to technology use, making this approach ideal for field identification[20–24]. Once the molecular analysis method has been designed and validated[25–27], it can be performed by any personnel with basic molecular biology training. Among the different portable tools available, real-time PCR on DNA sequences is one of the cost-effective choices[28]. The combination of a portable device, together with customized and validated molecular analysis, allows verification of botanical species and ingredients outside the laboratory, such as in farms and botanical material warehouses, reducing the time and costs associated with traditional methods.

The goal of this protocol is to introduce a method for botanical identification in situations where access to laboratory equipment and expertise is limited or unavailable, using a portable qPCR system. The method is demonstrated on a field of *Matricaria chamomilla* (**Figure 3A**), commonly known as German chamomile, widely used for its anti-inflammatory and antioxidant properties[29]. It can be confused with related species of similar appearance or odor, especially from the genera *Chamaemelum*, *Tanacetum*, and *Chrysanthemum*[30–32]. Among the related species, *Chamaemelum nobile*, also known as Roman chamomile, is a noticeable one with comparable production levels in commerce (**Figure 3B**). The method demonstrated was designed to not only identify the target botanical species *M. chamomilla*, but also detect its close relative, *C. nobile*, based on specific amplification of DNA sequences.

This article explains, in detail, how to perform field botanical identification of *M. chamomilla* using intercalating dye-based qPCR and melt curve analysis on a portable device. The protocol includes the collection of botanical samples from the field, on-site DNA extraction, and set up of real-time PCR reaction. To ensure a valid conclusion, target botanical *M. chamomilla* and non-target botanical *C. nobile* genomic DNA, pre-extracted from certified botanical reference materials, are used as positive control. The specificity of this method is demonstrated by performing both *M. chamomilla* and *C. nobile* identification tests individually on samples and controls. Non-template negative control is used to exclude false positive results caused by PCR contamination.

PROTOCOL:

1. Sample collection

1.1. Set up a testing area in the field with a flat and horizontal surface.

1.2. Identify a representative plant that reflects the characteristics of majority of the plants in the chamomile flower field (**Figure 4**).

1.3. Pick a flower head from the representative plant using sterile gloves.

1.4. Place the sample into a 2.0 mL collection tube.

1.5. Repeat steps 1.3 to 1.4 and collect a leaflet (approximately 0.5–0.7 cm long) from the same plant.

NOTE: *M. chamomilla* flower and leaf are small enough to sit at the bottom of a 2.0 mL collection tube. For other botanicals with larger surface area, a paper punch or scissors (rinsed in 70% ethanol prior to use) can be used to isolate tissue samples for testing. When multiple sampling is required, rinse the paper punch or scissors between handling of different samples.

2. DNA extraction

2.1. Preheat the dry bath incubator to 95 °C.

2.2. To each collection tube, add 100 µL of the extraction solution from the plant DNA extraction kit (listed in **Table of Materials**). For better DNA extraction efficiency, grind the tissue sample in the extraction solution using a tissue pestle.

2.3. Close the tube. Ensure that the botanical tissue is covered with the extraction solution throughout the extraction process.

2.4. Place the collection tubes in a preheated dry bath incubator and incubate the collection tubes at 95 °C for 10 min.

2.5. After 10 min, take the tubes out of the dry bath incubator.

2.6. Add 100 µL of the dilution solution from the same DNA extraction kit and mix the solution by pipetting up and down several times.

2.7. Repeat the same step for leaflet extraction.

2.8. Shake to mix the solution further. The plant tissue usually does not appear to be degraded after this treatment. The liquid color may change and become cloudy.

NOTE: The diluted solution can be stored at room temperature overnight if not proceeding immediately. It is not necessary to remove the cellular debris from plant tissue before storage. The liquid in the tubes holds the DNA templates for downstream PCR amplification.

3. PCR reaction setup

3.1. Configure the qPCR instrument thermocycling conditions according to the manufacturer's specifications. Apply the PCR thermocycling profile listed in (**Table 1**), which starts with a constant temperature step for initial denaturation, followed by 25 cycles of amplification, and

ends with temperature ramping to obtain a high-resolution melting curve.

3.2. Thaw the qPCR Master Mix and primers (**Table 2**) at room temperature prior to use.

3.3. Plan the reaction that will be loaded in each well: wells containing positive control with target species, positive control with non-target species, samples, and negative control (**Figure 5**).

3.3.1. In this example, ten wells are used – five for the German chamomile identification test and the remaining five for the Roman chamomile identification test. For each type of species identification test, one well contains positive control with DNA extracted from targeted species' reference material, one well contains positive control with DNA extracted from non-targeted species' reference material, two wells are filled with flower and leaf DNA samples extracted from the field, and one well is allocated for a negative control. **Table 3** describes each well type.

3.4. Prepare a reaction master-mix according to the manual for each botanical species identification test. A typical reaction master-mix contains universal qPCR Master Mix (2x), forward and reverse species-specific primers, and nuclease-free water. **Table 4** lists the reaction system composition.

NOTE: If not using immediately, store the qPCR reaction master-mix at +2 °C to +8 °C in a cooler or mini-fridge.

3.5. Thoroughly mix the reaction master-mix by pipetting before use.

3.6. Place the qPCR cartridge face-up on a flat and stable surface.

3.7. Load 18 µL of the reaction master-mix configured in step 3.4 into the cartridge wells according to the wells defined in step 3.3. For this demonstration, add the German chamomile identification test reaction master mix into wells labeled for GC test (GCT in wells 1, 3, 5, 7, 9) and the Roman chamomile identification test reaction master-mix into wells labeled for RC test (RCT in wells 2, 4, 6, 8, 10) (see **Figure 5**).

3.8. Transfer 2 µL of sample DNA from the supernatant of DNA extraction tubes and pre-extracted DNA positive controls into cartridge wells preloaded with qPCR master mix. After adding each DNA template to the qPCR master mix, gently mix the solution by pipetting.

NOTE: Avoid floating cellular debris when transferring DNA from the DNA extraction tube. Use minicentrifuge to separate the supernatant and cellular debris, if necessary.

3.9. Carefully seal the cartridge with adhesive film. Load the cartridge onto the thermocycling chamber and close it.

3.10. Set the qPCR instrument to run.

REPRESENTATIVE RESULTS:

Following the protocol described in section 1, botanical DNA from flower head and leaf were extracted into the supernatant after heat incubation of the collection tube at 95 °C for 10 min. In the current study, the supernatant showed a yellow and greenish color for both flower and leaf, indicating that a variety of natural compounds were released into the supernatant with botanical DNA (**Figure 6**). Although reliable PCR amplification was achieved later in triplicate for all field extracted DNA template, DNA quality assessment was performed in the laboratory as reference. The concentration of flower head DNA extract, determined by fluorometry, ranged from 3.69–5.36 ng/µL, while the concentration of leaf DNA extract ranged from 6.42–9.29 ng/µL. The A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios of flower and leaf DNA extracts were measured by spectrophotometry. However, due to the overlap between DNA and phytochemical UV absorption spectrum, these ratios could not be reliability measured (data not shown).

Intercalating fluorescent dye was used to monitor the amplification of target fragments in real-time. Since both the specific primers *M. chamomilla* and *C. nobile* target the internal transcribed spacer 2 (ITS2) region, which has tens to hundreds of copies in the plant genome, 25 PCR amplification cycles are sufficient to generate enough amplicons for the identification of chamomile species. In **Figure 7**, the Ct value for *M. chamomilla* positive control in *M. chamomilla* identification test was less than 25 (GCP_GCT), while after 25 amplification cycles, the fluorescence of the same control in *C. nobile* identification test was below the detection threshold (GCP_RCT). On the other hand, after 25 cycles, the fluorescence for *C. nobile* positive control in *M. chamomilla* identification test was below the detection threshold (RCP_GCT), while the Ct value for the same control in *C. nobile* identification test was less than 25 (RCP_RCT). The amplification of target and non-target positive controls in their respective assays demonstrate the specificity of the *M. chamomilla* identification assay. For sample DNA, field flower head and leaf DNA extract yielded Ct values of 15.18 and 19.41 in *M. chamomilla* identification test, respectively (Sample1(FLOWER)_GCT and Sample2(LEAF)_GCT). Both of these samples were not amplified in *C. nobile* identification test (Sample1(FLOWER)_RCT and Sample2(LEAF)_RCT). The amplification patterns of both the samples matched the amplification pattern of *M. chamomilla* positive control. Negative controls were not amplified in both *M. chamomilla* and *C. nobile* identification tests (NC_GCT and NC_RCT), excluding the possibility of false positives caused by PCR contamination. To further confirm specific amplification in positive controls and samples, fractions of PCR end product from each well were run on 2% agarose gel in the laboratory (**Figure 8**). For *M. chamomilla* identification test, both field samples yielded amplicons running at the same position as the *M. chamomilla* positive control with an estimated size slightly above 100 bp (theoretical size 102 bp). For *C. nobile* identification test, non-target species *C. nobile* positive control yielded a band between 50 and 100 bp, fitting the theoretical size of 65 bp. The rest of the lanes showed no specific amplification product, which was in agreement with the absence of fluorescent signal for these wells, as observed in field testing.

Following PCR amplification, a melting curve analysis was performed to assess the dissociation characteristics of double-stranded DNA (dsDNA) during heating. As the temperature ramped up during the final cycle for melt curve analysis, increases in temperature caused the double-strand amplicons to dissociate. The intercalating fluorescent dye was gradually released into the solution, decreasing fluorescence intensity (**Figure 9A**). The inflection point of the first derivative curve was used to determine the melting temperature (Tm) (**Figure 9B**), which depends mainly on DNA fragment length and GC content. Combining Ct value with melting temperature can increase the specificity of qPCR analysis. In the current study, the melting temperature peak of *M. chamomilla* positive control PCR amplicon occurred at 85.6 °C (GCP_GCT) and it was distinct from the melting temperature peak of *C. nobile* positive control PCR amplicon at 79.1 °C (RCP_RCT). The PCR amplicon from field flower head and leaf produced melting temperature peaks at 85.2 °C and 84.8 °C, respectively (Sample1(FLOWER)_GCT and Sample2(LEAF)_GCT). To assess melting temperature variations measured by the portable qPCR system, additional datapoints were collected to confirm that sample melting temperatures were always close to the melting temperature obtained from *M. chamomilla* positive control (within 2 °C) and were far away from the melting temperature of *C. nobile* positive control amplicon (**Figure 10**). Melting temperature peaks were sometimes reported in other wells. However, their Ct values were not less than 25 and melting temperature peaks were not close to *M. chamomilla* or *C. nobile* positive control (more than 2 °C apart).

In summary, field *M. chamomilla* identification test can be interpreted based on decision rules summarized in **Table 5**. With all the positive controls testing positive for the putative botanical species, negative for the other species, and negative controls testing negative, both field samples were determined to contain *M. chamomilla* but not *C. nobile*. In addition, to align field testing results with other analytical techniques, field conclusions were further confirmed by a previously validated DNA barcoding method[25] (data not shown).

FIGURE AND TABLE LEGENDS:

Figure 1: Morphological identification of botanical materials. (**A**) *Hibiscus rosa-sinensis* flowers, *Curcuma longa* roots, *Malva Sylvestris* leaves, *Rosmarinus officinalis* leaves, *Coriandrum sativum* seeds, *Zingiber officinale* roots. (**B**) *Petroselinum crispum* and *Apium graveolens* flakes are difficult to differentiate.

Figure 2: Chemical identification of botanical materials. (**A**) HPTLC instrument and a representative HPTLC chromatogram. (**B**) HPLC instrument and a representative HPLC chromatogram.

Figure 3: *Matricaria chamomilla* and *Chamaemelum nobile* in the field. (**A**) *Matricaria chamomilla,* adapted from Wikipedia under CC BY-SA 3.0, https://en.wikipedia.org/wiki/Matricaria_chamomilla#/media/File:Matricaria_February_2008-1.jpg. (**B**) *Chamaemelum nobile,* adapted from Wikipedia under CC BY-SA 3.0*,* https://en.wikipedia.org/wiki/Chamomile#/media/File:Chamaemelum_nobile_001.JPG.

Figure 4: **Collecting *M. chamomilla* plant parts from the field.**

Figure 5: Layout of testing wells in the demonstration.

Figure 6: Field DNA extract in collection tubes. Botanical tissue remains in the original tube and is covered by yellowish DNA extract.

Figure 7: Fluorescence plot showing the accumulation of PCR products over 25 cycles of thermocycling. *M. chamomilla* positive control and *C. nobile* positive control show Ct values less than 25 in *M. chamomilla* and *C. nobile* identification tests, respectively. The field flower and leaf samples were amplified by *M. chamomilla* identification test with Ct values of 15.18 and 19.41. The rest of the wells were not amplified.

Figure 8: Gel electrophoresis of field PCR amplification products.

Figure 9: Melting temperature analysis. (**A**) The fluorescence signal in each well decreases with the increasing temperature. (**B**) The identity of the PCR products was confirmed by the melting temperature peak in melting curve analysis. The field flower and leaf samples show peaks at 85.2 °C and 84.8 °C. These are close to the peak produced by *M. chamomilla* positive control. The *C. nobile* positive control produced a peak at 79.1 °C, which is different from the other three samples.

Figure 10: Melting temperature peak variation between positive control and field samples.

Table 1: qPCR thermocycling conditions for *M. chamomilla and C. nobile* identification tests.

Table 2: Primer pairs for *M. chamomilla* and *C. nobile* identification tests.

Table 3: Well types and descriptions for *M. chamomilla* and *C. nobile* identification tests.

Table 4: Master-mix composition for *M. chamomilla* and *C. nobile* identification tests.

Table 5: Rules for qPCR result interpretation.

DISCUSSION:

The design of primers and template selection are the crucial steps in obtaining an efficient and specific qPCR amplification. After identifying a suitable template, primer design software is typically used to aid selection of primers based on design variables such as primer length, melting temperature, and GC content[33,34]. Optimization and validation can be performed under the expected experimental conditions of the assay to ensure specificity, sensitivity, and robustness of a PCR reaction[35]. Sub-optimal primer design may result in primer-dimer formation, wherein primer interactions produce non-specific products[36].

The no template controls (NTC) used in this study check for both DNA contamination and the

presence of primer-dimers that could affect the assay. Results showed no amplification, a good indication that both DNA contamination and primer-dimers are not a concern. DNA contamination and primer-dimers are manifested in melt curves through no template controls, and as extra peaks in melt curves of positive controls. Typically, the melt curve of a positive control is expected to contain a single peak, unless AT-rich subdomains in the template cause uneven melting. Double peaks could be predicted by simulating melting assays using the uMELT software[37]. In this study, the gold standard of running the PCR product on agarose gel was used to confirm the presence of target PCR product and absence of contamination and primer-dimers.

A considerable challenge in botanical material molecular analysis is obtaining good-quality DNA following the botanical DNA extraction process. Botanical materials are traded and consumed for the active chemical compounds that are associated with health benefits. In the process of DNA extraction, these chemical compounds will also be released into the DNA extraction solution, potentially causing PCR inhibition, thereby resulting in failures in PCR amplification. Various plant DNA purification kits using organic solvents and columns have been developed to remove chemical compounds derived from botanicals[38]. However, fume hood and high-speed centrifuge required to assist these kits are not available in the field.

In the current protocol, the simplified DNA extraction method uses a commercial plant DNA extraction kit (see **Table of Materials** for details). It had the ability to neutralize common inhibitory substances for reproducible results and produced consistent results for *M. chamomilla* and *C. nobile.* Both *M. chamomilla* and *C. nobile* flower heads and leaves yielded PCR amplicons with specific melt peaks, indicating that the presence of PCR inhibitors was not a concern. For other plants with higher levels of PCR inhibitors, amplifying DNA in their original extraction may be less efficient. To reduce inhibition and improve amplification efficiency, with access to the whole plant, other plant parts with lower polysaccharide and polyphenol content can also be used for identification purposes. If there is limited access to different plant parts, younger leaves or petals dissected from flower heads, which typically have lower phenolic content[39], may offer a better chance of success. Since DNA sequences are consistent across the whole plant, any plant part may be used to confirm species identity. If PCR amplification is still suboptimal, the original DNA extract can be further diluted before PCR, or more sophisticated laboratory purification protocols can be used.

Another challenge for PCR analysis is false positive results caused by DNA contamination, which can negatively impact data interpretation. It is usually controlled by active housekeeping, using dedicated equipment, and restricting work to designated areas. Using qPCR, all PCR analysis can be accomplished in a closed system, which greatly reduces the chance of PCR amplicon contamination in an environment that is not well controlled. Besides, environmental DNA should also not show a false positive due to the specificity of the assay, according to a previous validation study[40].

There is room for improvement. In the protocol presented here, intercalating dye was used to show target fragment amplification in real-time. The specificity of the method is further

confirmed by the characteristic melting temperature, which is distinct between *M. chamomilla* and *C. nobile* amplicons. Therefore, intercalating dye-based PCR can efficiently answer the question "What is this plant species?" in the field. However, in addition to the need of performing botanical identification on a single plant isolated from the field, in many circumstances, botanical powders or extracts in the warehouse will also benefit from an on-site rapid identity assessment. For these types of materials, additional questions may need to be addressed, such as "What is in this material?", "Does it contain the botanical species I am looking for?", "Does it contain adulterants I want to avoid?", and "Is it substituted wholly or partially by other botanical species that are harmful?". Instead of using intercalating dye, different qPCR probes can be designed to target amplicons from different botanicals in one reaction system, while maintain high specificity and efficiency of the assay. Development of probe-based qPCR and utilization of a portable qPCR system that offers multiple channel detection can further extend the application of field testing as a fit-for-purpose assay to a broader environment setting, such as botanical material warehouses and distribution centers to answer more complicated questions. In addition, using multiple probes also allows the user to include internal amplification in each reaction system, so that more information will be available when PCR inhibition is suspected.

The protocol presented here has the following advantages compared to existing technologies used for the same purpose. First, for traditional morphological and chemical identification methods, the procedure and its results need to be conducted and interpreted by experts. qPCR-based identification tests can be conducted by people with basic molecular biology training and interpreted in a more standardized manner. Second, compared to qPCR-based species identification and differentiation normally performed in the laboratory, the field identification protocol using a portable instrument does not require instruments with a large footprint, such as a high-speed centrifuge, DNA quality evaluation equipment, thermal cycler with fluorescence detector, and a computer running a special software. Thus, DNA-based species identification can be performed in any setting without delay. Third, searching for botanical materials is a task that requires a global operation. With advancements in cloud services and artificial intelligence, a portable device can potentially receive methods developed and validated by experts in the laboratory, be operated by non-experts in remote areas, and produce objective certifications from third parties. Therefore, this option is more compelling than ever with remote work becoming the trend.

In summary, the protocol here demonstrated field identification of *M. chamomilla* using a portable qPCR system. The successful application of this technique will generate highly accurate results on botanical identification and help botanical manufacturers and suppliers qualify botanical materials in a timely and cost-efficient manner.

ACKNOWLEDGMENTS:
We thank James Shan for his efforts in field video recording. We thank Jon Byron and Matthew Semerau for their work in video editing. We thank Ansen Luo, Harry Du, and Frank Deng for their support in locating the test field. We thank Maria Rubinsky for her valuable comments on the whole manuscript. All the acknowledged persons are employees of Herbalife International

of America, Inc.

DISCLOSURES:

We certify that Zhengxiu Yang, Zheng Quan, Tiffany Chua, Leo Li, Yanjun Zhang, Silva Babajanian, Tricia Chua, Peter Chang, Gary Swanson, Zhengfei Lu are employees of Herbalife International of America, Inc. We certify that Francesco Buongiorno, Isabella Della Noce, and Lorenzo Colombo are employees of Hyris Ltd. that produces the portable qPCR instrument used in this article.

REFERENCES:

1. Smith, T., Gillespie, M., Eckl, V., Knepper, J., Reynolds, C. Herbal supplement sales in US increase by 9.4% in 2018. *HerbalGram*. **123**, 62–73 (2019).
2. Israelsen, L. D. The challenge of regulation, globalization and climate change on botanicals and traditional medicines: respecting tradition while embracing change. *Planta Medica.* **74** (3), S-36 (2008).
3. Cardellina, J. H. Challenges and opportunities confronting the botanical dietary supplement industry†. *Journal of Natural Products*. **65** (7), 1073–1084 (2002).
4. Foster, S. Exploring the peripatetic maze of black cohosh adulteration: a review of the nomenclature, distribution, chemistry, market status, analytical methods and safety. *HerbalGram*. **98**, 32–51 (2013).
5. Vanherweghem, J.-L. Misuse of herbal remedies: The case of an outbreak of terminal renal failure in Belgium (Chinese herbs nephropathy). *The Journal of Alternative and Complementary Medicine*. **4** (1), 9–13 (1998).
6. Vanherweghem, J.-L. et al. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *The Lancet*. **341** (8842), 387–391 (1993).
7. Khan, I. A., Smillie, T. Implementing a “quality by design” approach to assure the safety and integrity of botanical dietary supplements. *Journal of Natural Products*. **75** (9), 1665–1673 (2012).
8. Applequist, W. *The Identification of Medicinal Plants: A Handbook of the Morphology of Botanicals in Commerce*. Missouri Botanical Garden Press (2006).
9. Upton, R., Graff, A., Jolliffe, G., Länger, R., Williamson, E. *American Herbal Pharmacopoeia: Botanical Pharmacognosy - Microscopic Characterization of Botanical Medicines.* CRC Press (2016).
10. Frommenwiler, D. A. et al. Comprehensive HPTLC fingerprinting for quality control of an herbal drug - the case of angelica gigas root. *Planta Medica*. **84** (6–7), 465–474 (2018).
11. Heyman, H. M., Meyer, J. J. M. NMR-based metabolomics as a quality control tool for herbal products. *South African Journal of Botany*. **82**, 21–32 (2012).
12. Lazarowych, N. J., Pekos, P. Use of fingerprinting and marker compounds for identification and standardization of botanical drugs: strategies for applying pharmaceutical HPLC analysis to herbal products. *Drug Information Journal*. **32** (2), 497–512 (1998).
13. Tankeu, S. et al. Hyperspectral imaging and support vector machine: a powerful combination to differentiate black cohosh (actaea racemosa) from other cohosh species. *Planta*

Medica. **84** (6/7), 407–419 (2018).
14. Rodman, J. E., Cody, J. H. The taxonomic impediment overcome: NSF's Partnerships for Enhancing Expertise in Taxonomy (PEET) as a model. *Systematic Biology*. **52** (3), 428–435 (2003).
15. Chen, S. et al. A renaissance in herbal medicine identification: from morphology to DNA. *Biotechnology Advances*. **32** (7), 1237–1244 (2014).
16. Li, X. et al. Plant DNA barcoding: from gene to genome. *Biological Reviews*. **90** (1), 157–166 (2015).
17. Madesis, P., Ganopoulos, I., Sakaridis, I., Argiriou, A., Tsaftaris, A. Advances of DNA-based methods for tracing the botanical origin of food products. *Food Research International*. **60**, 163–172 (2014).
18. Newmaster, S. G., Ragupathy, S., Janovec, J. A botanical renaissance: state-of-the-art DNA bar coding facilitates an automated identification technology system for plants. *International Journal of Computer Applications in Technology*. **35** (1), 50–60 (2009).
19. Parveen, I., Gafner, S., Techen, N., Murch, S. J., Khan, I. A. DNA barcoding for the identification of botanicals in herbal medicine and dietary supplements: strengths and limitations. *Planta Medica*. **82** (14), 1225–1235 (2016).
20. Agrawal, N., Hassan, Y. A., Ugaz, V. M. A pocket-sized convective PCR thermocycler. *Angewandte Chemie International Edition*. **46** (23), 4316–4319 (2007).
21. Almassian, D. R., Cockrell, L. M., Nelson, W. M. Portable nucleic acid thermocyclers. *Chemical Society Reviews*. **42** (22), 8769–8798 (2013).
22. Benítez-Páez, A., Portune, K. J., Sanz, Y. Species-level resolution of 16S rRNA gene amplicons sequenced through the MinION portable nanopore sequencer. *Gigascience*. **5** (1), 4 (2016).
23. Emanuel, P. A. et al. Detection of Francisella tularensis within infected mouse tissues by using a hand-held PCR thermocycler. *Journal of Clinical Microbiology*. **41** (2), 689–693 (2003).
24. Quick, J. et al. Real-time, portable genome sequencing for Ebola surveillance. *Nature*. **530** (7589), 228 (2016).
25. Lu, Z. et al. Single-laboratory validation of a two-tiered DNA barcoding method for raw botanical identification. *Journal of AOAC International*. **102** (5), 1435–1447 (2019).
26. Sgamma, T. et al. DNA barcoding for industrial quality assurance. *Planta Medica*. **83** (14/15), 1117–1129 (2017).
27. Wallinger, C. et al. Rapid plant identification using species-and group-specific primers targeting chloroplast DNA. *PLoS One*. **7** (1), e29473 (2012).
28. Newmaster, S. G. et al. Recommendations for validation of real-time PCR methods for molecular diagnostic identification of botanicals. *Journal of AOAC International*. (2019).
29. Singh, O., Khanam, Z., Misra, N., Srivastava, M. K. Chamomile (Matricaria chamomilla L.): an overview. *Pharmacognosy Reviews*. **5** (9), 82 (2011).
30. Mills, S. Y., Bone, K. *The Essential Guide to Herbal Safety*. Elsevier Health Sciences. 325 (2004).
31. Avula, B. et al. Quantitative determination of phenolic compounds by UHPLC-UV–MS and use of partial least-square discriminant analysis to differentiate chemo-types of Chamomile/Chrysanthemum flower heads. *Journal of Pharmaceutical and Biomedical Analysis*. **88**, 278–288 (2014).

32. Guzelmeric, E., Vovk, I., Yesilada, E. Development and validation of an HPTLC method for apigenin 7-O-glucoside in chamomile flowers and its application for fingerprint discrimination of chamomile-like materials. *Journal of Pharmaceutical and Biomedical Analysis*. **107**, 108–118 (2015).
33. Dieffenbach, C. W., Lowe, T. M., Dveksler, G. S. General concepts for PCR primer design. *Genome Research*. **3** (3), S30–S37 (1993).
34. Untergasser, A. et al. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research*. **35** (Web Server issue), W71–74 (2007).
35. Bustin, S., Huggett, J. qPCR primer design revisited. *Biomolecular Detection and Quantification*. **14**, 19–28 (2017).
36. Brownie, J. et al. The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Research*. **25** (16), 3235–3241 (1997).
37. Dwight, Z., Palais, R., Wittwer, C. T. uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. *Bioinformatics (Oxford, England)*. **27** (7), 1019–1020 (2011).
38. Heikrujam, J., Kishor, R., Mazumder, P. B. The chemistry behind plant DNA isolation protocols. *Biochemical Analysis Tools - Methods for Bio-Molecules Studies* (2020).
39. Blum-Silva, C. H., Chaves, V. C., Schenkel, E. P., Coelho, G. C., Reginatto, F. H. The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of Ilex paraguariensis aqueous extracts. *Revista Brasileira de Farmacognosia*. **25** (1), 1–6 (2015).
40. Lu, Z. et al. Validation of a targeted PCR method for raw and processed botanical material identification: an example using matricaria chamomilla (chamomile). *Journal of AOAC International*. **102** (6), 1787–1797 (2019).

A

Hibiscus rosa-sinensis Flowers

Curcuma longa Roots

Malva sylvestris Leaves

Rosmarinus officinalis Leaves

Coriandrum sativum Seeds

Zingiber officinale Roots

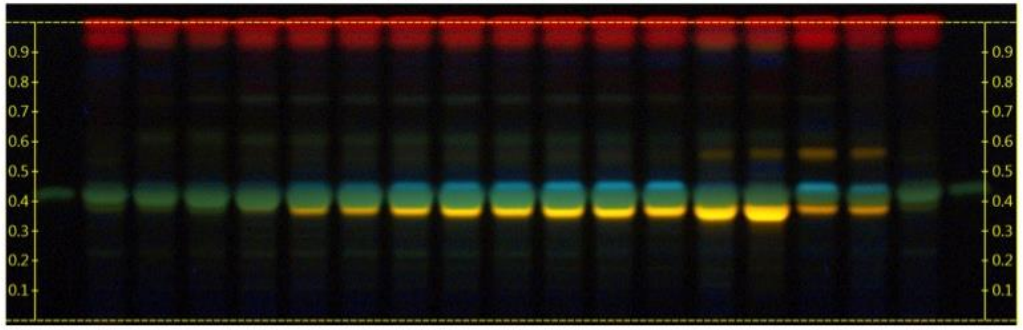
B

Petroselinum crispum Leaves

Apium graveolens Leaves

A

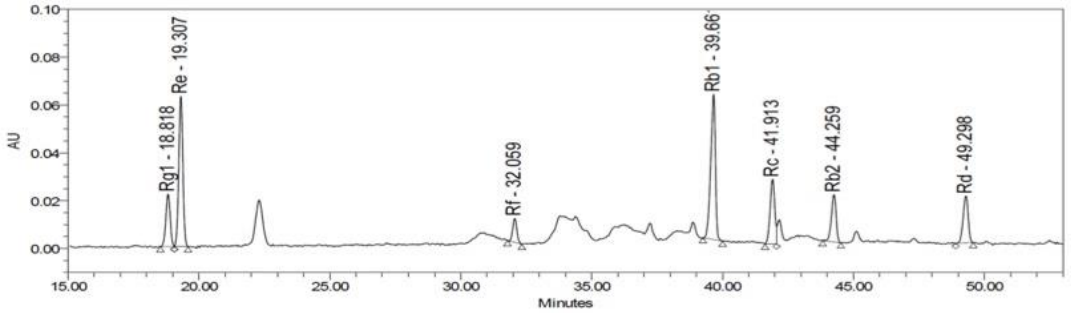
HPLTC instrument



Representative HPLTC chromatogram

B

HPLC instrument

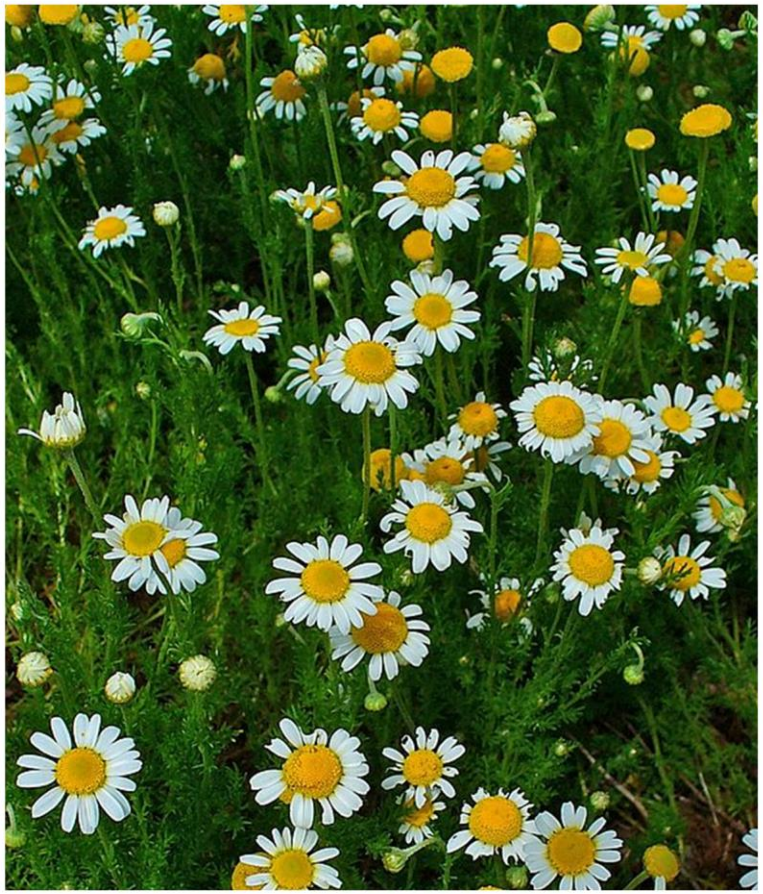


Representative HPLC chromatogram

A

Matricaria chamomilla
German Chamomile

B



Chamaemelum nobile
Roman Chamomile

Figure4

Figure5

Click here to access/download;Figure;Figure5.pdf

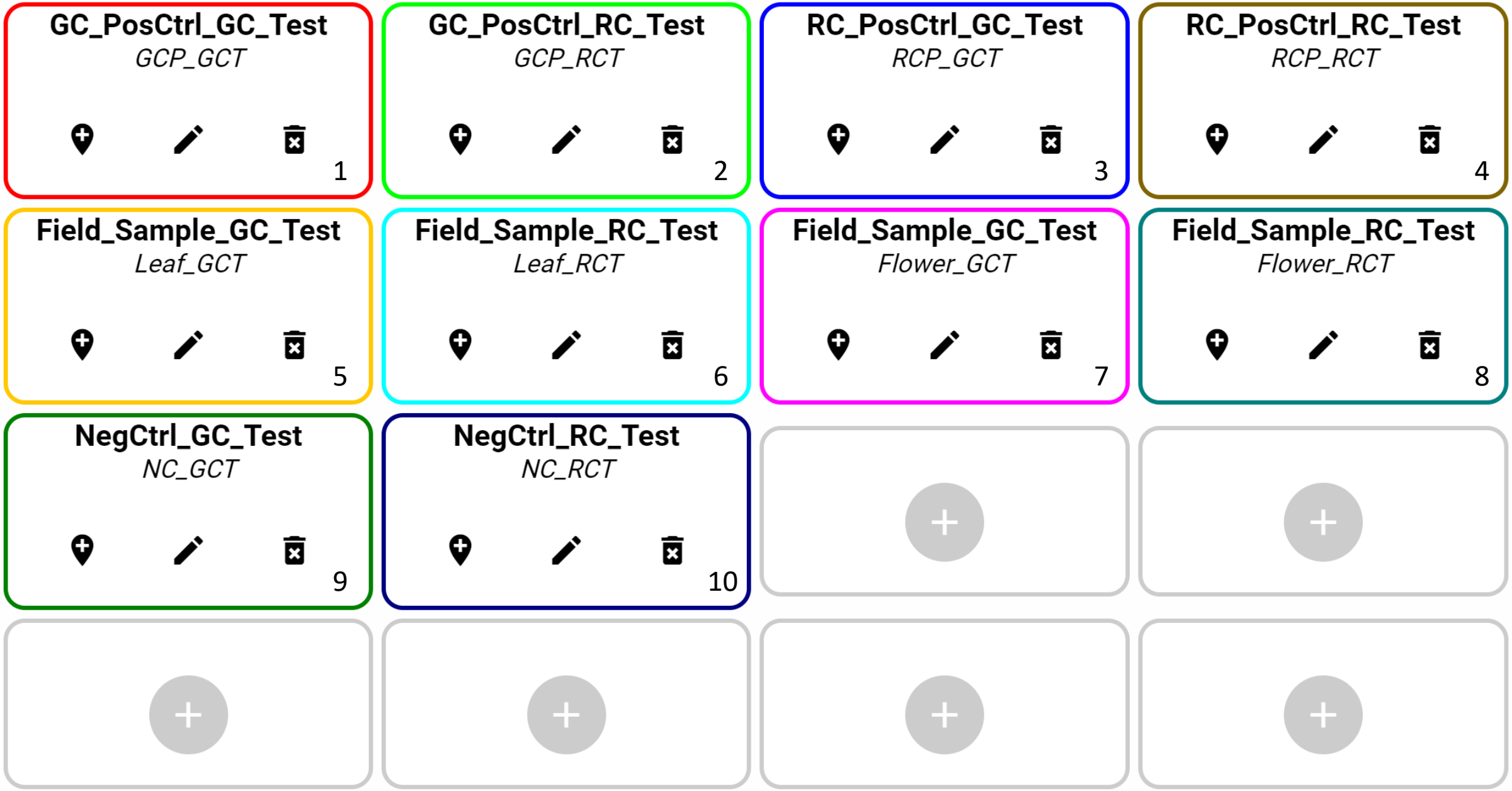


Figure6

Click here to access/download;Figure;Figure6.pdf

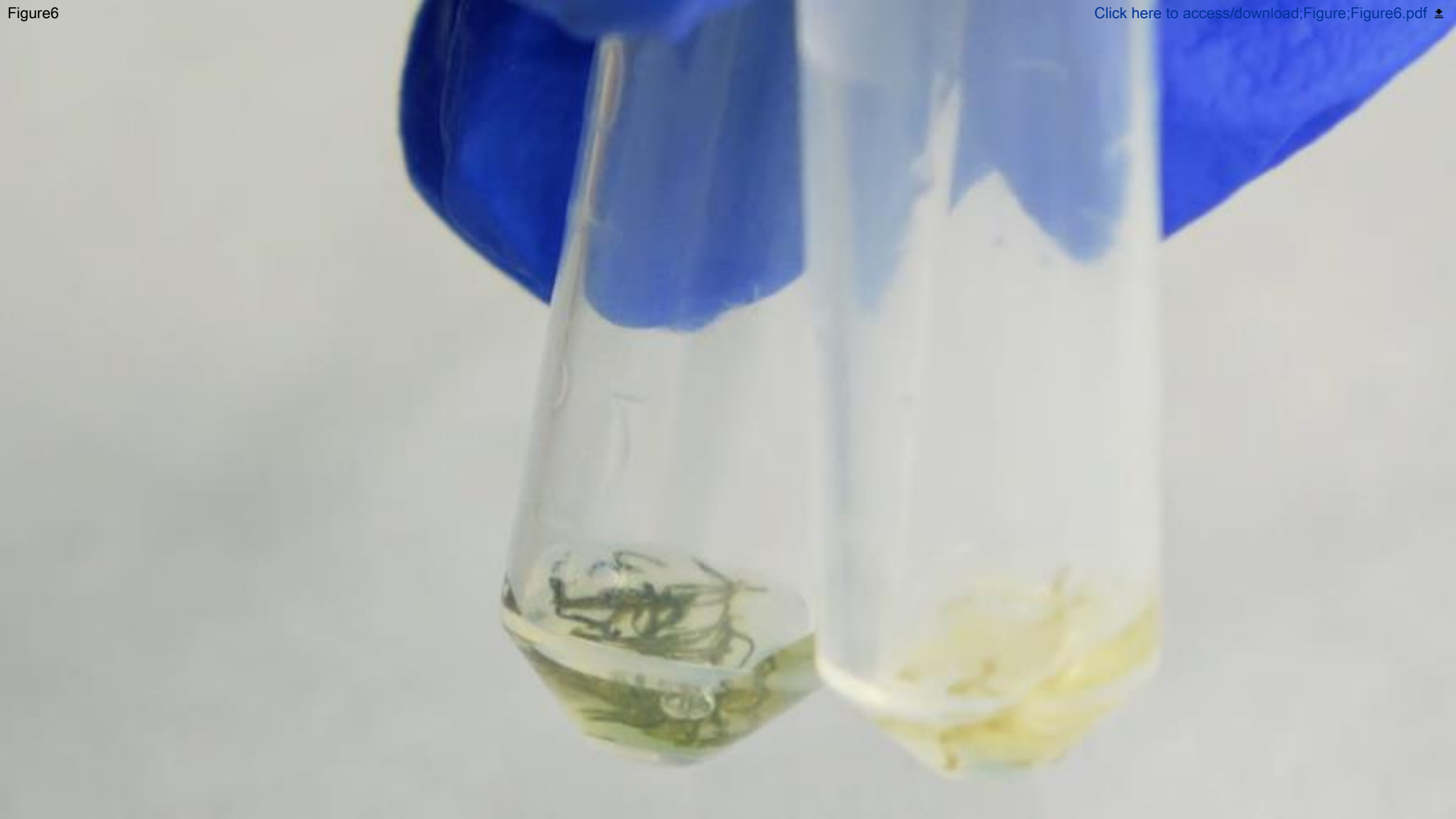


Figure7

Click here to access/download;Figure;Figure7.pdf

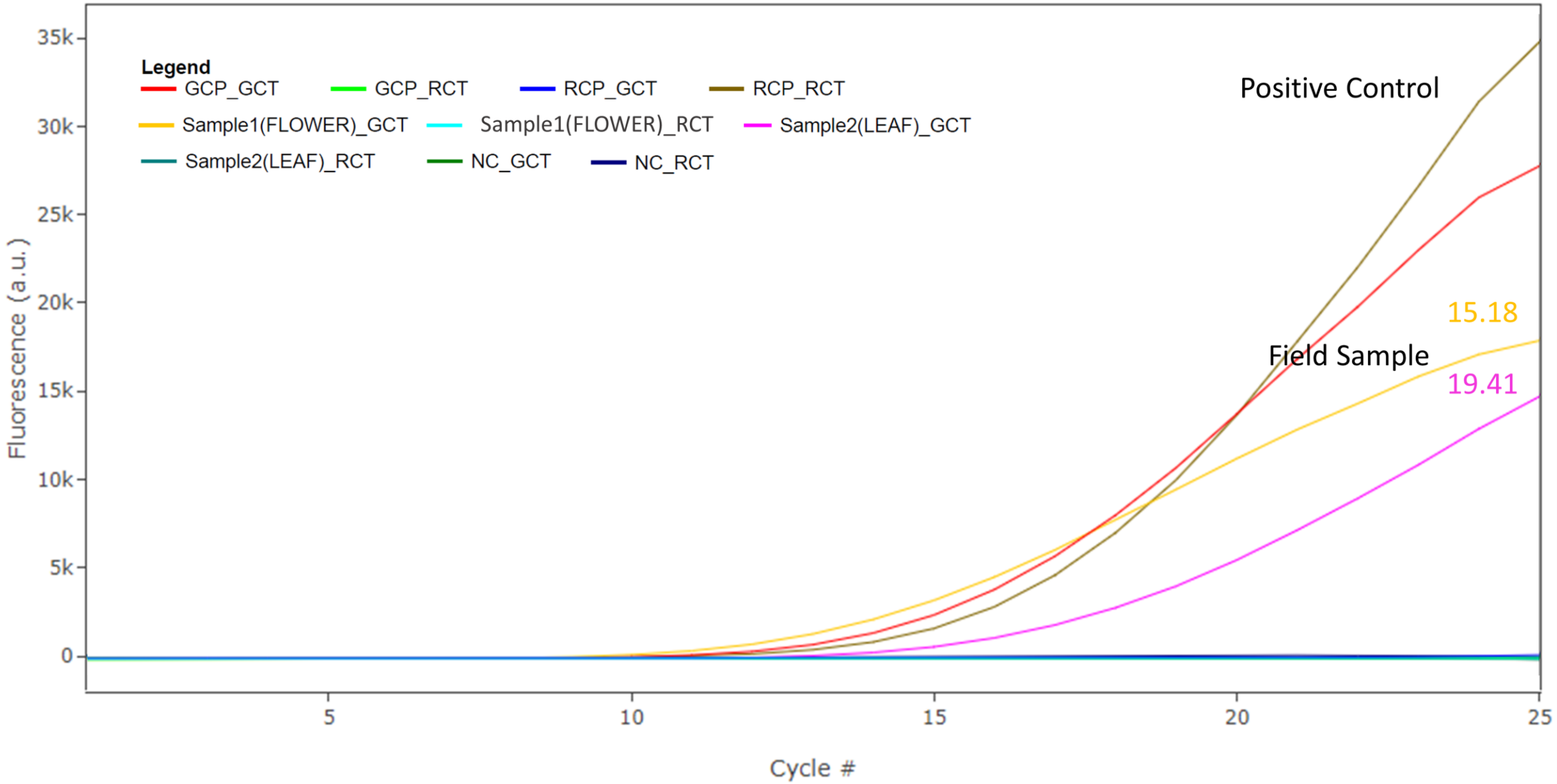


Figure8

Click here to access/download;Figure;Figure8.pdf

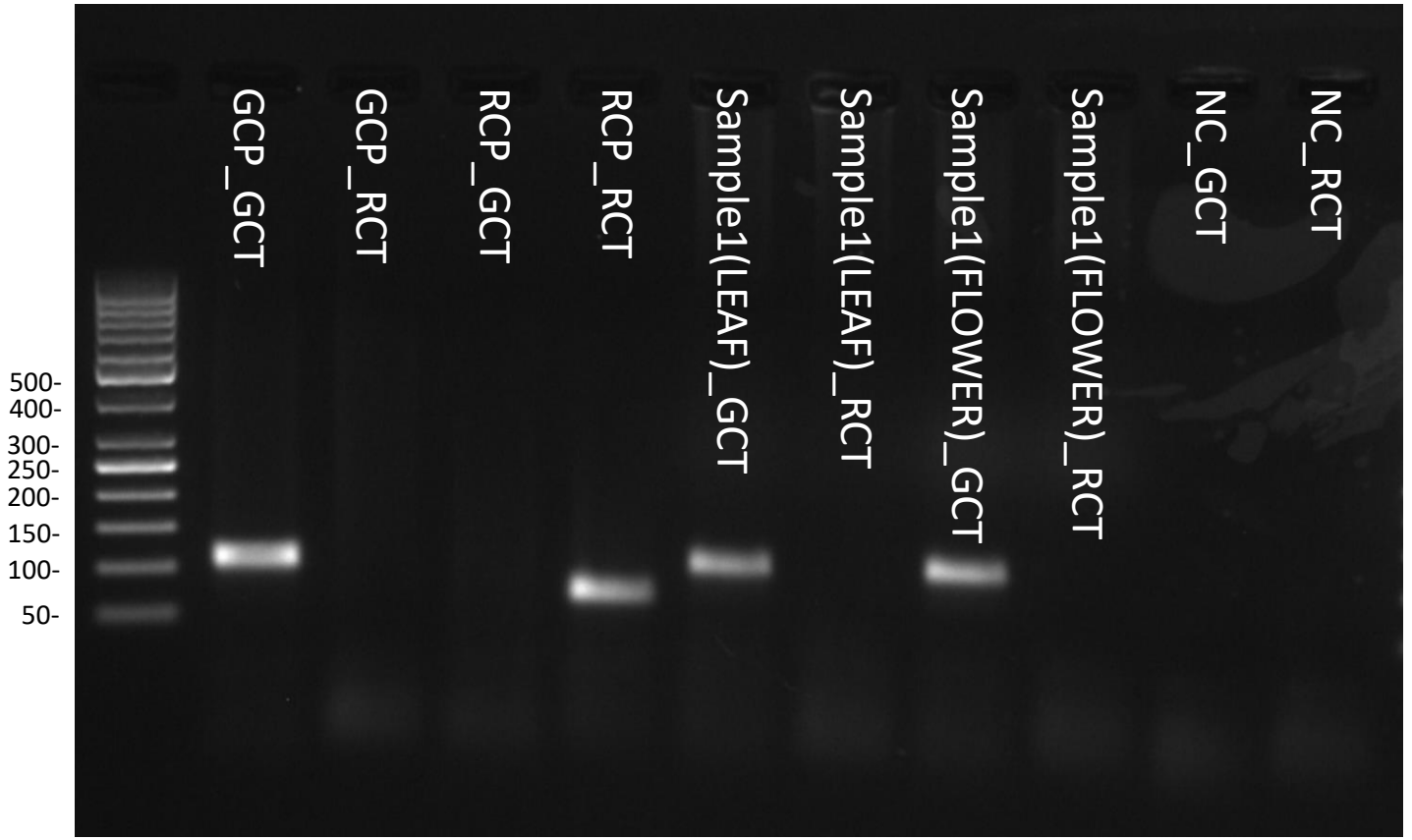
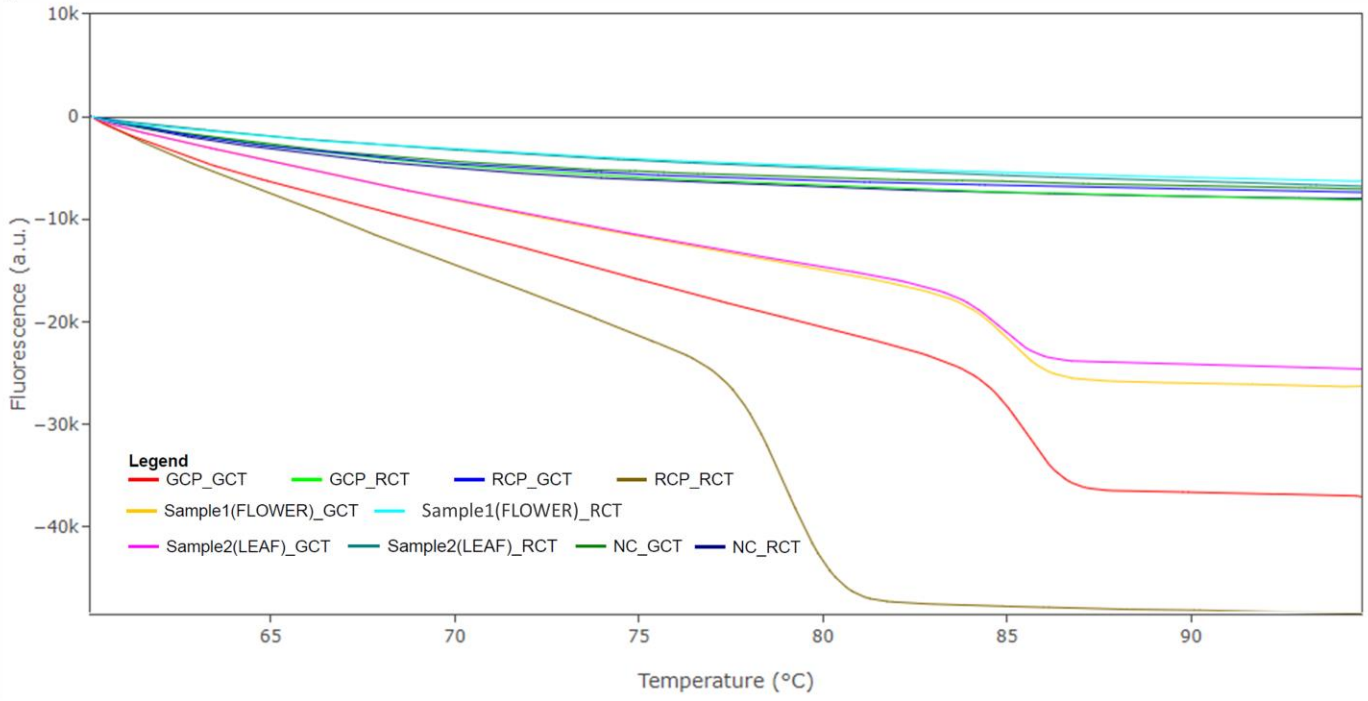


Figure9

A



B

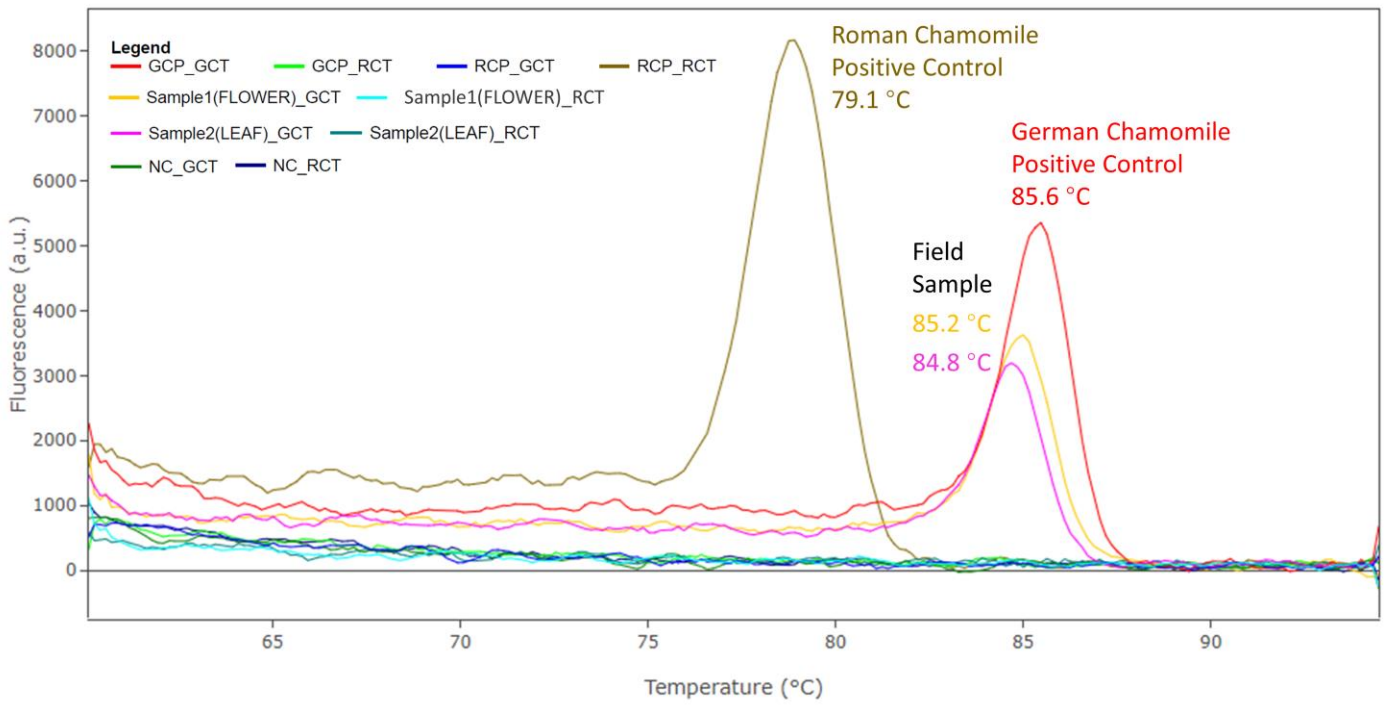


Figure10

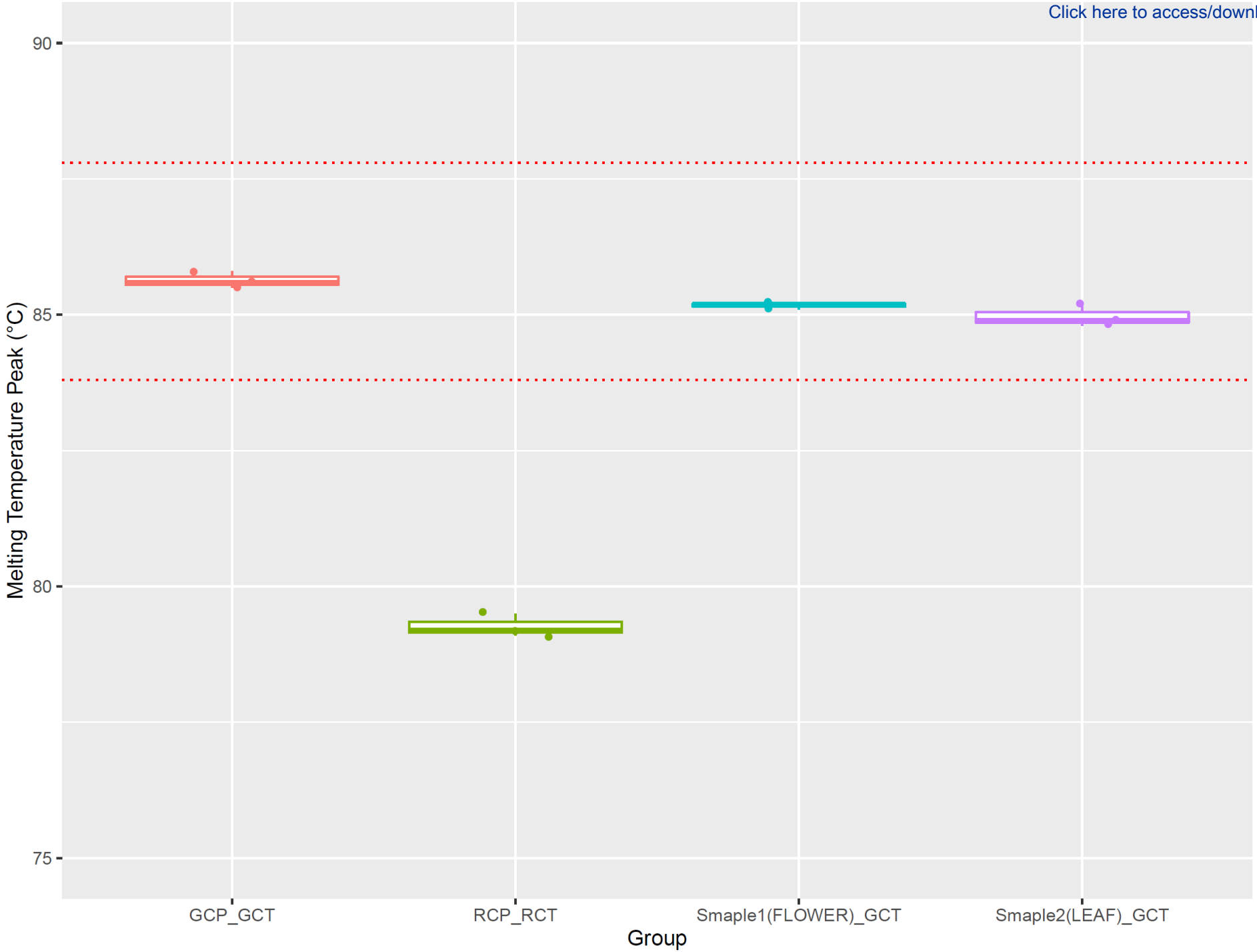


Table1

Click here to access/download;Table;Table 1. Thermocycling conditions.xlsx

Stage	Cycle	Temperature	Time
Constant Temperature	1	95℃	60s
Amplification	25	95℃	30s
		60℃	30s
Melting Curve	1	60℃	Ramp 0.05℃/s
		95℃	

Assay	Primer name	Sequence 5'-3'	Position	Region
Matricaria recutita	ZL3	TCGTCGGTCGCAAGGATAAG	Forward	ITS2
	ZL4	TAAACTCAGCGGGTAGTCCC	Reverse	
Chamaemelum nobile	ZL11	TGTCGCACGTTGCTAGGAAGCA	Forward	ITS2
	ZL12	TCGAAGCGTCATCCTAAGACAAC	Reverse	

Amplicon Size

102 bp

65 bp

Table3

Click here to access/download;Table;Table 3. Well types and descriptions.xlsx

Well position	Well name
1	GC_PosCtrl_GC_Test
2	GC_PosCtrl_RC_Test
3	RC_PosCtrl_GC_Test
4	RC_PosCtrl_RC_Test
5	Field_Sample_GC_Test
6	Field_Sample_RC_Test
7	Field_Sample_GC_Test
8	Field_Sample_RC_Test
9	NegCtrl_GC_Test
10	NegCtrl_RC_Test

Description

German chamomile positive control under GC Test
German chamomile positive control under RC Test
Roman chamomile positive control under GC Test
Roman chamomile positive control under RC Test
Sample of leaf tissue under GC Test
Sample of leaf tissue under RC Test
Sample of flower tissue under GC Test
Sample of flower tissue under RC Test
Negative control sample under GC Test
Negative control sample under RC Test

Table4

Click here to access/download;Table;Table 4. Master-mix composition.xlsx

Reagent	GC_Test	RC_Test
Universal qPCR Mix*	10 µL	10 µL
ZL3 primer (10 µM)	0.4 µL	NA
ZL4 primer (10 µM)	0.4 µL	NA
ZL11 primer (10 µM)	NA	0.4 µL
ZL12 primer (10 µM)	NA	0.4 µL
H_2O (Nuclease-free)	7.2 µL	7.2 µL

* contains Hot Start Taq DNA Polymerase

Table5

Click here to access/download;Table;Table 5. Rule for automatic qPCR result interpretation.xlsx

Well Name	Expected Result
GC_PosCtrl_GC_Test	Detected
GC_PosCtrl_RC_Test	Not Detected
RC_PosCtrl_GC_Test	Not Detected
RC_PosCtrl_RC_Test	Detected
Field_Sample_Leaf_GC_Test	Present
Field_Sample_Leaf_RC_Test	Absent
Field_Sample_Flower_GC_Test	Present
Field_Sample_Flower_RC_Test	Absent
NegCtrl_GC_Test	Not Detected
NegCtrl_RC_Test	Not Detected

Positive Result Criteria	Negative Result Criteria
Detected / Present	**Not Detected / Absent**
Ct < 25 and 84 <= Tm <= 86	-
-	No Ct value within 25 cycles
-	No Ct value within 25 cycles
Ct < 25 and 79 <= Tm <= 81	-
Ct < 25 and 84 <= Tm <= 86	No Ct value within 25 cycles
-	No Ct value within 25 cycles
Ct < 25 and 84 <= Tm <= 86	No Ct value within 25 cycles
-	No Ct value within 25 cycles
-	No Ct value within 25 cycles
-	No Ct value within 25 cycles

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Battery	TNE	78000mAh	Provide field power supply
bCUBE	HYRIS	bCUBE 2.0	Portable qPCR instrument
Cartridges(16 Well)	HYRIS	NA	Consumables for bCUBE
Electric pipette	Eppendorf	NA	Handling liquid
Extract-N-Amp™ plant PCR kit	SIGMA	XNAP2-1KT	Plant DNA extraction kit
nile (*Matricaria chamomilla L*) flower botanical re	AHP	2264	Used as positive control
Mini dry bath	Yooning	MiniH-100L	For DNA extraction
Nuclease-free water	AMBION	AM9937	qPCR reaction
Primer	o Fisher Sci	NA	qPCR reaction
mile (*Chamaemelum nobile)* flower botancial refe	ChromaDex	ASB-00030806-005	Used as positive control
Luna universal qPCR master mix	NEB	M3003L	qPCR reaction

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

All edits are based on "60940_R2.docx" downloaded from journal portal.

2. Please address all the specific comments marked in the manuscript.

All comments have been addressed. Please see the all changes in tracked version.

3. Since one of the authors is from the UK, please select I agree to UK ALA in the additional section page of the submission site. This is important.

Agree to UK ALA has been selected.

4. Please check with your funding source regarding PMC deposition. We do not deposit articles into PubMed Central on behalf of the authors. However, authors can self-deposit into PMC if required by their funding source.

The project is not funded by NIH and there is no requirement for depositing into PubMed.

Video:
1. Please include the chapter title cards for the protocol section.

Additional title cards for the protocol section has been added.

2. Please correct the spelling of Labeled in the video. Please ensure American English is used throughout.

"Labelled" has been changed to "labeled". Two places changed. We also reviewed and updated the video narratives to ensure American English is used throughout.

3. Please bring down the volume of the narration.

The volume of narration has been brought down by 5 dB.

Once done please ensure that the video is no more than 15 min in length. Please upload the video at: https://www.dropbox.com/request/s3VnyKmX8RgPcP9eQ07A?oref=e.

The video is 12:07 mins long.

Zhengfei Lu, PhD

Dr. Zhengfei Lu is a Sr. Analytical Scientist in the Center of Excellence (COE) Quality Control Laboratory at Herbalife International of America, Inc. He has over 15 years of experience in DNA research. Zhengfei shares his research and method with industry through publishing articles in prestigious peer-reviewed journals, including *Food Chemistry*, *Journal of AOAC International*, *Blood*, and *DNA Repair*. Prior to joining Herbalife, Zhengfei launched his career as a clinical genomic scientist at Vantari Genetics. Zhengfei holds a Ph.D. in Experimental Pathology from the University of Southern California and received a Medical degree from Peking University.

Zhengxiu Shawn Yang

Shawn Yang is a Scientist at Herbalife NatSource (Hunan) Natural Products Co., Ltd.

Zheng Quan

Zheng Quan is a Scientist at Herbalife International of America, Inc.

Tiffany Chua, PhD

Dr. Tiffany Chua is a consultant at Herbalife International of America, Inc.

Leo Li

Leo Li is a Sr. Manager at Herbalife NatSource (Hunan) Natural Products Co., Ltd.

Yanjun Zhang, PhD

Dr. Yanjun Zhang is a Principle Scientist at Herbalife International of America, Inc.

Silva Babajanian

Silva Babajanian is the Director of Center of Excellence (COE) Quality Control Laboratory at Herbalife International of America, Inc.

Francesco Buongiorno

Francesco Buongiorno is a Scientist at Hyris Ltd.

Isabella Della Noce

Isabella Della Noce is a Scientist at Hyris Ltd.

Lorenzo Colombo

Dr. Lorenzo Colombo is a Scientist at Hyris Ltd.

Steven Newmaster, PhD

Dr Newmaster has been a botany & genomics professor at the University of Guelph, Canada for 18 years and has more than 120 scientific publications. Currently he is conducting research on molecular diagnostic tools for identity testing botanical ingredients. His R&D has result in the development of novel biotechnology and impacted international trade of botanicals as he advises on the authentication of herbal products to regulators and is development of new QA/QC industry standards. He co-founded

the world's first ingredient identity certification program (TRU-ID), which has processed over 30 million natural ingredients in the USA, Canada, E.U and Asia.

Tricia Chua

Tricia Chua is a Sr. Director at Herbalife International of America, Inc.

Peter Chang

Peter Chang is a Vice President at Herbalife International of America, Inc.

Gary Swanson

Gary Swanson is a Sr. Vice President at Herbalife International of America, Inc.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Field Identification of Matricaria chamomilla using a portable qPCR system

Author(s): Shawn Yang, Zheng Quan, Tiffany Chua, Leo Li, Yanjun Zhang, Silva Babajanian, Francesco Bongiorno, Isabella Della Noce, Lorenzo Colombo, Steven Neumaster, Tricia Chua, Peter Chang, Gary Swanson, Zhengfei Lu

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

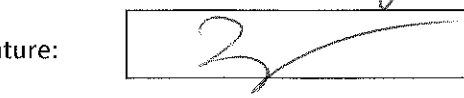
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Zhengfei Lu
Department:	Corporate Quality Laboratory
Institution:	Herbalife International of America, Inc.
Title:	Sr. Analytical Scientist
Signature:	
Date:	10/14/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140