

SPECIAL SECTION

Recommendations for Validation of Real-Time PCR Methods for Molecular Diagnostic Identification of Botanicals

STEVEN G. NEWMASER, DHIVYA SHANMUGHANANDHAN, PRASAD KESANAKURTI,

HANAN SHEHATA, and ADAM FALLER

University of Guelph, College of Biological Sciences, NHP Research Alliance, Guelph, ON, Canada N1G 2W1

ISABELLA DELLA NOCE

Hyris Biotech R&D, Lower Ground Floor, One George Yard, EC3V 9DF London, United Kingdom

JUNG YEOP LEE and PAWEŁ RUDZINSKI

NBTY, Global Analytical and Quality Control, 105 Orville Dr, Bohemia, NY 11716

ZHENGFEI LU, YANJUN ZHANG, and GARY SWANSON

Herbalife International, 990 190th St, Torrance, CA 90502

ROBERT HANNER

University of Guelph, Department of Integrative Biology, Guelph, ON, Canada N1G 2W1

SUBRAMANYAM RAGUPATHY

University of Guelph, College of Biological Sciences, NHP Research Alliance, Guelph, ON, Canada N1G 2W1

Background: PCR methods are the most commonly used DNA-based identity tool in the commercial food, beverage, and natural health product markets. These methods are routinely used to identify foodborne pathogens and allergens in food. Proper validation methods for some sectors have been established, while there are none in other markets, such as botanicals. **Results:** A survey of the literature indicates that some validation criteria are not addressed when developing PCR tests for botanicals. **Objective:** We provide recommendations for qualitative real-time PCR methods for validating identity tests for botanical ingredients. **Methods:** These include common criteria that underpin the development and validation of rigorous tests, including (1) the aim of the validation test, (2) the applicability of different matrix variants, (3) specificity in identifying the target species ingredient, (4) sensitivity in detecting the smallest amount of the target material, (5) repeatability of methods, (6) reproducibility in detecting the target species in both raw and processed materials, (7) practicability of the test in a commercial laboratory, and (8) comparison with alternative methods. In addition, we recommend additional criteria, according to which the practicability of the test method is evaluated by transferring the method to a second laboratory and by comparison with alternative methods. **Conclusions and Highlights:** We hope that these recommendations encourage further publication

on the validation of PCR methods for many botanical ingredients. These properly validated PCR methods can be developed on small, real-time biotechnology that can be placed directly into the supply chain ledger in support of highly transparent data systems that support QC from the farm to the fork of the consumer.

Targeted PCR has become the accepted method for DNA-based identity analysis of food ingredients in QC systems. This molecular diagnostic tool supports good manufacturing practices that address several concerns, including food security, safety, brand damage, and labeling accuracy. The definition of targeted PCR needs to be placed in the context of the numerous other PCR methods. PCR consists of three basic steps: (1) Double-stranded DNA is denatured into single strands at 95°C, (2) primers anneal to denatured single strands at temperatures between 50 and 60°C, and (3) primers extend to copy the target DNA. These three steps are repeated in 30–40 cycles to exponentially amplify, making billions of copies of target DNA (1). PCR methods can broadly be divided into the following two groups, depending on how amplified DNA is analyzed: endpoint PCR, in which DNA is analyzed at the end of the reaction through gel electrophoresis, and real-time PCR (RT-PCR), which analyzes the target DNA in “real time” during the process of reaction using fluorescence chemistry. Endpoint/conventional PCR involves several postprocessing steps, such as visualization of the product, purification, and sequencing, which may take several days as they are labor-intensive (2). However, RT-PCR methods are more sensitive and less laborious, as they do not require any postprocessing steps, and the results can be analyzed instantaneously (3, 4). Based on the method of fluorescence emission, RT-PCR methods can be divided into non-sequence-specific and sequence-specific techniques (5). In non-sequence-specific methods, DNA dyes called fluorophores are commonly used; they emit more fluorescence when they intercalate with double-stranded DNA than unintercalated dyes (6). Dye-based techniques are cheaper to use; however, they generate fluorescence even during a nonspecific reaction,

Invited by Joseph Betz (National Institutes of Health) and Paula Brown (British Columbia Institute of Technology) for a special section on DNA authentication of botanicals. This paper was originally presented at the American Herbal Products Association's 6th Annual Botanical Congress on September 29, 2017 in Las Vegas, NV.

We would like to acknowledge support from the NHP Research Alliance and critical reviews from Sara Handy (U.S. Food and Drug Administration, College Park, MD).

Corresponding author's e-mail: snewmast@uoguelph.ca

DOI: <https://doi.org/10.5740/jaoacint.18-0321>

irrespective of whether the target sequence is amplified or not. To overcome this problem, melting curve analysis is performed on amplified DNA fragments in the same reaction tube. During this analysis, amplicons are subjected to higher temperatures, allowing the disassociation of hydrogen bonds present between nucleotides of double-stranded DNA. Depending on the type of nucleotides present in a sequence, melting temperatures (T_m) vary between different amplicons, and the T_m value for the target sequence is determined through multiple rounds of melting analysis (7, 8). However, sequence-specific techniques offer more specificity, as fluorescence emission happens only if the target sequence is amplified. The TaqMan assay is one of the most widely used probe-based, sequence-specific RT-PCR techniques in molecular diagnostics (9). In this assay, a fluorescently labeled, target-specific probe is designed between primers. The probe binds to the target sequence downstream of one of the primers and is displaced by the cleavage activity of Taq polymerase during primer extension, releasing the fluorescence. The intensity of the fluorescence is increased with exponential growth of the amplicon. As the probe cannot bind to the nontarget sequence, no fluorescence is emitted; thus, only the target sample is identified. However, both dye- and probe-based methods are equally used in diagnostics, depending on the requirements of sample testing procedures. Dye-based methods are used when low levels of specificity (genus-level) and sensitivity are not a concern and when there is more time for optimization because they are easy to design and less expensive. In contrast, probe-based methods are preferred for highly specific and sensitive assays, although they take more time to design and are costlier. Overall, because of the ease of sample analysis and quick turnaround time, RT-PCR methods are more commonly used in molecular diagnostic identifications than conventional PCR.

A variety of PCR methods have been successfully commercialized and used extensively for species identity and QC systems in many different industry sectors. Applications include identification of raw and cooked meat mixtures (10), fish and seafood (11, 12), animal feed (13, 14), animal byproducts (15), allergens (16–18), foodborne pathogens (19–22), probiotics (23, 24), clinical infectious microbial diseases (25–27), and genetically engineered (GE) or genetically modified organisms (GMOs; 28, 29). These industry sectors have adopted molecular techniques into their QA programs during the past decade because they are rapid, less laborious, and more sensitive, specific, and efficient compared with conventional methods (30, 31). Targeted PCR methods (32) are the accepted, standardized tool within these industries because alternative molecular diagnostic methods (33; e.g., next generation sequencing) have yet to be thoroughly validated. Targeted molecular diagnostic methods are designed assays that ask the question, “Is species *X* present in the sample?” Nontargeted methods seek to ask the question, “What species are present in a sample?” Other interpretations include the terms active (targeted) and passive (nontargeted; 34). It is possible to design a good test assay with positive and negative controls for targeted molecular diagnostic methods. Nontargeted assays lack positive controls and, therefore, are not fit-for-purpose. These are two very different approaches to species identification using DNA-based tools, of which the targeted approach has been scientifically validated in the literature for commercial use (33, 35, 36). Although nontargeted DNA barcoding has been developed and validated for fish (37)

as a commercial tool, there are no validated methods published for botanical ingredients.

The use of DNA-based methods for identification of botanical ingredients is complementary to other methods used in the industry. Traditional methods are based on microscopic identification of plant parts, cells, and tissues or phytochemical analyses, both of which are limited in some cases in which there are no defining taxonomic characteristics or species-specific phytochemicals. These methods are particularly limited in the identification of some processed plant materials such as powders and extracts, which are prevalent in the food and natural health product (NHP) supply chains. This poses a considerable challenge for QA programs and has resulted in cases of product adulteration, which lower consumer confidence and may present considerable health risks. DNA-based biotechnology presents another tool for ingredient identity and the detection of adulterants, as demonstrated in several marketplace studies (38–42). Although many DNA-based methods have been proposed for the authentication of botanical ingredients, these methods must be validated for identity testing in commercial QC programs. It would be prudent to develop the standard validation procedures for DNA-based identity of botanicals, such as those already established within many other commercial species in the other sectors mentioned above (e.g., foodborne pathogens).

Guidelines for the validation of qualitative RT-PCR methods for molecular diagnostic identification have not been developed for botanicals. Conversely, guidelines have been developed for the identification of foodborne pathogens (21, 33), meat, fish and seafood (10, 12), and GE organisms (28, 29). Consequently, there are rigorous guidelines in place so that our food is safe from toxic pathogens. A survey of the literature (Table 1) indicates compliance with all validation criteria within research papers concerning the development of PCR tests to identify a specific pathogen. Meat, fish, and allergen PCR validation studies are less rigorous but are still founded on considerable criteria, of which the only criterion lacking was the need for a reproducibility study. The few studies we looked at on probiotics and botanicals lacked some criteria, particularly for botanicals, which would impede proper validation. Some of the validation criteria that were assessed in this botanical study did not fully comply with appropriate standards for criteria such as specificity and sensitivity. This is not surprising given that there are no guidelines for validation studies for botanicals. This gap in the literature is not addressed by animal or microbial PCR validation guidelines because molecular diagnostics in plants have unique attributes (e.g., secondary metabolites, evolutionary processes, and breeding systems) that must be considered within the criteria for proper validation of PCR methods.

The objective of this article is to provide recommendations for the validation of qualitative RT-PCR methods for molecular diagnostic identification of botanical ingredients. We expect that these methods will evolve to include quantitative PCR and multiplex methods. We hope this article inspires a series of validation studies using these recommendations as a framework for developing targeted, species-specific identity tests for use in the QC and supply chain management systems. We have outlined these recommendations as follows.

Aim

The aim of the validation tests is focused on the correct species identification for authentication of botanicals in

Table 1. PCR validation criteria assessed within several published journal articles for different commercial applications

Validation criteria	Foodborne pathogens			Meat, fish, and seafood			Allergens				Probiotics			Botanicals		
Ref.	(43)	(44)	(20)	(11)	(10)	(45)	(18)	(17)	(16)	(24)	(46)	(47)	(48)	(49)	(50)	(51)
Standard RM	✓	✓	✓	✓			✓			✓	✓	✓		✓		✓
Specificity	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sensitivity	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓			✓	✓
Repeatability	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	
Reproducibility	✓	✓	✓	✓	✓						✓					
Practicability	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓					✓	
Comparison with alternative methods	✓	✓	✓	✓			✓			✓	✓			✓		

NHPs on miniature RT-PCR platforms that are handheld and portable, providing on-site identity testing that is relatively inexpensive, fast, and easy to use. The validation trials aim to evaluate the performance of an assay in correctly identifying the species of interest while not identifying other nontarget species. Analytical validation criteria include (1) the aim of the validation test, (2) the applicability of different matrix variants, (3) specificity in identifying the target species ingredient, (4) sensitivity in detecting the smallest amount of the target material, (5) repeatability of methods, (6) reproducibility in detecting the target species in both raw and processed materials, (7) practicability of the test in a commercial laboratory, and (8) comparison with alternative methods. To determine the performance of the assay, tests on target and nontarget samples must be carried out in replicates by different operators and on different devices.

Applicability

Different matrix variants must be considered, as they utilize different extraction methods that should be used on both raw and processed material (e.g., botanical extracts), respectively. The botanical target samples may need to utilize different genomic DNA extraction methods because of the nature of the target materials. Botanical targets include herbals, medicinal plants, spices, plant oils, seeds, fruits, juices, beverages, and other plant-based food and/or agricultural commodities. Samples should be tested in triplicate. The exact number of samples to be used for validation must be assessed for each assay, depending on the availability of samples with certified identity from verified botanical reference material (RM) and on the possible cross-specificity of the assays as it has been evaluated in the research and development activities during the development of the assay. Certified RM includes specific criteria (Table 2) to ensure that the DNA standards used for developing molecular diagnostic tools such as PCR are reliable and authentic.

DNA Extraction

The type of extraction method and material can affect the quality and quantity of DNA, which in turn affects the analytical sensitivity of the assay. Raw materials can utilize simplified protocols that only use lysis and incubation steps but yield good-quality DNA. Highly processed samples may need more elaborate DNA extraction methods (e.g., cetyltrimethylammonium

Table 2. Criteria for preparing a botanical reference material (RM) standard required for developing molecular diagnostic tools

Criterion	Description
Taxonomic identity	Identified by a professional taxonomist; PhD in botany with evidence of published taxonomic works
Herbarium voucher	Professionally mounted using standard herbarium techniques and curated in a registered herbarium
Leaf voucher archive	Leaf vouchers (100 g) stored in silica gel within plastic pouches (other plant parts may be stored depending on mode of utility)
Data archive	Include sample data as follows: geographic/ collection location, species ID, ecosystem, date, taxonomists name, collector

bromide, hexadecyltrimethylammonium bromide, or enzymes) with longer incubation times that utilize a multistep protocol to get high-quality DNA. We recommend DNA quantification of samples using a fluorometer.

Analytical Specificity

The specificity of the assays is determined based on their ability to amplify only the target species, whereas known adulterants and closely related nontarget species should not be amplified (52). The challenge of identity is founded on the fact that there is a gradient of taxonomic uncertainty among plant species. We must comprehend that species are not strictly defined, but rather species are hypotheses based on phylogenetic relationships. These relationships are defined by heredity and result in variation among species (interspecific) and within species (intraspecific). Capturing this diversity is critical and requires adequate sampling of populations across areas of known cultivation and from the wild species' known geographic distributions (i.e., from floristic distribution maps), which must be recorded as botanical RM (BRM) standards. These are used for developing phylogenetic concepts for species based on the monophyletic hypothesis, by which we identify nucleotide signatures and the respective primers for use in PCR assays. For each assay, nontarget species should be selected from close genetic relatives (same genus or family as the target species) and from commonly used industry adulterants; published phylogenetic trees for the family of the target taxa can used

to pick closely related species within the same generic clade. Given that there is a degree of taxonomic uncertainty associated among different taxa, phylogenies should be developed to comprehend the level of uncertainty among the target species and the next closely related species. Because genetic relatedness is a key criterion for assay validation, the first test must validate the specificity of each assay by checking to confirm that each assay does not amplify any nontarget species, including species used in the commercial supply chain of the target species. Each assay should be performed on a sample of target species samples from different geographic origins, if possible, and a sample of nontarget species including all known adulterants, some closely related species, and a standard BRM with a taxonomic herbarium voucher. The correct sample size is based on saturation curves (53), in which the number of haplotypes is plotted on the x-axis and the sample size on the y-axis; sampling is complete when the curve levels or reaches a horizontal asymptote. These sample sizes should provide 95% confidence according to the simplified Cochran approach (54).

If dye-based chemistry is used, then an additional parameter called T_m needs to be documented in addition to the quantification cycle (C_q) value. Amplicons from botanical species have specific T_m because of their differences in the respective target sequences (7). The target species T_m can be obtained from the amplicon DNA during the specificity test and should be within the interval nominal $T_m \pm 1^\circ\text{C}$ (36).

The specificity of an assay is determined by the set of oligonucleotides (i.e., primers and/or probe) that react only with the target species and do not amplify any nontarget species (which may include closely related species from the same genus or other plant species from different genera) as well as PCR conditions. The specificity test results can be conveyed as a percentage of false positives or negatives (54); the percentage of false-positive or false-negative results should be zero. See formulae below:

$$\text{True Positive} = \frac{100 \times \text{number of correctly classified known samples}}{\text{Total number of known positive samples}}$$

$$\text{False Positive} = \frac{100 \times \text{number of misclassified known negative samples}}{\text{Total number of known negative samples}}$$

$$\text{False Negative} = \frac{100 \times \text{number of misclassified known positive samples}}{\text{Total number of known positive samples}}$$

In some cases, species amplification curves are noticed for nontargets at late C_q values. In such cases, a twofold approach can be followed, depending on the level of industrial processing from raw material to finished extract. For raw botanicals, a threshold for the C_q value can be determined to define false positives and false negatives. The specificity threshold should be lower than the C_q value of nontargets. Both targets and nontargets are tested at the same DNA concentration, and the lowest C_q value of nontargets can be used to set the threshold for specificity. We documented that, when testing targets and nontargets at the same concentration (normalized), there was always a clear difference in their C_q values, which supports the specificity requirement of an assay. However, if the sample is a processed material, it is preferable not to have any thresholds because it reduces the sensitivity of the assay, which is often required for testing processed samples.

Analytical Sensitivity

This parameter measures the lower limit of the target that can be detected reliably by the assay. Factors like the DNA extraction method and the type of material will affect the detection range of the assay. We recommend using two extraction methods for two types of material (e.g., leaf/root, etc.) to determine the analytical sensitivity range. Sensitivity is defined as the LOD, i.e., the lowest concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure (52). Theoretically, the most sensitive LOD is three copies per PCR assuming a Poisson distribution, a 95% chance of including at least one copy in the PCR, and single-copy detection (52). We recommend determining the range of analytical sensitivity of the assay using dilution series of target DNA (extracted using two different methods and materials). At least two different starting concentrations and five dilution points, each in triplicate, should be tested for each extraction type and material, for a total of 120 reactions. A standard curve can be plotted for each extraction type and material. A minimum of 80 to 120% efficiency is required for each standard curve (36). Further research is needed to develop methods for quantitative estimates of the fraction of the botanical extract that is derived from the targeted species in relation to other botanical ingredients. Our lab is engaged in this research so that we might advance these recommendations for quantitative PCR.

Repeatability

This parameter measures the percentage of agreement of results obtained for replicated samples analyzed in the same laboratory by the same operator on the same device. At least seven target samples and three nontarget samples should be tested in triplicate by the same operator on the same device on two different days for a total of 60 reactions. These sample sizes (N_o) should provide 95% confidence according to the simplified Cochran approach (a 95% confidence level gives us Z values of 1.96, per the normal tables; 54):

$$N_o = Z^2 pq / e^2$$

where e = desired level of precision; p = estimated proportion of the population; and $q = 1 - p$.

Reproducibility

This parameter measures the percentage of agreement of results obtained for replicated samples analyzed in two laboratories or by two operators. At least seven target samples and three nontarget samples should be tested in triplicate by two different operators for a total of 60 reactions. These sample sizes should provide 95% confidence according to the simplified Cochran approach (54).

Practicability

The practicability of the test method is evaluated by transferring the method to a second commercial laboratory, where the experiment can be performed under reproducibility conditions. We recommend the use of RM-vouchered samples and industry-processed samples. The new method can be

combined with other PCR-based methods that are already used in the laboratory and can be run under the same conditions. The evaluation of the resource cost, including the amount of training required for the technical staff, must be reported. A method can be considered as passing practicability if the cost of testing is low, training of staff is limited, testing equipment is affordable and assessable, and the operations are easy to complete and document.

Comparison with Alternative Methods

The performance of the assays should be compared with the performance of a gold-standard alternative method, if available (55). Other methods may include orthogonal methods such as morphological identity, the assessment of macroscopic and microscopic taxonomic characters, or analytical chemistry (e.g., TLC, HPTLC, and NMR). Digital droplet PCR can provide a very rigorous estimate of LOD and LOQ, as it performs 10 000 droplet experiments in one run and is more robust to inhibition (56, 57). At a minimum, the specificity can be further validated by confirming the identity of the targeted/amplified sequence by sequencing the amplicon (58).

Discussion

The validation of DNA-based test methods is well-developed for some sectors of industry in which it underpins strict regulations. Although many DNA tools exist, the most rigorous test assays have been developed for PCR methods, which has made it the preferred test for commercial applications and the assessment of serious QC issues. For example, PCR test assays were developed for the control of the bovine spongiform encephalopathy outbreak, and these tests were under strict regulatory oversight by the European Union (EU; EC 2002). DNA testing using qualitative PCR is the recommended method to monitor banned mammalian bone meal as a feed ingredient for farmed animals and for testing animal feed for compliance with these strict regulations (59). DNA testing for GMOs is governed by regulations based on very precise qualitative PCR assays in the food supply chain in accordance with good manufacturing practices in both North America and the EU (28, 29, 35, 36). Perhaps the most compelling evidence of DNA-based compliant test methods with strict regulations is in food safety methods, in which PCR is the dominant tool for regular testing with statistical confidence for the identification of foodborne pathogens (21, 33, 60). Regulatory agencies and industry have agreed that, at this time, the PCR method provides huge advantages in terms of precision, sensitivity, specificity, reliability, labor savings, and practicability over the previous method or novel molecular methods that are yet to be validated (15, 33, 36, 61). More recently, there has been a move to regulate the identity of probiotics because of a market study that identified considerable (42%) issues, including incorrectly listed target species in the ingredients (24). Subsequently, the United States Pharmacopeia (USP) developed a working group and commissioned USP General Chapter 64 on probiotic DNA-based testing methods. These USP methods will be the foundation for regulatory oversight in the botanical and probiotic industry for QC for species ingredient authentication and proper labeling. The Australian government has already

ordered the accurate labeling of consumer products containing live microbials with species-level identity and viability (62). More regulatory oversight is on the way in North America and the EU, as a more recent marketplace survey of probiotics discovered that almost all (15/16) products tested had incorrect label claims, leading to some cause for concern by consumers (63).

Commercial molecular diagnostic test methods for the authentication of botanical species ingredients is not regulated in any country. The World Health Organization (64, 65) advocates international harmonization for the quality, safety, and efficacy of herbal medicines (66). This is a considerable challenge given that the supply of botanicals is a global market with complex entry regulations (67) that vary considerably among countries (68). The regulatory framework is very different in Canada (69) than in the EU (70) or the United States (71, 72). Canada and the EU have focused on strict labeling requirements. In the United States, dietary supplements include botanical food supplements, which are regulated by the Dietary Supplement Health and Education Act (DSHEA) of 1994 (73). The DSHEA forbids the marketing of adulterated, misbranded, or mislabeled products. It requires manufacturers and distributors to comply with the DSHEA and places the responsibility of ensuring the safety of products and compliance with labeling requirements on the U.S. Food and Drug Administration (74). Checks should occur throughout the supply chain, as adulteration and mislabeling can occur in raw materials, during manufacturing, or even in the counterfeiting of high-end brands traded on global markets. QA requires continuous monitoring by national authorities as well as careful adherence to good manufacturing practices by industry members throughout the product process lifecycle.

We recommend that targeted PCR-based tools need to be developed for many botanicals used in commerce. This should follow the well-established methods that are already used to validate ingredient identity throughout the supply chain in many sectors, including foodborne pathogens (22), probiotics (24), GMOs (29), meat (10), fish (12), animal feed (14), and allergens (18). We can establish DNA-based technology in the supply chain that is affordable for industry at the level of testing every batch. This would provide an alternative species ingredient identity verification method in support of QA programs. Quantitative PCR methods are in development, with the goal of new methods that will provide an accurate estimate of the target ingredient relative to adulterants and contaminants. Contaminant DNA is unavoidable, as botanical ingredients come from farms and incidental DNA is almost certainly found in every product at low levels; this incidental DNA is not a health hazard because it represents botanical ingredients in negligible amounts. Further research in our lab is determining how common incidental DNA is in food and NHP products and what is an acceptable limit for incidental DNA in products.

PCR methods are the mainstay of the industry and could be used to empower a transparent, consumer-focused QA system underpinned by real test data. Unfortunately, only few botanicals have had properly validated PCR methods developed, as the focus has been on foodborne pathogens and allergens. We hope that this recommendation document will inspire a surge of PCR validation studies for important botanicals in commerce that have considerable value for consumer health. We advocate that national authorities and industry should attempt to shift

consumers' mindsets on food safety concerns directly onto the products they purchase; consumers should be able to dictate the distribution of corporate market share based on evidence of QA of botanical ingredients. Consumers could have access to transparent QA test data generated by validated PCR tests of botanicals used in their desired NHPs or food supplements. Rather than expecting botanical ingredients and products to be authentic or safe, consumers should be empowered to verify that the products they purchase have been adequately tested. Transparency can be enabled through real-time testing with on-site diagnostic tools, such as PCR, in which the results can be provided in a blockchain-like ledger. Ideally, this could be accessed by a number of stakeholders, including all members of the industrial supply chain from the farm to the fork of the consumer. Regulators could peer into this transparent model of test-based evidence that ensures QA. Perhaps this is a large step for industry, but in this manuscript, we have provided recommendations for PCR-based biotechnology that would make possible this vision for a transparent food chain quality system that crosses the void from industry to consumers.

References

- Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51**, 263–273. doi:10.1101/SQB.1986.051.01.032
- Kramer, M.F., & Coen, D.M. (2006) *Curr. Protoc. Cytom.* **37**, A.3K.1–A.3K.15. doi:10.1002/0471142956.cya03ks37
- Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993) *Bio/Technology* **11**, 1026–1030
- Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., & Strömbohm, L. (2006) *Mol. Aspects Med.* **27**, 95–125. doi:10.1016/j.mam.2005.12.007
- Gibson, N.J. (2006) *Clin. Chim. Acta* **363**, 32–47. doi:10.1016/j.cccn.2005.06.022
- Zipper, H., Brunner, H., Bernhagen, J., & Vitzthum, F. (2004) *Nucleic Acids Res.* **32**, e103. doi:10.1093/nar/gnh101
- Ririe, K.M., Rasmussen, R.P., & Wittwer, C.T. (1997) *Anal. Biochem.* **245**, 154–160. doi:10.1006/abio.1996.9916
- Pryor, R.J., & Wittwer, C.T. (2006) in *Clinical Applications of PCR*, Y.M.D. Lo, R.W.K. Chiu, & K.C.A. Chan (Eds), Humana Press, New York, NY, pp 19–32. doi:10.1385/159745074X
- Livak, K.J. (1999) *Genet. Anal.: Biomol. Eng.* **14**, 143–149
- Cammà, C., Di Domenico, M., & Monaco, F. (2012) *Food Control* **23**, 400–404
- Rasmussen, R.S., & Morrissey, M.T. (2008) *Compr. Rev. Food Sci. Food Saf.* **7**, 280–295. doi:10.1111/j.1541-4337.2008.00046.x
- Naaum, A., & Hanner, R. (2016) *Seafood Authenticity and Traceability: A DNA-Based Perspective*, Academic Press, Cambridge, MA
- Gizzi, G., von Holst, C., Baeten, V., Berben, G., & van Raamsdonk, L. (2004) *J. AOAC Int.* **87**, 1334–1341
- Loncarevic, S., Økland, M., Sehic, E., Norli, H., & Johansson, T. (2008) *Int. J. Food Microbiol.* **124**, 154–163. doi:10.1016/j.ijfoodmicro.2008.03.032
- Fumière, O., Dubois, M., Baeten, V., von Holst, C., & Berben, G. (2006) *Anal. Bioanal. Chem.* **385**, 1045. doi:10.1007/s00216-006-0533-z
- Palle-Reisch, M., Wolny, M., Cichna-Markl, M., & Hohegger, R. (2013) *Food Chem.* **138**, 348–355. doi:10.1016/j.foodchem.2012.10.055
- Garino, C., De Paolis, A., Coisson, J.D., Bianchi, D.M., Decastelli, L., & Arlorio, M. (2016) *Food Chem.* **194**, 980–985. doi:10.1016/j.foodchem.2015.08.114
- Linacero, R., Ballesteros, I., Sanchiz, A., Prieto, N., Iniesto, E., Martinez, Y., Pedrosa, M.M., Muzquiz, M., Cabanillas, B., Rovira, M., Burbano, C., & Cuadrado, C. (2016) *Food Chem.* **202**, 334–340. doi:10.1016/j.foodchem.2016.01.132
- Heller, L.C., Davis, C.R., Peak, K.K., Wingfield, D., Cannons, A.C., Amuso, P.T., & Cattani, J. (2003) *Appl. Environ. Microbiol.* **69**, 1844–1846. doi:10.1128/AEM.69.3.1844-1846.2003
- Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., & Helmuth, R. (2004) *Appl. Environ. Microbiol.* **70**, 7046–7052. doi:10.1128/AEM.70.12.7046-7052.2004
- Dwivedi, H.P., & Jaykus, L.-A. (2011) *Crit. Rev. Microbiol.* **37**, 40–63. doi:10.3109/1040841X.2010.506430
- Adzitey, F., & Corry, J. (2011) *Trop. Life Sci. Res.* **22**, 91–98
- Postollec, F., Falentin, H., Pavan, S., Combrisson, J., & Sohler, D. (2011) *Food Microbiol.* **28**, 848–861. doi:10.1016/j.fm.2011.02.008
- Morovic, W., Hibberd, A.A., Zabel, B., Barrangou, R., & Stahl, B. (2016) *Front. Microbiol.* **7**, 1747. doi:10.3389/fmicb.2016.01747
- Khot, P.D., & Fredricks, D.N. (2009) *Expert Rev. Anti-Infect. Ther.* **7**, 1201–1221. doi:10.1586/eri.09.104
- Kourkoumpetis, T.K., Fuchs, B.B., Coleman, J.J., Desalermos, A., & Mylonakis, E. (2012) *Clin. Infect. Dis.* **54**, 1322–1331. doi:10.1093/cid/cis132
- Vuong, J., Collard, J.-M., Whaley, M.J., Bassira, I., Seidou, I., Diarra, S., Ouédraogo, R.T., Kambiré, D., Taylor, T.H., Jr., & Sacchi, C. (2016) *PLoS One* **11**, e0147765. doi:10.1371/journal.pone.0147765
- Mazza, R., Soave, M., Morlacchini, M., Piva, G., & Marocco, A. (2005) *Transgenic Res.* **14**, 775–784. doi:10.1007/s11248-005-0009-5
- Holst-Jensen, A. (2009) *Biotechnol. Adv.* **27**, 1071–1082. doi:10.1016/j.biotechadv.2009.05.025
- Magistrado, P., Garcia, M., & Raymundo, A. (2001) *Int. J. Food Microbiol.* **70**, 197–206. doi:10.1016/S0168-1605(01)00537-2
- Keramas, G., Bang, D.D., Lund, M., Madsen, M., Bunkenborg, H., Telleman, P., & Christensen, C.B.V. (2004) *J. Clin. Microbiol.* **42**, 3985–3991. doi:10.1128/JCM.42.9.3985-3991.2004
- Lorenz, T.C. (2012) *J. Visualized Exp.* **63**, e3998. doi:10.3791/3998
- Rohde, A., Hammerl, J.A., Boone, I., Jansen, W., Fohler, S., Klein, G., Dieckmann, R., & Al Dahouk, S. (2017) *Trends Food Sci. Technol.* **62**, 113–118. doi:10.1016/j.tifs.2017.02.006
- Simmons, M., Tucker, A., Chadderton, W.L., Jerde, C.L., & Mahon, A.R. (2015) *Can. J. Fish. Aquat. Sci.* **73**, 76–83. doi:10.1139/cjfas-2015-0262
- Hübner, P., Waiblinger, H.-U., Pietsch, K., & Brodmann, P. (2001) *J. AOAC Int.* **84**, 1855–1864
- Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosens, N., & Morisset, D. (2014) *Trends Food Sci. Technol.* **37**, 115–126. doi:10.1016/j.tifs.2014.03.008
- Handy, S.M., Deeds, J.R., Ivanova, N.V., Hebert, P.D., Hanner, R.H., Ormos, A., Weigt, L.A., Moore, M.M., & Yancy, H.F. (2011) *J. AOAC Int.* **94**, 201–210
- Newmaster, S.G., Grguric, M., Shanmughanandhan, D., Ramalingam, S., & Ragupathy, S. (2013) *BMC Med.* **11**, 222. doi:10.1186/1741-7015-11-222
- Posadzki, P., Watson, L., & Ernst, E. (2013) *Eur. J. Clin. Pharmacol.* **69**, 295–307. doi:10.1007/s00228-012-1353-z
- de Boer, H.J., Ichim, M.C., & Newmaster, S.G. (2015) *Drug Saf.* **38**, 611–620. doi:10.1007/s40264-015-0306-8

- (41) Han, J., Pang, X., Liao, B., Yao, H., Song, J., & Chen, S. (2016) *Sci. Rep.* **6**, 18723. doi:10.1038/srep18723
- (42) Shanmughanandhan, D., Ragupathy, S., Newmaster, S.G., Mohanasundaram, S., & Sathishkumar, R. (2016) *Drug Saf.* **39**, 1211–1227. doi:10.1007/s40264-016-0459-0
- (43) Chen, R., Bi, Y., Yang, G., Liu, Z., Liu, Z., Zeng, B., & Tong, T. (2010) *Diagn. Mol. Pathol.* **19**, 172–179. doi:10.1097/PDM.0b013e3181d8c241
- (44) McGuinness, S., McCabe, E., O'Regan, E., Dolan, A., Duffy, G., Burgess, C., Fanning, S., Barry, T., & O'Grady, J. (2009) *Meat Sci.* **83**, 555–562. doi:10.1016/j.meatsci.2009.07.004
- (45) Soares, S., Amaral, J.S., Oliveira, M.B.P., & Mafra, I. (2013) *Meat Sci.* **94**, 115–120. doi:10.1016/j.meatsci.2012.12.012
- (46) Leuschner, R.G., Bew, J., Fourcassier, P., & Bertin, G. (2004) *Syst. Appl. Microbiol.* **27**, 492–500. doi:10.1078/0723202041438464
- (47) Vitali, B., Candela, M., Matteuzzi, D., & Brigidi, P. (2003) *Syst. Appl. Microbiol.* **26**, 269–276. doi:10.1078/072320203322346128
- (48) Wang, H., Sun, H., Kwon, W.-S., Jin, H., & Yang, D.-C. (2010) *Mol. Biol. Rep.* **37**, 1053–1057. doi:10.1007/s11033-009-9827-5
- (49) Ciarmiello, L.F., Piccirillo, P., Pontecorvo, G., De Luca, A., Kafantaris, I., & Woodrow, P. (2011) *Mol. Biol. Rep.* **38**, 1237–1249. doi:10.1007/s11033-010-0223-y
- (50) Hupfer, C., Waiblinger, H.-U., & Busch, U. (2007) *Eur. Food Res. Technol.* **225**, 329–335. doi:10.1007/s00217-006-0418-6
- (51) Sharma, S., & Shrivastava, N. (2016) *Acta Pharm. Sin. B* **6**, 253–258. doi:10.1016/j.apsb.2016.02.003
- (52) Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., & Wittwer, C.T. (2009) *Clin. Chem* **55**, 611–622. doi:10.1373/clinchem.2008.112797
- (53) Egeland, T., & Salas, A. (2008) *PLoS One* **3**, e3988. doi:https://doi.org/10.1371/journal.pone.0003988
- (54) Cochran, W.G. (1977) *Sampling Techniques*, 3rd Ed., John Wiley & Sons, Inc., Hoboken, NJ
- (55) CODEX (2010) Codex Alimentarius Commission, Food and Agriculture Organization of the United Nations and World Health Organization, Rome, Italy, CAC/GL 74-2010
- (56) Zhao, Y., Xia, Q., Yin, Y., & Wang, Z. (2016) *PLoS One* **11**, e0159004. doi:10.1371/journal.pone.0159004
- (57) Shehata, H.R., Li, J., Chen, S., Redda, H., Cheng, S., Tabujara, N., Li, H., Warriner, K., & Hanner, R. (2017) *PLoS One* **12**, e0182872. doi:10.1371/journal.pone.0182872
- (58) *ISO 21569:2005: Foodstuffs—Methods of Analysis for the Detection of Genetically Modified Organisms and Derived Products—Qualitative Nucleic Acid Based Methods*, International Organization for Standardization, Brussels, Belgium
- (59) European Union (2003) *Off. J. Eur. Union L* **173**, 6–13
- (60) Law, J.W.-F., Ab Mutalib, N.-S., Chan, K.-G., & Lee, L.-H. (2015) *Front. Microbiol.* **5**, 19
- (61) Prado, M., Berben, G., Fumière, O., van Duijn, G., Mensinga-Kruize, J., Reaney, S., Boix, A., & von Holst, C. (2007) *J. Agric. Food Chem.* **55**, 7495–7501. doi:10.1021/jf0707583
- (62) Hammett, R. (2008) *Therapeutic Goods Order No. 78: Standard for Tablets and Capsules*, Federal Register of Legislation, F2008L04287, <https://www.legislation.gov.au/Details/F2008L04287> (accessed on June 4, 2018)
- (63) Lewis, Z.T., Shani, G., Masarweh, C., Popovic, M., Frese, S.A., Sela, D.A., Underwood, M.A., & Mills, D.A. (2016) *Pediatr. Res.* **79**, 445–452. doi:10.1038/pr.2015.244
- (64) World Health Organization (2010) *WHO Monographs on Medicinal Plants Commonly Used in the Newly Independent States (NIS)*, World Health Organization, Geneva, Switzerland
- (65) World Health Organization (2009) *WHO Monographs on Selected Medicinal Plants*, Vols 1–4, World Health Organization, Geneva, Switzerland
- (66) Mahady, G.B. (2001) *J. Nutr.* **131**, 1120S–1123S. doi:10.1093/jn/131.3.1120S
- (67) Shao, A. (2016) in *Developing New Functional Food and Nutraceutical Products*, D. Bagchi & S. Nair (Eds), Academic Press, Cambridge, MA, pp 279–290
- (68) Low, T.Y., Wong, K.O., Yap, A.L.L., De Haan, L.H.J., & Rietjens, I.M.C.M. (2017) *Compr. Rev. Food Sci. Food Saf.* **16**, 821–834. doi:10.1111/1541-4337.12289
- (69) Health Canada (2013) The Approach to Natural Health Products, https://www.canada.ca/content/dam/hc-sc/migration/hc-sc/dhp-mps/alt_formats/pdf/prodnatur/nhp-psn-eng.pdf (accessed June 2018)
- (70) Silano, V., Coppens, P., Larrañaga-Guetaria, A., Minghetti, P., & Roth-Ehrang, R. (2011) *Food Funct.* **2**, 710–719. doi:10.1039/c1fo10105f
- (71) Avigan, M.I., Mozersky, R.P., & Seeff, L.B. (2016) *Int. J. Mol. Sci.* **17**, 331. doi:10.3390/ijms17030331
- (72) Swann, J.P. (2016) *Drug Test. Anal.* **8**, 271–282
- (73) National Institutes of Health (1994) Dietary Supplement Health and Education Act of 1994, https://ods.od.nih.gov/About/DSHEA_Wording.aspx (accessed July 2018)
- (74) U.S. Food and Drug Administration (2016) Dietary Supplements, <http://www.fda.gov/Food/DietarySupplements/> (accessed July 2018)