USP & FDA Co-Sponsored DNA Standards for Botanical Identification Workshop

Discussion Summary, Day 1

Tuesday, August 21, 2018

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- New York Botanical Garden Herbarium Specimens
  - Over 7.5 million specimens are housed in the botanical gardens.
  - Specimens serve two primary purposes:
    - Document observations of the natural world published in scientific literature and allow findings to be independently verified by other researchers.
    - Immense store of extractable data that can be used to gain new insights into natural world, such as:
      - Measure of morphological characteristics
      - Specimen observation under the microscope to see structures not visible to naked eye
      - DNA sequencing
      - Examination of metadata (e.g., place and time specimen was collected)

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- New York Botanical Garden Herbarium Specimens, cont'd
  - Other patterns can be extracted from specimens aside from geographic distribution
    - Time of year reproduction occurs
    - Detailed description of morphological features
    - Evolutionary history
    - Variation within and between species
- Peer-Reviewed Specimen Summaries
  - Botanical Gardens' primary research product
  - Demand for summaries is greater than ability to produce them

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- Peer-Reviewed Specimen Summaries, cont'd
  - Production of summaries is highly manual
    - Individual measurements are calculated manually
    - Measurements are stored in a database that lacks summary capabilities
      - Data are extracted, condensed, and summarized with various computer programs
      - Process is inefficient and error prone
- Importance of DNA Identification
  - Non-specialists can identify specimens
  - Morphologically deficient or incomplete specimens can be identified

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- Q: PCR-based methods are not good. A lot of traditional testing is based on PCR, or more or less based on Blast. How do you put your results in context with the long history of things like the use of qPCR?
  - A: To have that work, you have to have a negative control that includes the part you are targeting. If there is a TaqMan probe, you have to make sure that piece is present. You can design a PCR failure-based assay.
  - Look at internal controls to look for inhibitions. Properly designed qPCR assays can get around this. We do not have the ability to control for all possible targets.

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- Q: What happens in a commercial test that is being offered that does not have a negative control?
  - A: If you do not have a negative control, there is no way to determine where the DNA you are picking up is coming from (to ensure none of the DNA that you see came from the laboratory, a technician, or was caused by some error in the facility). Without a negative control, you do not know where it came from and as a result, you cannot say if the source was your product or contamination.

Keynotes

Damon Little, PhD., New York Botanical Gardens

What We Heard

- Q: The use of molecular methods in industry is problematic because we are not working in a sterile environment. Plants have been contaminated and mutated and data in GenBank is unreliable. Can you bottom-line things from a commercial setting perspective, given we do not have perfectly preserved samples/tissues?
  - A: GenBank is like Wikipedia. Every plant we use came from somewhere, and contamination is a fact that a user will have to contend with. It is possible that your product will have some of the foreign product DNA in it. DNA is murky, but you have to be looking at the right four pieces. Liquid extracts that are aqueous with gunk usually work fine. Those that are aqueous and are clear material will not produce much of anything.
- Q: Can you explain more about negative controls, would I have to have negative controls for all species I want to eliminate as a special target?
  - A: You need a set of controls for what you want to eliminate, then the others in a batch. At each stage, you add a little more negative control.

Keynotes

Caroline Howard, PhD., British Pharmacopeia, National Institute for Biological Standards and Control (NIBSC)

What We Heard

- *British Pharmacopeia*
  - Includes all monographs and texts of the *European Pharmacopeia*
  - Published yearly in August
  - Effective January 1 of following year
- DNA Barcoding
  - DNA testing within plant material
  - Identity is the most fundamental measure of quality
  - DNA cannot detect everything

Keynotes

Caroline Howard, PhD., British Pharmacopeia, NIBSC

What We Heard

- Plant ID
  - Several PCRs designed
  - Difficult to design
  - qPCR methods
    - Long store past 35 cycles
    - We can use this to account for testing for the absence
- Bacterial DNA
  - Could be useful
  - Need consensus of opinion to create reliable database
  - New methods should be considered
    - Comparing draft genome to transcriptome
    - LCN marker development
      - New method; gets around many DNA issues
  - Feedback from industry is needed
    - What would be most useful?
    - What should we focus on?
    - Industry and stakeholders should communicate with each other on what the best way forward will be

Keynotes

Caroline Howard, PhD., British Pharmacopeia, NIBSC

What We Heard

- Q: It is useful that *BP* has started to link sequences with certain plants. It is hard to figure out how to do this. We run into issues when traditional barcodes cannot distinguish because they are not diagnostic of a particular species. Do you include those sequences or do you just say here are the traditional barcodes, this is what the sequences are for these plants and leave it at that?
  - A: We try to stick to the traditional barcodes as much as we can. Ideally we'd like to have a platform that is easily understandable, easily accessible, and can be used in the same way for as many different herbal drugs as possible; that doesn't always work. We might look outside the methods. At the moment, those are the methods we use within the laboratory, but we have not published those methods yet.

Keynotes

Caroline Howard, PhD., British Pharmacopeia, NIBSC

What We Heard

- Q: Regarding the reference sequence you are publishing, how far from that base of variance do you go?
  - A: Depends on what the sequence is like. What we aim to do is anchor the snip with as many bases as possible. If a different number is used or has shifted, we would be confident that it would still align and show that difference.

Keynotes

Caroline Howard, PhD., British Pharmacopeia, NIBSC

What We Heard

- Q: Regarding the depth of the contaminants you are finding. Do you know the level of what is present and how low can you detect?
  - A: We didn't go through to looking at the quantitation of those issues. In order to do the quantitation, we would have to have a known DNA standard, and we don't have that. What we can talk about is relative abundance, which gives us an idea or impression, but it is not 100% quantitative.
- Q: Do manufacturers in the UK have to perform the test if it is in the *BP*? Have you rolled this out yet and has there been compliance?
  - A: Once the method is in the monograph, it is enforceable. This method has not been rolled out as yet.

Keynotes

Caroline Howard, PhD., British Pharmacopeia, NIBSC

What We Heard

- C: Using PCR-based NGS can be problematic. We tried for herbal mixtures. Some sequences are over and some underreported. We tried two diff loci, but some of the species cannot be bound. There could be PCR bias.
  - A: I agree this is a major issue. The method is in the very beginning stages. There are ways to look at methods without using PCR. Those can be quite expensive. Overamplification of some species, like bacteria, would be an issue.
- Q: Developing reference libraries that are fit for purpose is crucial. Is this an extract or a genome?
  - A: It is just a synthesized DNA molecule.

Industry Perspective

Pawel Rudzinski, Nature's Bounty

What We Heard

- Dietary supplements are a regulated industry and GMPs are followed.
- FDA does not use DNA sequencing alone to analyze herbal extract for phytochemicals.
- Scientific methods are needed that are precise and fit for purpose.
- As we are working on method development, application is key.
- There is no one definition of herbal ID testing.
- Phase 1 Testing
  - Assess accuracy and specificity of lab data
  - If data is inconclusive, use same solution if possible and try to analyze again.
  - If you have an out-of-specification investigation, you cannot retest. You must have justification for your results.
    - You cannot cherry pick your results.

Industry Perspective

Pawel Rudzinski, Nature's Bounty

What We Heard

- Per ICH:
  - ID testing should optimally be able to discriminate between compounds of closely related structures that are likely to be present.
- Key Takeaways
  - Testing must be appropriate and scientifically valid.
  - While DNA techniques cannot ID a targeted phytonutrient and quantify it, they can help ID herbal species.

Industry Perspective: Q&A

Pawel Rudzinski, Nature's Bounty

What We Heard

- Q: If you send a sample to a laboratory and they say their test cannot measure DNA, is that tantamount to an OOS result or do you decide you cannot use DNA and use something else?
  - A: Prior to applying a DNA technique, you have to prove you can do it. Otherwise, this implies that DNA sequencing was not done. If the test is inconclusive, you have to rely on other results. You have to do something with your results. There are broader implications. You have to investigate. It is a bigger picture we have to have in mind.
- Q: What percentage of cases did you employ DNA techniques when another method was not able to determine?
  - A: The percentage was very small. We have a large portfolio of test methods.

Methods to Characterize Highly Processed Materials

Zhengfei Lu, PhD., Herbalife

What We Heard

- Conventional Botanical Authentication
  - Morphological and chemical methods are used for this type of authentication.
    - Limitations to these methods:
      - Loss of physical features in processed products (e.g., extracts)
      - Variation of botanical chemical profile due to seasonal and geographical differences
- DNA Testing
  - A major challenge to this technique is DNA degradation.
    - This type of testing yields inconsistent results.
- DNA Visualization
  - Quantifies DNA degradation.
  - Helps determine the right technique to do testing.

Methods to Characterize Highly Processed Materials

Zhengfei Lu, PhD., Herbalife

What We Heard

- Adapter Ligation + PCR Amplification (LA-DNA)
  - Has the potential to non-selectively amplify the “invisible” dsDNA across different lengths.
  - Helps visualize all fragments between 50 to 900 bp, when the amount of input DNA used is between 10 and 100 pg.
  - Helps identify which botanical samples are feasible for DNA-based techniques.
    - Allows for better understanding of materials that will be tested.
  - Determining the size of “invisible” DNA by LA-DNA analysis aids in designing appropriate test methods for botanical authentication (e.g., processed materials).
  - Developing reliable DNA-based methods will lead to more consistent results.

Methods to Characterize Highly Processed Materials

Zhengfei Lu, PhD., Herbalife

What We Heard

- Q: How do you prevent chimera formation during the ligation reaction?
  - A: The ends are staggering; I blunted the ends. I put some nucleotides to fill in the ends and adding A to the tail. Adapters will have a T. It is not about preventing, but trying to reduce chimera formation as much as possible.
- Q: What method was used to extract DNA?
  - A: We used a commercial kit.
- Q: Were any other botanicals tested? If so, which?
  - A: In terms of species, we tested more than 10. Ginseng is one that was a very good example, also ginger, chamomile, and parsley, for the mixture. We tested the extract, but the testing was very limited.

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition (CFSAN), Office of Regulatory Science (ORS)

What We Heard

- Center for Food Safety and Applied Nutrition
  - Responsible for regulating foods, dietary supplements, and cosmetics.
    - Regulates $417 billion worth of domestic food, $49 billion of imported foods, and over $60 billion dollars worth of cosmetics
  - Promoting and protecting public health by ensuring that nation's supply is safe, sanitary, wholesome, and honestly labeled
- Office of Regulatory Science
  - Staffed by more than 100 scientists
  - Supports CFSAN's regulatory duties
  - Provides lab science for CFSAN's regulatory, policy, and compliance and enforcement programs

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., CFSAN, ORS

What We Heard

- DNA-Based Methods for Species Identification
  - DNA sequencing (Sanger)
  - PCR
  - RT-PCR
  - DNA microarray
  - Next-generation (next-gen) sequencing = high throughput sequencing
    - Whole-genome shotgun
    - Amplicon-based
  - In finished products, it is difficult to prove what is not there.
  - If you can't get DNA out of the sample, you cannot use a genomic method.
  - Also, you may get a positive result, but if it is not from the right plant part, it could still be wrong.
  - Is the right database available for the question you have?

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., CFSAN, ORS

What We Heard

- Chemical methods provide quantitative information on the composition of dietary supplement products.
- Chemical fingerprinting methods are needed.
- Traditional DNA barcoding alone cannot confirm the identity of a finished product.
- Chloroplast genome data is important.
- GenomeTrakr CP
  - Shotgun sequenced on Illumina MiSeq
  - Botanical species of FDA interest
    - In foods/dietary supplements
    - Known toxin producers or allergens
    - Known contaminants or adulterants
    - Closely related to any of the above
  - Chloroplast genomes released to date: 40
    - Open to the public
- Allergen Detection
  - Develop real-time PCR-based assays for peanut/tree nut allergen detection in foods
  - Assay design/testing for various regions of chloroplast genome to determine which targets yield the best specificity and sensitivity

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., CFSAN, ORS

What We Heard

- Q: In initial studies, when you were looking at isoflavones versus DNA barcoding, it seems like you were making the assumption that if isoflavones are there, it was legitimate. But in previous talks, there was a lot of evidence of spiking. Can you comment on that?
  - A: That is a very valid point. Just because you see the compound there, doesn't mean the product is correct. A lot of companies do not have the money to do this. The best approach is to compare some of the chemical methods and some of these DNA-based methods. The technology is constantly changing. Some of the methods we just heard might help in some of these instances where the extracts didn't give us usable DNA to start, but maybe they would after some treatment, at least .
    - Inhibitor removal can do a lot for bringing signal up.

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., CFSAN, ORS

What We Heard

- Q: A lot of the research at CFSAN focuses on food safety. One of the things that we are talking about is fitness for purpose. We see potential utility of DNA methods alongside chemical, physical methods. But one of the challenges I'm concerned with as we move into an NGS framework, is just because we detect a DNA sequence from *E. coli* bacteria does not necessarily mean that there's a health risk because it could have been sterilized. How do we go from a list of a thousand things in our food microbiome to assessing what is actually a potential health risk beyond just simple quantification in terms of more of these biologically infectious agents?
  - A: It is definitely something we have to be careful about. We can at least pin down quickly who the culprit is and it works really well in bacterial systems, at least with pathogens. Probiotics are a bit different. You are right, however, that using some of these big scans can raise more questions. That said, it is good to know what all is in there, when you see a weird effect and you don't know why.

Genomic Approaches Used by FDA to Investigate Botanicals

Sara M. Handy, Ph.D., CFSAN, ORS

What We Heard

- Q: As you move through a bunch of different techniques and you are heading toward a shotgun approach using beyond next-gen approaches, we have heard from a various talks that you have to validate methods before it can be in commercial use. How do you validate that method and how do you deal with not having a negative control?
  - A: Some of these methods, it is really important to have validated methods. So with these DNA ones, especially next-gen, it is really hard to do. Researchers are working toward a way of validating these methods to standardize the microbes. That may lend information to us on how we can then do the same and ensure everything is cross-checked the way it should be. Validating next-gen methods is going to be tricky and require some conversation within the community.

Development and Validation of Real-Time PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Food Allergies
  - Important health and safety concern in North America
  - Affects ~2% of adults and 5% of the infant population in the US
- Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA)
  - Food groups that account for ~90% of food allergies in the U.S. must be declared on the label:
    - Milk
    - Eggs
    - Fish
    - Crustacean shellfish
    - Tree nuts
    - Peanuts
    - Wheat
    - Soybeans

Development and Validation of Real-Time PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Detection Methods
  - Traditional methods used to detect allergenic nuts: ELISA and Western Blot.
  - Mass spectrometry and biosensors have also been used, but with significant limitations (i.e., sensitivity, specificity).
  - PCR assays have also been developed using the allergen gene.
    - Nuclear gene are inherently low copy number regions.
    - ITS also used, but resulted in high levels of cross-reactivity.

Development and Validation of Real-Time PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- DNA in Food Analysis
  - Using DNA in food analysis is not new.
    - Considered controversial at times
    - More cost effective and suitable than protein-based analyses
      - DNA as a molecule is more stable
- Chloroplast Genome
  - Highly conserved structures and organization of content
  - Includes 120–130 genes
    - Primarily participating in photosynthesis, transcription, and translation
    - Hundreds of potential markers that could be used for DNA detection and identification
  - Size varies between species (range: 107–218 kb)

Development and Validation of Real-Time PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

- Chloroplast DNA for Allergen Detection
  - Novel and systematic approach
  - Targets 3 different, high-copy number regions in a single reaction
  - Highly sensitive
  - Allows for detection in difficult and unique commodities

Development and Validation of Real-Time PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Reference libraries (e.g., GenomeTrakrCP), are valuable resources for method development in food safety.
- Chloroplast regions are useful markers to develop qPCR assays to detect peanut and other allergenic plants.
- Further assays will be developed for tree nuts in foods, including dietary supplements.
- Other methods (e.g., target enrichment, ddPCR, and NGS) could potentially provide new alternatives to detect food allergens and other applications.

Development and Validation of RT-PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Q: Have you done any dilution experiments, especially comparing ELISA with qPCR, RT-PCR, or other quantitative methods? Because it could relate back to those thresholds that have already been set through regulations.
  - A: Yes. We are working on that. For the peanut assay, it has been done and it is being tested in different peanut varieties as well. The ELISA results in different peanut varieties is being compared because differences are expected between the ELISA results. For this PCR assay, we do not expect differences.
- Q: Do you have any initial results to get at what the R^2 values would be? Because we have been doing some of these methods, not for allergens, but comparing methods and qPCR is very accurate.
  - For the peanut comparison, data are not available yet, but the results will be published.

Development and Validation of RT-PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Q: With which instruments are you running your experiments and are you using melting curves for validation?
  - A: We are running this on the BioRAD platform.
- Q: Which labeling are you using?
  - I use Texas Red, Cy5 and others.

Development and Validation of RT-PCR Assays for Allergen Detection: Examples in Peanut and Tree Nuts

Caroline Puente-Lelievre, Ph.D., CFSAN

What We Heard

- Q: Is there a standardized DNA extraction method for these products?
  - A: All the DNA extractions were done with the QIAGEN plant kit. I got an incredible amount of DNA from chocolate. The DNA is degraded; the fragments that are amplified with this assay are ~100 base pairs (bps). It is expected that DNA from tomato sauce would be degraded, but this is overcome by the fact that I am targeting amplicons that are ~100 to 110 bps.

Next-Generation Sequencing as a QA Tool

Jesse D. Miller, Ph.D., Director, NSF Applied Research Center & NSF Authentechnologies

What We Heard

- DNA is evolving, moving quickly
- DNA-Based Methods for Botanical ID
  - Differentiate between close relatives
  - Detect and identify expected and/or unexpected contaminants/adulterants
  - Identify more than one species in a mixture
  - Provide repeatable, precise results
- There are no global sequencing standards
  - ISO Group 25 is working on global sequence data

Next-Generation Sequencing as a QA Tool

Jesse D. Miller, Ph.D., Director, NSF Applied Research Center & NSF Authentechnologies

What We Heard

- Having a solid reference database is important
- Q and run scores are important.

- Next-Gen Sequencing (NGS)
  - NGS can be used to determine what is in a sample
    - Even trace contaminants
  - Only as powerful as the database it references
- Universal Testing
  - Use of PCR primers that will amplify a wide range of species, including both target and non-target species
  - Best when unprocessed sample is unknown

Next-Generation Sequencing as a QA Tool

Jesse D. Miller, Ph.D., Director, NSF Applied Research Center & NSF Authentechnologies

What We Heard

- Whole-Genome Sequencing
  - Breaks genome into small fragments for sequencing with digital output file of many short sequences.
  - Bioinformatics can be used to piece these fragments back together to get the whole genome sequence.
  - The whole genome can be used, or smaller non-traditional regions can be used for analysis.
- Chloroplast Sequencing
  - In early stages for broad botanical identification
  - Could potentially be useful for finding non-traditional gene regions that can differentiate closely related species or varieties.

Next-Generation Sequencing as a QA Tool

Jesse D. Miller, Ph.D., Director, NSF Applied Research Center & NSF Authentechnologies

What We Heard

- Q: How valuable has it been to integrate DNA testing into your certification program? What percentage of the time is it figuring out stuff you could not figure out through other methodologies?
  - A: We currently do not use sequencing in any actual certification operations. We consider it a "test-only" case. In other words, I will run the sample for you, give you your sequence results, and then we can go deeper on that if you would like. What we found a lot of times is it depends on the problem you are trying to solve. You might have *x* number of sequences that are *Camellia sinensis*, then there are "other sequences" in there. There are other sequences that pop up when you do next-gen and the question is, what are those other sequences and what are the utility of those sequences? A lot of times, these other sequences when using NGS are incidental contaminants. So it's important that when you're doing that more broad sequencing work application, you look at the next couple of sequences that are coming in to determine if there are red flags of concern.

Guidelines for Botanical Reference Materials & Validation of Molecular Diagnostic Methods

Steven Newmaster, PhD., University of Guelph

What We Heard

- Sample Collection and ID Tests
  - A gold standard is needed (e.g., DNA reference material library)
  - Collection of closely related species is needed
  - Multiple populations need to be collected
    - Many vouchers need to be collected from these populations
    - Populations are needed across a range
- Building a Good Database
  - Requires partnering with industry members, producers, farmers
  - Database can be built on vouchers that can be sampled in many different ways
  - NMR models are encouraging for below-species level
    - NMR has been used to ID different species in various origins across the world

Guidelines for Botanical Reference Materials & Validation of Molecular Diagnostic Methods

Steven Newmaster, PhD., University of Guelph

What We Heard

- Molecular diagnostic (MD) tests can be either targeted or non-targeted.
- Targeted MD tests seek to determine if a certain species is present in a sample.
  - Include a positive and negative control
- Non-targeted tests seek to determine what species are in a sample.
  - Have only negative controls
  - Difficult to perform
- Not having a negative control for commercial tests is a big problem in the industry.
- Methods will be published soon based on an enzymatic approach.
  - Based on high-quality, small sequence DNA

Guidelines for Botanical Reference Materials & Validation of Molecular Diagnostic Methods

Steven Newmaster, PhD., University of Guelph

What We Heard

- Validated methods, species by species, are needed
- A guidelines document was recently submitted to AOAC for validation and DNA methods
- For a test to be valid, it must possess the following qualities:
  - Specific
  - Sensitive
  - Repeatable
  - Reproducible
  - Practical
- There is no one tool that can be used across all species.

Guidelines for Botanical Reference Materials & Validation of Molecular Diagnostic Methods

Steven Newmaster, PhD., University of Guelph

What We Heard

- Q: All the DNA test methods I've seen so far are proprietary. Can you speak on the transparency aspect of using these highly specialized methods versus more classical/traditional validation approaches?
  - A: Several people here are working on General Chapter <563> *Identification of Articles of Botanical Origin.* That will provide transparency because the authors of the chapter will provide the methodology and the approach for DNA molecular diagnostics in the general chapter. Additionally, scientific peer-reviewed literature will provide transparency. In the last 4 days, four papers were submitted to AOAC Standards. Those papers will be public and available for any commercial laboratory or researchers to use. These are the channels to use to ensure transparency in industry.

Quantification of Type I and Type II Errors in Genomic Assessment of Botanical Authentication

David Erickson, PhD., DNA4Technologies

What We Heard

- Errors can be false positive (Type 1) or false negative (Type 2)
- DNA database is required to correctly interpreting results
- The less DNA we have, the more we will star elevating type 1 errors

- Sources of Error
  - PCR
  - Sequencing
  - Analysis challenges
    - Species not in database
    - Species incorrectly assigned
    - Authenticity and Purity

Quantification of Type I and Type II Errors in Genomic Assessment of Botanical Authentication

David Erickson, PhD., DNA4Technologies

What We Heard

- Q: When we do validation, at what rate do we have type 1 or type 2 errors and how do we develop a number to report out?
  - A: Off the top, it's hard to say. Replication and looking at the rate at which you repeat assay and get different results, you start from the known material and cannot replicate, you can tell at what rate you start identifying these errors.
- Q: What would be depth of experience that you're comfortable in? How many samples do we need to put together?
  - A: Depends on the confidence you have that you have enough data to answer the question. We don't always get the saturation, or something close to it.

New Approaches in Genetic Testing and Data Management for Botanicals: A Roadmap For Viable Genomic Identification Tests for QC Applications

Stefano Lo Priore – Hyris Ltd./Roberto Pace – Indena Spa

What We Heard

- Traceability/Authenticity
  - Key elements to support consistency of the herbs' composition.
  - Minimum control is necessary to ensure herb quality and consistency.
  - Traceability and identification can be measured in QC one of three ways: botanical ID, chemotaxic ID, and genomic ID.
- Genomic ID
  - The latest entrant in this arena.
  - Methods that are available and reliable are more often than not quick and cheap.
  - Validation of genomic ID methods is time- and resource-intensive.
- Genomic ID at Indena
  - In use since 2010
  - Traditional test methods are used; samples are sent to contract laboratories.

New Approaches in Genetic Testing and Data Management for Botanicals: A Roadmap For Viable Genomic Identification Tests for QC Applications

Stefano Lo Priore – Hyris Ltd./Roberto Pace – Indena Spa

What We Heard

- Genomic ID at Indena (cont'ed)
  - Many contract laboratories may not work under GMP environment; therefore, tests may not be used for product release.
- bCube
  - In use at Indena since 2016; allowed for in-house quality control in their supply chain.
  - Portable technology that includes artificial intelligence module that will read the results automatically.
  - Performs real-time PCR
  - Results can be uploaded to the cloud for remote access
  - Validation kits, known as Bkits, will soon be available industrywide
    - Goal is to give these kits the capability for use in the field with any apparatus
  - There are two options for use of this technology
    - Quality control, which allows for in-house testing for supply chain and process control
    - Validation certificates: can be issued once data is uploaded to the server

New Approaches in Genetic Testing and Data Management for Botanicals: A Roadmap For Viable Genomic Identification Tests for QC Applications

Stefano Lo Priore – Hyris Ltd./Roberto Pace – Indena Spa

What We Heard

- Q: How long does it take to develop a specific BKit?
  - A: It depends on the plant material; that determines how much time it takes. We started with Panax genus: it takes 1 year. The time to develop kits generally ranges 3 to 9 months. Our industrial and academic partners dictate what kits we develop. If you are interested in developing a kit that is not available, we quantify it not by how much it will cost to develop a kit, but how much will you need of this test once the kit is developed.

New Approaches in Genetic Testing and Data Management for Botanicals: A Roadmap For Viable Genomic Identification Tests for QC Applications

Stefano Lo Priore – Hyris Ltd./Roberto Pace – Indena Spa

What We Heard

- Q: What protocols did you use and which methodology did you follow? Is this technology compliant from a data integrity perspective?
  - A: We are following cGMP requirements; it is not a common analytical methodology. With regard to data integrity, it is a closed system. A big driver for in-sourcing this analysis is because we cannot certify the cGMP status of outside laboratories.

Globally Recognized Definition of Voucher Specimens

Wendy Applequist, Ph.D., Missouri Botanical Garden

- There is no globally recognized definition of voucher specimens
  - Working definition: A preserved specimen that represents and documents a single observation of, or collection of material from, a single population.
- The same voucher is not used for multiple plants (one voucher per plant)
  - A voucher only speaks to the moment the specimen was collected; a future specimen of a plant cannot be covered by an old voucher.
- Vouchers should be botanically identifiable, but are not necessary.
- Just because a voucher is mounted on piece of paper does not mean it is correct,

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Wendy Applequist, Ph.D., Missouri Botanical Garden

What We Heard

- Q: How many samples is enough to identify a species?
  - A: sometimes you can describe something as new from one specimen. For taxonomic purposes, you will want to look at a fair number of specimens.
- Q: In the context of the discussion we heard today, people are very interested in the importance of trying to establish at least some baseline genetic data for some markers for validly described species. Given that museums and herbaria typically aren't too friendly toward destructively sampling type material, what type of guidelines would you give to a community that is interested in building a referenced, sequenced library?
  - A: It should rarely be necessary to sample type material. If you are at the point where you can morphologically identify something with DNA, it should be morphologically cohesive. If you have the taxonomy sorted out, you could come to us and ask for samples of material and they would be provided. We would not need the type because those vouchers would be adequately identified.

Globally Recognized Definition of Voucher Specimens

Wendy Applequist, Ph.D., Missouri Botanical Garden

What We Heard

- Q: When you collect the plant, season plays a key factor and impacts the profile. Does the voucher specimen always refer to DNA or morphological characteristics or is there any consideration of the chemical profile?
  - A: That speaks to fitness for purpose. If you have a tree in your front yard and you cut a piece of it and make a voucher of it today, that is documentation of its identity and we can be confident that if we come back and look at that tree in October, it will have the same identity. But we cannot say that we can use this voucher specimen to do a chemical analysis in October. It is not useful for that purpose. If you wanted to take a sample every month year round, you could use that one herbarium specimen to document the identity of that tree.

Database of Molecular Sequences: GenBank and RefSeq

Karen Clark, Ph.D., NCBI/NLM/National Institutes of Health

- GenBank is likened to Wikipedia: submissions uploaded are available for everyone to use.
- Once a genome is submitted to GenBank, it goes through three screens: foreign contamination screen, evaluation size; validation/discrepancy screen.
  - All three screens are based on what is already in GenBank.
  - A submission could pass the screens simply because it does not exist in GenBank.
- Foreign contamination screen is done very conservatively so as not to exclude correct data inadvertently.
- Genome size is generally expected to be within 4 standard deviations of the median size of the genomes of that species that are already in GenBank.
  - Test requires manual review by the taxonomist.
- In some cases, a submitter may leave the wrong name for a submission. In those cases, GenBank will not verify the sample.

Database of Molecular Sequences: GenBank and RefSeq

Barbara Robbertsche, Ph.D., NCBI/NLM/National Institutes of Health

- In RefSeq, we can pick and choose which records we want to preserve.
- Identifying fungi can be very challenging.
- Type specimen is required in a species description in the literature when published.
- Working with source material that has a validly published name is valuable and powerful.
  - Sequence can more readily be identified.
- RefSeq only works with records where the source material is present in a public culture collection or herbarium.
  - Allows researcher to reuse the data since the biosample type identifier in the record and you can go specifically to that
- It is important to ID where your queries should fall.
- You can obtain the whole dataset on the RefSeq FTP site.
- Any taxonomy changes made will also change in the record.

Database of Molecular Sequences: GenBank and RefSeq

Karen Clark, Ph.D., NCBI/NLM/National Institutes of Health

- Q: When will the probiotic strains be visible at the NCBI?
  - A: We have the reports on an FTP site. Maybe by the end of the year, we will have gone through that 4%. They are in progress now.
- Q: About a month ago, I downloaded 24 closed genomes of 1 species and 17 of those were not correct.
  - A: Send that back to us. We really want that input.

Database of Molecular Sequences: GenBank and RefSeq

Karen Clark, Ph.D., NCBI/NLM/National Institutes of Health

What We Heard

- Q: Would it be possible to have one species, one name, and one sequence that is curated?
  - A: There are some species that have internal variation within each genome. Usually only 1 ITS sequence is represented per species, but for some of the basal fungi, that is not possible. The whole range of variation has to be shown within the genome within one single organism within the range of what you would expect to see. So it is not possible to do just one sequence. Also, this type specimen is the original biomaterial that a person used to associate a name with. It is not representative of the species as a whole. There is variation within species. For the ITS region, most of the time it works well. There is not much internal variation in the ITS percentage identity.

Database of Molecular Sequences: GenBank and RefSeq

Karen Clark, Ph.D., NCBI/NLM/National Institutes of Health

What We Heard

- Q: GenBank is a great platform, but there is a lot of noise out there. How do we curate the data in such a way that the data can be referenced and there can be confidence in the referenced data?
  - A: You would use a curated dataset, not just all of GenBank.
- Q: It is my understanding that a curated folder can be created as a repository of information. Is that available for others?
  - That is the submitter's own data. So when the genomes and the chloroplast genomes come in, they have a biosample and they have a bio-project, which is way to cluster the information. When people submit their sequence, they would have their own bio-project. We are considering offering a RefSeq select and offering these collections of data. Genbank is a primary archive. Some of the stuff is noise. The stuff way back in 1999 is probably better than 2005. We have in our minds to go back and add the quality tests run against existing records so we can lift better ones out of the mass. That is a long-term project.

Selection of Molecular Markers to Distinguish Closely-Related Species

Natasha Techen, PhD., University of Mississippi

What We Heard

- Identification Via Metabarcoding
  - Probably ideal to sequence every DNA piece in a sample
  - Compare with whole genome (chloroplast, mitochondria, nucleus)
  - Only a few genomes have been published to date
  - Hybrids are difficult to identify
  - Showed limited success on damaged/ fragmented DNA (dietary supplements)
    - DNA in processed plant material is degraded.
- Universal Primers
  - Capable of amplifying from a large plant variety
  - Barcode amplification from processed plant material may fail using this approach

Selection of Molecular Markers to Distinguish Closely-related Species

Natasha Techen, PhD., University of Mississippi

What We Heard

- Species-Specific Primers
  - Sometimes not enough differences between the sequences to design very specific primers
  - Absence/presence of a PCR product may not be reliable diagnosis
  - Genus-specific primers are a better option; can amplify from a variety of species
- Commercial Sample Analysis
  - Various species of commercial material were tested.
    - Familiy Ranunculaceae: DNA dilutions showed inhibitors may be present.

Selection of Molecular Markers to Distinguish Closely-related Species

Natasha Techen, PhD., University of Mississippi

What We Heard

- Q: How did you test the primers to ensure you did not have cross-reactivity with other families? What did that panel look like?
  - A: We haven't had a chance to test with other families. We still have many markers for more samples.
- Q: When you design these primers, they must not work with any other families. How many other families did you test? How did you do that?
  - A: The DNA mix was made up of 11 other DNAs. I looked at the phylogenetic tree and looked at plants that were far away from Ranunculaceae to see if it worked on them and it did not work on them. It definitely needs to be tested on more families.

Application of Molecular Techniques to Authenticate Herbal Medicine Granules

Dr. Pang Chui Shaw

What We Heard

- Background
  - Granules are a time-saving, convenient alternative to consumption of fresh herbs, which are typically boiled for 1-2 hours prior to consumption.
  - Product is produced by several companies, each with their own manufacturing process.
- Authentication Challenges
  - Granules cannot be organoleptically inspected and no microscopic characteristics are available because of its destructed morphology.
  - Goal was to determine if molecular techniques could be applied to this highly processed material.

Application of Molecular Techniques to Authenticate Herbal Medicine Granules

Dr. Pang Chui Shaw

What We Heard

- Key Findings
  - DNA with size <200 bp may be amplified fro granules and identified in many cases.
  - Adaptor ligation-mediated PCR provides a potential universal method for species determination.
  - Granules from the same company may be quantified by qPCR.

Application of Molecular Techniques to Authenticate Herbal Medicine Granules

Dr. Pang Chui Shaw

What We Heard

- Q: On the three batches of granules where you saw different levels of DNA expression, did you do comparative chemistry by any chance to see if there was any correlation in chemical quality.
  - A: We have not compared the chemical profiles. There are similar comparisons published. Published results indicate that researchers obtained different granules from different manufacturers and found they had different profiles.
- Q: Do you have some idea on what the level of dilution is? Do you have some dilutions to get at limit of detection?
  - A: We have not yet done this test.

Q&A Discussion

Joseph Betz, PhD., Member, USP Non-Botanical Dietary Supplements Expert Committee (EC)

What We Heard

- Q: If you pick up a plant, do you ask “What is this?” or do you say, “Is this such and such?”
  - A: Most of the time, the answer will be “What is this?” If you have a species that you’re looking for, then you’ll be asking the latter.
  - A: It can be a targeted approach, where you’re looking at the same species across different geographic locations. Ultimately, it depends on the purpose of the collection.
- The answer to the question, “What is this?,” is a nominal question.
  - You can answer the question, “Is this such and such?,” with a binary answer: yes or no. The statistics when you’re doing validation for a nominal property are very different from a binary question.

Q&A Discussion

Joseph Betz, PhD., Member, USP Non-Botanical Dietary Supplements EC

What We Heard

- Q: What approach are you using when it comes to validation?
  - A: For a validation study, the first step is specificity. In specificity, we are identifying the target, but not other closely related species or adulterants or any other species. We have to be able to design that test. Just to go through those experiments, it would take two postdocs 6 months for each species.
  - It seems like the basic science has already been applied. The money investment is tied to going through species by species and applying the tools we already have. That is going to take a lot of time and money.
  - Besides specificity, you have to consider the sensitivity measures. You can in theory do both at same time since you are just running the sample through the same pipeline. Sensitivity is not talked about as often, but it is a key component in medical tests. It is not a bad metric in terms of measuring the efficacy, particularly in assay, to measure the component of a metric.

Q&A Discussion, cont'd

Joseph Betz, PhD., Member, USP Non-Botanical Dietary Supplements Expert Committee

What We Heard

- You cannot extract every product the same way or run the same test.
- Validation will vary from manufacturer to manufacturer, since some may not have the same tools or use the same techniques as others.
- Unknown samples from third-party laboratories would be useful to run validation tests against.
- There is no absolute ruler available for measuring validation.
- From a pharmacopeial point of view, the question is, "Is this such and such?" not "What is this?"
- The challenge with the "What is this?" question is it has to do with how well our methods do their job.
- Level of validation will differ based on the ingredient.
  - Validation level is critical as to how we triage and what we're going to validate.
- The test method is extremely important and it has to be validated.
- In some cases we know more than in other cases. Everything is risk based.

Q&A Discussion, cont'd

Joseph Betz, PhD., Member, USP Non-Botanical Dietary Supplements Expert Committee

What We Heard

- Plants don't care what you call them, that is a human construct. The names and identity are what is important.
- From a regulatory requirements perspective, an identification test is not explicitly stated.
- For the purpose of validation studies, we look at the possible adulterants and other substances that might interfere with the analysis of the sample and use them as positive or negative controls.
- There is a difference between validating chemical components versus validating material.
- Fitness for purpose is important. A different technique might be appropriate depending on the needs.
- There is a need to develop and validate methods that are more broad screens.

Q&A Discussion, cont'd

Joseph Betz, PhD., Member, USP Non-Botanical Dietary Supplements Expert Committee

What We Heard

- Most people use botanicals for health reasons. One of the major reasons WHO supports botanicals is because they're cheaper. The more we go down this higher path of sophistication, the more we price these out of the market and we eliminate the reason why these are valued worldwide. We need to remember why we're doing what we're doing.
- We need sophisticated testing due to nature of modern marketplace. There are drugs in the market now manufactured in factories that weren't around 2000 years ago.
  - The need for sophisticated methods is partly driven by the marketplace: costs rise as the marketplace evolves.

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