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

Discussion Summaries

Wednesday, August 22, 2018

Learnings from the USP Project Team on Botanical Library for DNA-based Identification: Use of Orthogonal Test Methods

Nandu Sarma, PhD., and Ning Zhang, PhD., USP

What We Heard

- Key Takeaways
  - Pilot studies on species-specific test methods using authenticated and commercial samples demonstrate the ability to discriminate.
    - Validation is needed for routine screening.
  - The studies demonstrate value of orthogonal methods for cross-validation of the identification methods.
  - Orthogonal tests increase confidence on identity based on unique attributes.
  - Out of specification (OOS) investigation protocols are needed to address false negative tests due to matrix (such as red ginseng or extracts), which impact DNA extraction or false positives due to contamination.

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What We Heard

- If you don't get DNA from your extraction, you stop. There may come a point where the method you are using is not fit for the purpose for the product you are using.
  - In cases such as this, system suitability controls would come into play, to determine whether there was some inhibition of the DNA extraction, for example.
- While visualization of an amplicon is a quick and easy tool, it is not definitive. We have more sensitive methods to determine whether we get DNA from extraction rather than looking at PCR amplification of the target.

- Q: For ginseng, did you consider using mini barcodes instead of these long regions?
  - A: We will.

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- Q: Is there any concern about the use of gels for widespread use in relation to the possibility for contamination of the environment and have you been thinking about using enclosed methods like qPCR or so to avoid this problem?
  - A: No we did not consider, but that is a good point for consideration.

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What We Heard

- Q: Which method are you choosing from USP? Can you explain what method you're using? How are assays selected? Is it for ID?
  - A: The current compendial methods are based on multiple ID methods—based on microscopy, macroscopy, HPLC, or HPTLC. These are orthogonal. If there is one test that discriminates from the others, that is ideal. But we need orthogonal methods to increase the level of confidence.
  - Each test is based on validation over several samples, based on fitness for the purpose. If the objective is to test for the ID, the attribute of specificity is required, for example, test methods for American ginseng should be able to identify American ginseng and discriminate from other closely related species or adulterants. We have some publications that describe how we do that and what the data requirements are for developing monographs. They are available out on our website and in the public domain. We will gladly share that.

Learnings from the USP Project Team on Botanical Library for DNA-based Identification: Use of Orthogonal Test Methods

Nandu Sarma, PhD., and Ning Zhang, PhD., USP

What We Heard

- Q: In regard to your DNA assay that you are selecting, how does USP select assays from different publications?
  - A: Our basic expectation is to discriminate the target species. We could choose many different gene regions and the approach that we are taking is the species-specific PCR approach. Depending on the complexity of it, we may have to go for some of the sequence-based identification.

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What We Heard

- Q: In regard to your DNA assay that you are selecting, how does USP select assays from different publications? (cont'd)
  - What is important to indicate is that the analytical method needs to ID the true members of the set and discriminate from the ones that are not, that we want to discard. We need to have two sets of authenticated samples. The ones that are authenticated are the true members of the monograph and the analytical procedure needs to 100% of the time indicate positive results for those members of that set. Then we have another which are the potential confounders: the potential suspects that may be confounded with the article under test and an appropriate procedure should give a negative result. And that is true for the chromatographic procedures, for the HPTLC procedures; for the DNA procedures, it should be the same thing. That is how we really validate the specificity of the analytical procedure for identity.

Learnings from the USP Project Team on Botanical Library for DNA-based Identification: Use of Orthogonal Test Methods

Nandu Sarma, PhD., and Ning Zhang, PhD., USP

What We Heard

- Q: In regard to your DNA assay that you are selecting, how does USP select assays from different publications? (cont'd)
  - You can make the assay very complicated but the resolution will also get influenced, but if you can invest more for sequencing, you are able to pick a region that could be validated and be able to distinguish multiple species in one assay, in one test and have better resolution. So it all depends on what you want to do.
  - If you want to discriminate many species maybe sequencing is a better option and in terms of sequencing, if you want to detect multiple species in a mixture, maybe next gen sequencing will be better because it gives you single-molecule resolution, but if you only want to answer, is there Panax ginseng there, then maybe the PCR test is good enough.

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What We Heard

- Q: In regard to your DNA assay that you are selecting, how does USP select assays from different publications? (cont'd)
  - Multiplex is a good choice, but the problem is sometimes you have new knowledge that there is another adulterant, then you have to modify your method and redo the validation.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- National Institute of Standards and Technology (NIST)
  - Agency makes certified reference materials for a number of different products.
  - NIST holds a collection of plant DNA data available to the community for sampling and testing.
  - Organization runs laboratory intercomparison programs.
  - Primary focus is on quantitative aspect of what is in the supplement, but there is now a focus on the authenticity of materials.
- 2013 Study Led by NIST
  - Plants and extracts were collected in the field.
  - Samples were mixed with known adulterants, then analyzed.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- 2013 Study Led by NIST (cont'd)
  - Study sought to answer the following question: is this gingko or not?
  - A total of 26 labs signed up to participate in the study and 19 reported data.
    - Labs were very good at identifying adulteration if the adulteration was with another plant, but not at identifying if the adulteration was with something that didn't have chromatographic bands in it.
    - No genomic methods were used.

Botanical Authenticity Interlaboratory Studies

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What We Heard

- NIST Authenticity Program
  - Program was run in conjunction with a group of AHPA botanists.
  - AHPA program goal: focus on DNA methods.
  - NIST program goal: make materials that are good no matter what methods you are running.
  - NIST and AHPA shared program goal is data evaluation and interpretation.
  - Findings
    - DNA methods were mostly unable to identify extracts of Ginkgo.
    - The genomic methods were able to almost all the time in almost every lab identify presence of Ginkgo leaves.

Botanical Authenticity Interlaboratory Studies

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What We Heard

- Q: When you sent the samples and got the data, there was an underlying assumption that the methods are validated, that methods are fit for the purpose. So would you consider recommending some method—for example, the USP monograph methods? That way all the labs can, besides their own method, also provide the data on some validated method? So you may have you may have additional information that might drive toward the right answer.
  - That's an interesting idea. I really like the idea of asking labs to run some method that we could gauge their performance against. My only concern would be that we had some labs in that group that aren't going to have LC equipment or that aren't going to be able to run a genomic method or that don't have anyone qualified to do microscopy in their lab. So I don't know if there's a single method where we could do that. We could ask people to do that and the people who are available and had that capability could.

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What We Heard

- Q: Previous studies have shown that maybe 2 to 10% of the total DNA there is fungal or bacterial. So the genomic study that said, “yes, there's something there,” I'm sure they were right. How do you collect gingko without getting the fungi?
  - It’s true. These are natural products that are out in the world. To me the question there would be more, does that information hurt you or does it help you? Have you written your specifications to allow for that?
- Q: I noticed that mass spectrometry and NMR seem to be totally missing from the data presented. Flow injection mass spectrometry and NMR are certainly much more definitive and in many cases, much more fit for purpose than HPTLC, for example.
  - I would agree, but NIST does not dictate, “use this or this or this” category. No labs that are running those methods decided to participate.
  - A few NMR labs have been recruited for the for the next time we run something similar. What I would like to ultimately do is make small kits of different authentic materials that represent some diversity and an exclusion panel and have people test both of those.

Botanical Authenticity Interlaboratory Studies

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What We Heard

- C: The study seems to have been designed by scientists with a chemistry background because the gingko leaves or gingko tissue samples are adulterated with another species that's extracted. But in my point of view, when you extract DNA, the tissue itself and the extract behave differently. So that may explain why the methods to ID the for extract is not working very well.
  - R: I would agree with that. The design was actually set up by members of the industry, to mimic what they would actually see in their lab; they would actually see the tissue adulterated with the extract and with that specific extract. It's not the best example because out there in the world, in the market we're not seeing adulteration of Ginkgo leaves. It's the extract that has been adulterated. Your point is very well taken.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- Q: Will any of this supplemental information or metadata from this be available to look at? I know you're blinding the labs, but the precise extraction protocols, is any of that available?
  - A: So every lab reported their own data and we have as much data as any particular lab provided. We will make tables of all of that information available.
- Q: And in the future, if we wanted to get involved with this, would we just sign up on the website? Because we're interested in proficiency testing programs.
  - A: Email us.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- C: A qPCR-based test made to identify raw materials caught all the raw materials. Surprisingly, we caught a slight majority of the extracts as well although the intended purpose of the kit was to do a sample preparation based on raw materials. So it looks like it's completely overlapping with the concept that we're in a gray area with extracts. Our biologists ran a straight-up diagnostic tests that we do for clients based on raw materials. I cannot imagine a more plastic representation of why the two questions that we discussed yesterday—"is x in the sample or not" or "what is in the sample"—belong in two completely different domains. Even talking about them as genetic tests as opposed to the others risks creating some confusion because it's completely two different animals as we have seen here.
  - R: Absolutely agree. We look forward to any of your comments in terms of experimental design, how we can improve, suggestions for particular herbs, all of that.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- C: I would support the comment regarding the additional metadata that may help give a better understanding of what each one of the laboratories actually did. I think the most important part of that might be a set of acceptance criteria for each one of the laboratories. What they consider their acceptance criteria parameters. That may help also to understand the qualities of the different analytical procedures and how they make fit better for the purpose that we are looking.
  - R: In some of the reporting, especially from third-party labs that they didn't necessarily claim: “is this gingko? Yes or no.” “Is this contaminated? Yes or no,” but they gave the information for somebody else to make the call. So I think you're exactly right.
  - The way it's been set up in all the exercises in the past is you may ask the question to the lab, what was your method, but we'll have to design a series of questions to get more into the genomic methods and how the approach is by each participating lab.

Botanical Authenticity Interlaboratory Studies

Catherine (Kate) Rimmer, PhD., NIST and Holly Johnson, PhD., AHPA

What We Heard

- C: We are one of the labs that participated in this exercise and we regularly do this because this is the best way to show proficiency testing in case of an FDA audit or any other audit. In this study, we were able to identify most gingkos. Our challenge was in identifying the stem and other parts because there are no reference materials.
  - R: That is why it's important to have a well-designed exclusion panel because people that work in the industry, know about this adulteration with stem or pother parts, and with *Sephora japonica*; so you include that in your exclusion panel because it's a known problem in the industry. In this case ginkgo doesn't have any super close relatives or things that might be mistakenly harvested for it. So it was a pretty small exclusion panel. That needs to be part of the validation data for specificity for any type of new methods that are going to be used for identity.

Multitude of Methods: Guidelines to Compare Methods

Robert Hanner, Ph.D., University of Guelph

What We Heard

- Background: University of Guelph
  - Headquarters for the International Barcode of Life project
- Key Learnings/Conclusions
  - It's important to distinguish between metabarcoding and deep sequencing.
  - Short gene sequences can be used to tell species apart; there can be diagnostic nucleotide differences in their DNA sequences.
  - Positive controls are needed.

Multitude of Methods: Guidelines to Compare Methods

Robert Hanner, Ph.D., University of Guelph

What We Heard

- Key Findings/Learnings, cont'd
  - Whole genome amplification offers us an opportunity to make up a kind of standard reference material that can go into proficiency panels and things of that nature.
  - There are ways that we can make biological reference materials and sufficient quantity to support the molecular diagnostic community.
  - Methods are needed; reporting the identity of a particular plant is insufficient for these problems of potential spiking.
  - A number of studies that have shown that we can get DNA sequence motifs out of extracts.
  - Our active tests can provide actionable information. We can couple that with NGS to help look at some type of horizons scans and think about what other things we want to put in our panels, but we're still trying to figure out how to interpret our data.

Steps Needed for Actionable DNA Data: Method Specificity & Certificates of Analysis

Tyler Daniels, Thorne Research

What We Heard

- Business value of DNA Data
  - For botanical ingredients, identity is the taxonomic characteristics of the starting material.
  - What is not detected can be just as important as what is.
  - Quality programs for dietary supplements is a balance of analytical testing, understanding your ingredient, and removing uncertainty.
  - DNA is objective and its sequence is consistent.
  - DNA describes the biological basis for change.
- Challenges: Regulatory Perspective
  - Commercial service offerings must be able to show that their methods are scientifically valid and fit for purpose.

Steps Needed for Actionable DNA Data: Method Specificity & Certificates of Analysis

Tyler Daniels, Thorne Research

What We Heard

- Exclusion Panel Design
  - Design should list all necessary botanical variants that should provide a positive identification.
    - Species, varieties, geographic or seasonal variants, and other variants that are believed to possibly associate with identification performance
  - Materials that might accidentally or intentionally be used to replace or augment the target material(s) are of prime interest.
  - Exclusivity list should include botanical materials that are closely related taxonomically, morphologically, or phenotypically.

Steps Needed for Actionable DNA Data: Method Specificity & Certificates of Analysis

Tyler Daniels, Thorne Research

What We Heard

- Method Validation and Evaluation: USP's Role
  - Methods should be subject to validation, and each of the following should be explicitly stated and the appropriateness of the data in the specified context should be evaluated.
    - Fit for purpose of the selected technology
    - Inclusion/Exclusion panels
    - Locus/loci selected with primer sequences

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways
  - Developing an approach for these types of methods is challenging. It raises the following questions: How do we validate? What's enough data?
  - The different levels of complexity that are needed to achieve the right result to support a specification is different with each different herb or set of exclusion panels. When you think of it in terms of a general chapter, how would that work? It might be a completely different platform or assay that is needed to achieve the result within a given exclusion panel. Therefore, USP will really need consider in terms of going forward.
  - Harmony across methods within industry is important, but may not be easy.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - We don't want to create specifications for quality that lock out a significant portion of the industry. We have to make sure that the methods are fit for purpose. We have to make sure that we have orthogonal methods because there is no one answer to this. Orthogonal methods are needed to treat the different aspects of it.
  - It's important to learn how to deal with multiple ingredient products because more than half of all the dietary supplements in the US market are combination products. It's important the methods established are capable of dealing with complex mixtures.
  - USP may consider a good summary of this presentation in a general chapter that can then at future points be linked to individual monographs.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - As we move forward with modernizing the monographs, it's really important to make sure this information is well captured, but not set out any requirements that are really going to create challenges for industry while providing a solution to something.
  - There's a great need for curated, sequence data out there. Finding a way to provide that to the community in a way that will be both collaborative but also useful and accessible is the challenge.
  - Testing labs or industry can go to GenBank to access raw data that may be relevant to their purpose. Stakeholders should keep that in mind as a possible source of data for tools that could be used or made.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - As we move forward with modernizing the monographs, it's really important to make sure this information is well captured, but not set out any requirements that are really going to create challenges for industry by providing a solution to something.
  - Having a curated database would be an extremely important and necessary tool; all of the NGS methods would rely on that database. The responsibility for that database should be considered? Where should the ownership be? Should it be open access in the public domain?
    - Database should be harmonized as well as curated.
    - A database in place would provide a baseline from which to develop simple, easily implemented, and affordable techniques that can be used and spread throughout industry without a major shake-up across laboratories.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - Developing a set of standards is key. Without consistent standards, it would be impossible to make a comparison across all tests held in a repository.
  - Questions to consider are as follows:
    - What is validation?
    - How does one go about it?
    - What are the parameters and what do you mean or think about when you say "a validated test."
  - USP has very high standards and validation criteria for their methods. As a brand holder or an ingredient company, you can feel confident in a USP method that comes from a specific monograph for your materials because you know it has been very well validated.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - In addition to comparing the same materials, storage and sampling are absolutely fundamental to arriving at consistent and comparable results, as samples change over time and are impacted based on storage conditions.
  - Characterizing variation within species is not necessary because industry and is mostly using herbs of commerce.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - Q: How do you get down to the plant part?
    - A: There is currently no genomic test that does that. It may be possible to develop something that works through DNA methylation.
  - Q: Is there a way to use a DNA-based method approach to distinguish between GMO and non-GMO plants?
    - A: Europe uses DNA-based methods to look for GMOs. That is what they mainly use. Our real-time PCR essays to look at finished products using some of these methods to look for the genome certifications. So it is done.
  - A determination is needed on whether the whole genome would be good as a tool may be a good resource or maybe botanical material may be a good tool to take the product/sample through the system suitability for the methods and to avoid the false positives/false negatives.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - Using the whole plant genome would be a large undertaking.
  - Having digital resources are a good idea in the sense that curated sequences are cheap to maintain once they are curated.
  - With respect to a materials repository, it is important to have something that's kind of close to a dried version of what occurs in nature (e.g., whole leaves), it's also important to have something that represents what happens after processing (e.g., leaves that have been extracted and dried).
  - With respect to the whole genome approach, the idea behind this approach is that if you provide the whole genomic DNA, the user could select their own test method and use it appropriately. An alternative is that we can give something like a PCR amplicon for a specific botanical for the specific approach.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - With respect to the British Pharmacopoeia approach, the approach NIBSC has taken is slightly broader, in the idea that we're trying to really control the process rather than provide a reference for each and every monograph and every kind of individual test in all sorts of different industries and all sorts of different applications.
    - Process control has become much more important than providing the correct answer to the question. That is part of the reason NIBSC chose to take the approach that it did.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - The question we're seeking to answer is: Does this method consistently give you the same results that you want to get? It's important to make sure that we're not talking about industry-type standards, but proficiency standards.
  - One of the key questions to answer is, what's the public health issue? What's the problem that has to be solved? We don't have to create solutions when there's not a problem.
  - From a prioritization perspective, an issue for USP to look at is what is the most commonly adulterated botanical in the United States. Within the *Dietary Supplement Compendium*, USP could identify the top 10 most adulterated botanicals, or the most likely contaminants that are going to cause a public health hazard. DNA methods are needed for this. We need to have an analytical exclusion.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - One of the things that USP has done in working through this last 5-year cycle with the modernization of the Dietary Supplement monographs is develop a set of criteria for prioritizing which of the monographs should we look at modernizing first based on questions like adulteration.
  - Transparency is an issue. It is unclear who owns the data and at present, the data are funneling into one monetized source and a pharmacopeia is not supposed to do that. A pharmacopeia is supposed to be accessible to the public, out there for public scrutiny and reproducibility.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - Few to none of the commercial DNA manufacturers make their methods available for all the botanicals they're working on. That has to be a top priority for USP and for the policymakers: whatever we do, it has to be transparent and cannot just be monetized. That is a real limitation, especially from an FDA perspective because if USP adopts a proprietary standard and puts it in the monograph, that creates a lockout standard. And if FDA regulates against that standard that creates a lockout standard. That is something that needs to be monitored closely.
  - The goal is not to detect all adulterants or other possible contaminants or other things that might be in there. The focus should be on supporting and developing identity specifications.

Panel Discussion: Application of Standards

Michael Ambrose, Moderator, Ph.D., USP

What We Heard

- Key Takeaways, cont'd
  - In the probiotic space, one SNP (single nucleotide polymorphism) in the whole genome is considered a different bacteria. This is also in line with the position of USP's Probiotics Expert Panel.
    - Yes, the Expert Panel made a recommendation that if there is a 1 SNP difference, it should be named differently; but in that case, it is named at the strain not at the species level. So whereas when we are talking about American ginseng, for example, that American ginseng, which we have shown to be very high in ginsenoside Rg1 is American ginseng, not American ginseng with another type.
  - So we are debating as a as a species in the case of botanicals and in some cases, the chemotype is such that may be multiple spaces may be combined into identification of the genus level like (e.g., Salix bark species).

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