

Host: This is the podcast from *Clinical Chemistry*. I'm Bob Barrett.

The scientific community continues to debate the quality of results produced in microarray experiments as evidenced by quality initiatives.

An article published in the June issue of *Clinical Chemistry* highlights an underlying concern in microarray based testing that relates to the quality of multiplexed assays.

The lead author in that study is Dr. George Klee, Professor of Laboratory Medicine and Pathology at Mayo Clinic College of Medicine and Chair of the Division of Experimental Pathology and Laboratory Medicine at Mayo Clinic in Rochester, Minnesota.

The same issue of *Clinical Chemistry* has an editorial on the topic by Dr. Stephen Master, Assistant Professor of Pathology and Laboratory Medicine at the University of Pennsylvania and Director of the Endocrinology Laboratory at the Hospital of the University of Pennsylvania. They're both our guests in this podcast.

Dr. Master, tell us why are multiplex assays important?

Dr. Stephen Master: Well, multiplex assays are important for at least three reasons. The first is efficiency, if the lab can measure, let's say 100 analytes at the same amount of effort as it takes to measure one analyte that's a good thing, particularly in this era of increasing cost constraints.

The second advantage, I think, is that in some formats such as microarrays, the multiplex assays allow us to measure many analytes with only a small amount of source material, whether that source material is tissue or body fluid or something else, so that's an additional efficiency.

The third and I think perhaps the most interesting reason is that multiplex assays have often been shown in the research literature to do a good job at distinguishing disease states.

So as a rule the more analytes that we can measure reliably in the clinical lab, the more detailed information we can give to clinicians about the underlying biological state of the individual patient.

Host: So what does the current study by Dr. Klee and his colleagues tell us about protein microarrays? Why exactly is that significant?

Dr. Stephen Master: Well, this is a very important type of study because it really highlights the kind of real world performance that we can expect from current protein-microarray technologies.

This group from the Mayo Clinic was able to make duplicate measurements of 15 proteins from a large sample of patients or to really try and get a handle on the reliability of measurements that were made in this protein microarray format, and based on their data they were able to identify pre-analytical challenges to reliability, they showed analytical measurement differences based on the individual antibodies that were used, and they also showed more global variation from plate-to-plate.

So all in all it was a very nice demonstration of the type of work that needs to be done to characterize these types of complex assays.

Host: Well those considerations sound impressive, but Dr. Klee are there any disadvantages to multiplex testing?

Dr. George Klee: The major disadvantages as I see it is that you can't really optimize each assay. When you have one sample for measuring one analyte, then you can really choose the formats you want to use, you can choose incubation time, you can choose whether or not you want to have multiple washes within that measurement system.

You can also choose different detection systems if that's more appropriate for the level of the analyte that you're looking at, whereas when you put many different tests together you have to reach some type of compromise and you can't really allow that to be optimal for any given test within the system.

A second disadvantage which we found to be quite important is the vast range of concentrations that different analytes have within the blood, and many times when we do testing, we are able to correct for that differences in range by using different amounts of sample or different dilutions.

So if you have some things that are 1000 pulls different in concentration, you'd probably use a larger dilution for the higher concentration than you would for the low concentration analyte. Whereas when you're measuring all of these within that same sample, your assay has to have a very large dynamic range to be able to cover the different ones or you have to reach some type of compromise.

Some of the things will be measured on the highest slope on the dose-response curve, whereas others will probably be measured down in the bottom or on the top part of the curves where the slope is often less, then the precision then

is less on that. So dilution of the sample is, I think, a major issue related to multiplexing that's going through there.

The third one is how do you quality control, when you've got multiple assays running on that same sample and that you then go through and analyze that and the quality control is run for multiple analytes and if one of those quality controls fail, you pretty much have to reject the whole assay.

So that becomes a problem, how do you retest or what do you do to make sure that we have the right quality control parameters keyed into these types of systems.

Host: How exactly does the number of false-positive quality control signals relate to the number of test multiplexed?

Dr. George Klee: Well, if we look at the traditional quality control where we're running like a two standard deviation limit, then you have like 5% false-positive rate. That's just the way that the system is set that if the things are in control there is a chance, of 1 in 20 that it would be out of control.

Same philosophy would apply if you look at a higher limit like 3 SD but you'd still have 6% that would be outside of it, and that's for one analyte.

If you're measuring multiple analytes these things compound together. For example, if you're measuring two at that 5% level, there 5% the first ones is out and 5% the second ones is out, so if you multiply 0.95 times itself you end up with 0.90 that is about 10% false positives would occur within that.

If you start to extrapolate that out and go 0.95 times 0.95 times 0.95 for three of them you've got about 86% or 14% would be out.

You take that up to 10 and multiply 0.95 to the power of 10 you'd end up having about 60% of those that would actually be reported or 40% false-positive rate.

So these concatenate together very dynamically as you're increasing the number of analytes that you're measuring in that single sample.

Host: Also tell us how do you retest if one or more tests were unacceptable on a multiplexed played assay?

Dr. George Klee: Now, that's an excellent question, and that's something that we're struggling with. You run the controls and you have this high probability that one is going to be out.

If we were doing it as a uniplex test you would just retest that particular analyte, but when you're testing multiple analytes you pretty much are obligated to go back and retest that whole plate because that's the only system that you've validated.

So if you had 40% false positive on the first one you're going to retest it, you get another 40% false positive. So when you put the two of them together you end up having a large percentage of them that will not ever get an answer, you end up in this infinite loop that you've always got some that you're going to be retesting.

So there is a not a good way to control for this and the number of false positives really starts to drive this thing such that you either have to have an alternate testing strategy for the ones that fail, but that means you have to have another analytical system as a uniplex to back-up the multiplex and many times the performance characteristics of the uniplex would be different than the multiplex.

So it's not valid to replace the answers from the first set of measurements with an answer from the second.

Host: So quality control for multiplex assays seems to be a particular challenge. Dr. Master, in your opinion, how can laboratories overcome these quality control problems?

Dr. Stephen Master: Well, I think the answer to this really depends on your diagnostic question. So if you think of a multiplex assay is just an efficient way to measure many analytes in parallel then the solution would be the limit that the total number of analytes that are measured.

So if one constructed, for example, an array measuring 15 proteins—this was done in the study by the Mayo clinic, it's quite feasible to adopt the same kinds of westguard rules that we use now in any individual analytes.

Now on the other hand, if the multiplex assay is really being used as a pattern to distinguish between people with higher low risk of disease for example, then perhaps the individual analytes won't matter as much because basically it is the whole assay is what you care about.

So in this case it becomes critical for the lab to understand the way in which the analytes are put together to come to a diagnosis and the quality assurance procedures would be at the level of samples in a known diagnostic category, rather than merely samples that are spiked with the known amount of some analyte.

I think the other thing to point out in this context is that the study by Dr. Klee and his group was able to distinguish entire plates that behaved well or poorly. So it's possible that we'll be able to use these kinds of approaches to perform quality control rather than necessarily focusing on individual analytes all the time.

Host: Have these assays already been widely adopted by clinical laboratories?

Dr. Stephen Master: Well, as of today, there's only a few examples of devices that are performing quantitative measurements of many analytes. There are a few notable examples, there is a multiplexed DNA microarray assay for predicting risk of breast cancer metastasis, but these assays have not yet spread widely into the clinical lab.

I think the real key to this will be for clinical labs to develop the kinds of QC tools that will allow adoption to happen. I think that this issue along with regulatory challenges that are before the FDA will really determine how quickly multiplex assays will reach the clinical laboratory.

Host: So do you think that clinical laboratories will be transitioning to protein microarrays anytime soon?

Dr. Stephen Master: Well, I think it's inevitable that a variety of highly multiplexed assays will, in fact, enter the clinical lab at some point. Whether the first wave will be protein microarrays or quantitative DNA microarrays or multiplexed massspectrometry or even something else, I think that remains to be seen.

Part of this will be driven by what particular diseases we're focusing on. If it turns out that a particular disease can be diagnosed by a small number of markers that may well drive the technology in one direction; on the other hand if it turns out that you need 100 or 1000 markers then that may drive us in a different direction.

Host: Keeping in mind the strengths and weaknesses of multiplexed platforms is there a maximum number of tests that can practically be multiplexed together, Dr. Klee?

Dr. George Klee: That is a very difficult question, if you look at the genomic testing, they're doing thousands of them, you're looking at some of these array type things where they're doing hundreds, these issues that I just alluded to in terms of false positives or optimization of assays, I think become overwhelming.

My recommendation would be to keep it down to an accountable number; if you count with your fingers say ten,

if you only want to count on one hand, five. Somewhere in that range I think is doable with these multiplexed assays, but once you get much higher than that, I think these other issues of optimization, of quality control, and another thing we didn't even talk about is cross reactivity between the different substances that are being tested in that same sample.

So I think there's some major issues once you get beyond ten analytes that you're trying to measure in one particular well.

Host: Well, lastly, Dr. Mater, based on your experience what advice would you give to those who are developing multiplexed devices?

Dr. Stephen Master: Well, I think there are several key lessons at this point, and the first is to know your question, because this is going to fundamentally affect the number of analytes that are chosen. So knowing the disease and the kind of answer you expect out of your device, I think, is going to be critical. On the one hand you might be focusing on individual analytes as a pure multiplex panel, and on the other hand you might be looking at a pattern as a whole.

I think the second lesson is really highlighted by the study by Dr. Klee's group, in which there were substantial variability from antibody to antibody, and I think the lesson here is that when you put things in a multiplexed context you really have to pay attention to the reagent quality that you're using, just putting things in a multiplexed format doesn't solve anything by itself.

Finally, I think the last lesson is to really engage the laboratories early. These are going to be complex paths run in a complex environment and if you are going to require real partnership between the manufactures, the laboratory technicians, physicians, to make sure that everyone is working together to make these devices work appropriately for good patient care.

Host: George Klee is a Professor of Laboratory Medicine and Chair of the Division of Experimental Pathology and Laboratory Medicine at the Mayo Clinic in Rochester, Minnesota.

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I'm Bob Barrett. Thank you for listening.

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