

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

Severe Combined Immunodeficiency, or SCID, describes a spectrum of primary immunodeficiency is of about 14 independent genetic conditions that produce severe defects in Cellular and Humoral Immunity. If undetected and untreated, SCID typically leads to death before a baby's first birthday.

SCID is often called "bubble boy disease," after the movie based on the true story of a boy with SCID who died at age 12 after spending his life in a plastic bubble because he was so vulnerable to infection.

An article published in the September issue of *Clinical Chemistry* reported that real-time quantitative PCR targeting a specific marker of functional T-cells, the T-cell-receptor excision circle, detects the absence of functional T-cells and has a demonstrated clinical validity for detecting infants with SCID.

Dr. Anne Comeau is an author of the report and Deputy Director of the New England Newborn Screening Program of the University of Massachusetts Medical School.

She joins Jacalyn Gerstel-Thompson, the Laboratory Supervisor of the Molecular Diagnostics and Research Laboratory at the New England Newborn Screening Program in Boston, Massachusetts, as our guests in this podcast.

So Dr. Comeau, what are some considerations about molecular testing that might be unique to newborn screening.

Dr. Anne Comeau: Well, it's very interesting. Newborn screening is a population-based screen and from a non-technical point of view, there are aspects of molecular testing that need to be considered for one thing, newborn screening is pretty much unrequested information. Even though there is a requisition for newborn screening, it's more of less behind the scene. And this stewardship that states have for newborn screening, basically means that all babies are tested for something under state authority, but the physician who is going to get the results of the testing hasn't necessarily ordered the test, unlike a molecular diagnostic test where the physician is looking for a particular allele or interested to know whether or not a baby has CF, that is not how newborn screening is run. It's population-based screening.

So to that and we need to be very careful with the kinds of information that are generated and molecular testing in the newborn screening arena needs to be very sensitive, but

also very specific and very much classified by the information that will be available. So we might not want to identify carriers, for instance, or we might want to limit the number of carriers that are identified, that comes under state stewardship and it really will have an impact on the particular test that are run and that's why newborn screening for Severe Combined Immunodeficiency, SCID, is so interesting, because it's a DNA-based screen that is looking for a DNA structure, as opposed to looking for allele.

So it's really the first population-based screen where DNA make sense, is easy, offers information for the physician to use, but doesn't carry all the problems of carrier identification.

From a technical point of view, newborn screening also is very interesting. Some of the considerations that we run in to is the fact of the kind of specimen that we utilize. Newborn screening which has been in place in the United State since the early 1960s, it first started in Massachusetts in 1962, is able to be done because it's run on a dried blood-spot. The dried blood-spots allow easy transport. They also allow small amounts of blood from neonates to be obtained and tested.

So for a molecular assay, that has to be extremely sensitive and specific. We are working with only three micro-liters of whole blood or less, and it's one thing to run an assay on three micro-liters of whole blood if that's the one assay that you are running for the day if you are running 300 to 1000 specimens that day on three micro-liters of whole blood, you better have a very robust assay.

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Also, because it's a population-based screen, we need to take into consideration when we are designing the test of the psychological differences in the population, which brings into question, challenges to interpretation of the result. Was the baby in NICU, was the baby receiving different medication, and do you have reference ranges for extremely low birth-rate infants?

So these are all typical quality assurance questions that newborn screeners are used to asking. It's not typically something that a DNA lab that's not involved in population-based screening would be prone to ask. Other technical issues that we need to take into consideration, again, because it's a population-based screen, involve some of the environmental differences in the population. Environmental being the method of collection of the blood from babies might be in neonatal intensive care units, and so bloods might be obtained off of a line, those bloods might be

heparinized or not, even though there are directions for how we ask the nurseries to obtain the bloods.

There are circumstances that varies, whether it's heparinized blood or whether the baby had multiple transfusions; all of these considerations need to be taken into play and design of an assay and in the quality assurance that we put into place to monitor the assay.

Host: Now you mentioned choosing to implement a multiplex controlled DNA assay into the newborn screening program as a model, rather kinds of newborn screening. Doesn't this add a degree of complexity and sophistication to this type of testing that's normally performed in a public health laboratory?

Dr. Anne Comeau: That's very interesting that you would put that question that way, and I think it's probably a perspective that many people have, but actually public health laboratories run some of the-most sophisticated tests in the country, and they do it well.

So part of the decision to implement a multiplex DNA assay for screening for Severe Combined Immunodeficiency, was to make sure that we had everything that we needed to have the quality controls in place, to make sure that the DNA was there, that it was amplifiable and that we would have some sense of a normalizer for the actual target, for the TRAC.

The multiplex reaction in this particular case where we were looking for babies who have an absence, sort of very low copy number of TRACs, the fact that we have an internal control tells us that the DNA is there, and that it's amplifiable.

The other aspect of choosing to run a multiplex test is that if as you may recall from the previous response, when we are running 300 to 1000 samples a day, one wants to have a high throughput streamlined protocol with very few exceptions. So the fact that we can run both assays, both the TRAC and the reference chain assay in one tube is wonderful. We don't need to invest in the labor of going back to retrieve a specimen for testing with an external control. We don't need to worry about retrieving the wrong specimens in order to have a controlled assay; everything is done in one tube.

Those are some of the major reasons for wanting to run such an assay. The other aspect of this is, just good lab quality assurance one wants internal controls, and we thought that the screen for Severe Combined Immunodeficiency was challenging enough, and that if we

were able to do it for this assay, it would be simple to do for other assays.

So it's a great model to show that it's successful and can be applied.

Host: With that in mind, what other conditions might be added to a newborn screening program panel that would take advantage to the protocols and place for SCID screening?

Dr. Anne Comeau: Basically any DNA-based assay that was amenable to newborn screening or applicable to newborn screening could take advantage of the protocols and place for SCID screening.

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When you ask about other conditions that might be added to a newborn screening panel, any condition added to a newborn screening panel would have to meet the generalizable criteria that the condition was treatable, that the assay for the condition was sensitive and specific enough for population-based screening with good predictive values, and that it met reasonable cost for the assay itself.

With that being said, the kinds of other conditions that are most likely to fall into a SCID-type of an assay would probably be conditions of an infectious disease type, where one might be looking to determine whether or not they were circulating quantities of DNA or RNA from an infectious agent, and to date, there are couple that are under consideration for newborn screening, but none that have risen to the top yet.

Any other genetic conditions or any condition that might look for a quantitative DNA or RNA sequence, such as SCID, where you have a piece of the chromosome that's been excised and one can then measure the likelihood of an infant having the condition by whether or not one sees that excision circle.

So quantitative assays would be the most diminable, but any assay. So we've gone forward with the most difficult kind of assay or quantitative assay, but a lot of what we have learned from doing SCID would be applicable to genetic assays, just the high-throughput DNA extraction, etcetera, etcetera.

Host: Regarding some technical issues with the calibrators, could you expand upon the quality assurance of the assay in a newborn screening setting. How do you do this, Ms. Thompson?

Gerstel-Thompson: The use of a standard curve in our research setting has different level of monitoring them, when you are looking at a standard curve for a clinical reporting; there is a need for ensuring the quality of that data for reporting purposes.

So we feel that we have addressed that with our standard curve, quality control monitoring. For example, we have in the standard curves; there are two targets for the TRAC RNase P. We have four common data points and in an assay that is running at 100% efficiency, if you have two samples that have the same copy number, you should basically have about the same CQ values for those two samples. So we compare the four common data points, and our expectation is that they should be within one CQ value of each other.

Some other areas where we look is when we make new standard curve lots, we compare those CQ values of the new standard curve lots with the average of numerous daily run CQ values. Each time we do a SCID run, we compare those CQ values with the average, the min and the max and our expectation is the new lot CQ values must be within that range that we see, and preferably run around the average of those other values. And if they run outside the values, which we rarely see, we fill that new lot of standard curves and we'll make another one.

We also monitor on a daily basis these. For every routine run we record and monitor the CQ values for each data point of both targets, and also we monitor and record the standard curve values for each target, and we use those values to make sure they fall within the expected ranges.

Host: Now, what about the decision to include a plasmid for the TRAC sequence calibrator in total human genomic DNA for the reference sequence calibrator, rather than inserting the RNase P sequence in a plasmid?

Gerstel-Thompson: We had considered putting the RNase P in the plasmid, which may be more cost effective in the long run, but to add the TRAC plasmid to total human genomic DNA was more closely similar to our actual samples, and we wanted to have a standard curve that was as close to the actual samples we were testing as possible.

Host: Do you linearize the TRAC plasmid or is it circular?

Gerstel-Thompson: We do not linearize the plasmid. We had considered that in the beginning in following of recent paper, but we felt that the TRAC itself which is a circular DNA, and the plasmid should be handled as similar as possible, in that respects we decided to keep the TRAC plasmid circular.

Host: Now, for the DNA extraction, Dr. Comeau, why extract it at all? Why not just run the assay with the dried bloodspot in place?

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Dr. Anne Comeau: Well, that's an excellent question, recall for newborn screening, these are tiny babies, and we might have for all of the newborn screening assays somewhere between 200 to 500 micro-liters of whole blood. If in the future of newborn screening, we are running a DNA assay per punch, we actually use a little paper punch to access the sample.

If every time we run a DNA assay, we have to do paper punch. We would quickly run out of specimen, especially if one is looking into the future and there might be a 100 DNA assays, 100 paper punches is running out of specimen and it's not a good use of sophisticated labor.

So instead, we wanted to do one DNA extraction from which we could utilize the extract for multiple different assays, and that's pretty much what we have described.

In addition to conservation of specimen, there are other considerations, in that when one is extracting DNA from a dried bloodspot, one has the opportunity to dilute inhibitors. This is very important, as I indicated before; there are environmental conditions that we cannot control in the collection of blood from the newborn that happens in the nurseries. So we certainly want to be able to dilute inhibitors.

Furthermore, there are assays and there are DNA-based assays for genetic markers or for the Severe Combined Immune Deficiency condition in which they are advertised as *in situ* assays where the dried bloodspot is "just dropped into a reaction mixture" and that does sound appealing if one is only looking for one condition. It sounds pretty simple, but the reality is that for an assay in which you are looking for a very low copy number sequence, you really are going to need to do some cleaning of the dried bloodspot anyway.

So all of these assays require some level of cleaning, and thus if you are going to go through any cleaning one might as well go forward and do more cleaning and get a DNA extract in which the DNA is present in a volume that can be used for multiple other assays.

Host: So do you normalize the DNA concentration prior to amplification? And with that in mind, Ms. Thompson, what about prevention of sample-to-sample contamination during

the step? Is this a point where using dried bloodspot alone would be a more straightforward approach?

Gerstel-Thompson: No, we do not normalize the DNA concentration, because the filter-paper cards, first off they have different levels of saturation of blood across that blood circle, and also we are looking for the presence or absence pretty much of the TRACs in the sample, and so we felt it was not really necessary. We also have in these multiplexing with the RNAes P and that does help us to normalize as far as if we were to consider the TRACed RNAes P ratios.

And as far as regarding sample-to-sample contamination, when we do our DNA extraction, we do them in the 96-well plates and prior to release of the DNA into the LUA. We hermetically seal to the tops of these plates a pierceable aluminum cover that we clip down an aluminum block to into prevent any pressure from bursting the top of the plate. And after we have basically boiled the samples, shaking them, we take a look at the plates and we know that each well has not been compromised or there has not been any of the wells that are no longer sealed, because you can see with each individual well there is a convex appearance, and if we do see that there is any kind of bubble on top of the plate, we will discard those samples and we start the DNA extraction on them.

And at that point the only time that these plates or these wells are now opened up is when we pierce them to transfer the DNA from the 96-well plate to 384-well plate.

And regards to the last question there is really nothing to be gained for contamination control just by using the dried bloodspot alone.

Host: For the reaction itself, you have got a good standard curve, good DNA, how do you know that the reaction is good for every sample, otherwise you could have many false-positives, right?

Dr. Anne Comeau: Not really, when we look at the results that we get from the real-time instrument, all our controls and each well is closely looked at to consider our controls our new blood samples.

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Which are our sample preparation blanks, and well the sample preparation blanks have no blood controls, we look at our negative, and we have reference samples on there that we also consider. And then for each sample itself, we look at the amplification curves for both targets, both the TRAC and the RNAes P for any strange or unusual type of amplification that you might see, and if we see that as one

of the amplification curves is not typical, then we will further investigate that by looking at the multi component plot and in some cases, when obviously there has been a problem in that particular well, we will discard that result and retest from the beginning.

Host: Let's go back to the issue of the dried blood spot as specimen and talk a little about the Multiplex and Multiplex Plus, Dr. Comeau.

Dr. Anne Comeau: Okay. So for people who don't do newborn screening, it's probably a little surprising to understand the vast variety of assays that can be done on the newborn screening dried blood spot. They range from amino assays to biochemical assays, including tandem mass spectrometry and DNA-based assays, hemoglobin types of assays, but when it comes to accessing the dried blood spot, or a DNA-based assay, the idea of adding multiple analytes or being able to evaluate analytes beyond the DNA from exactly the same paper punch is an interesting and very promising opportunity.

So for instance, for a Severe Combined Immune Deficiency, the DNA-based assay might be the TRAC assay, which we have described in our paper and an immunoassay might be an assay targeting CD3 CD45, such as is described in Dr. Ken Pass' paper in the same issue of *Clinical Chemistry*.

The possibility of being able to evaluate the quantity of DNA product and another analyte, such as an antibody, is really exciting. So the possibility of being able to do that is what I have tried to describe in the Multiplex Plus, and what that comes down to is that during the course of a DNA extraction, one first of all has to elute other whole blood components from the filter paper, and that elution contains all of the other analytes that one might want to evaluate.

So Multiplex Plus basically means that one does the paper punch and then in the course of the DNA extraction one retains the other blood components for evaluation by another assay. It really has the potential to be quite powerful to be able to analyze different kinds of analytes from the exact same small three micro-liters of blood. And possibly, put a multi-analyte profile into place for interpretation of a particular assay.

Host: Well, lastly, Dr. Comeau, how has this assay affected newborn screening nationwide?

Dr. Anne Comeau: So I expect that you mean by this assay, you mean the TRAC assay, the assay for severe combined

immunodeficiency and the multiplex assay that we have described, I think this is one of the most exciting developments in newborn screening in many, many years.

It's mobilized the nation's newborn screening programs to become part of a new development of DNA-based technology that is useful. So it's not just a few of the research newborn screening programs developing DNA-based technology, because they could. Instead, it's a very applicable assay that can be used by newborn screening programs for a condition that people believe should be included in newborn screening. It means that the newborn screening programs workforce now has to include people with varying degrees of expertise in molecular technology.

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And this is wonderful to just see a whole new cadre of molecular technologists included in the newborn screening arena, because these people have their own perspectives and expertise to bring in to this public health service.

The SCID assay, the TRAC assay, is something that with the development of our in-house assay, we've run some molecular training workshops. We have posted many states in our laboratory for training in our home-brew assay. The CDC has also run a couple of workshops, again, hosting a variety of states for training in the couple of different TRAC assays that there are out there, and it's just so heartening to see and participate with people who have similar interest and similar expertise in molecular biology coming together to apply it to newborn screening.

Some of the people in the public health service might have come from a Bioterrorism unit with molecular expertise, some people might be coming straight out of graduate school, but they now have a place to use it in newborn screening, and it's really quite wonderful to see this going forward.

Host:

Dr. Anne Comeau is Deputy Director of the New England Newborn Screening Program of the University of Massachusetts Medical School. Jacalyn Gerstel-Thompson is laboratory supervisor of the Molecular Diagnostics and Research Laboratory at the New England Newborn Screening Program in Boston, Massachusetts.

They have been our guest today in this podcast from *Clinical Chemistry*. I am Bob Barrett. Thanks for listening.

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