Molecular clonality testing for lymphoma

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May 2013—Most jobs in the kitchen or the home workshop can be done with a basic set of tools. But every once in a while you need something special—a zester, say, or a dremel—and in those situations it's nice to have that special tool on hand. Even more important, it's nice to know how to use it.

So, too, in hematopathology, where the special tool is clonality testing by PCR. Most cases of lymphoma can be diagnosed with a basic tool kit—morphology, with H&E staining in essentially all cases, plus some type of immunophenotyping, typically immunohistochemistry or flow cytometry. But in a small proportion of cases, molecular clonality testing is necessary.

"Molecular clonality testing is not needed for most cases [of lymphoma diagnosis]," Patricia J.T.A. Groenen, PhD, clinical molecular biologist in pathology at the Radboud University Nijmegen Medical Centre, the Netherlands, told CAP TODAY. Dr. Groenen is a member of the EuroClonality Group, which developed, validated, and generated guidelines for the most widely used PCR clonality kit, the EuroClonality/BIOMED-2 multiplex PCR kit, marketed in the U.S. by InVivoScribe. "Pathologists can diagnose leukemia or lymphoma and identify malignancy in most specimens," Dr. Groenen notes. She estimates that only 10 percent to 15 percent of suspected lymphoma cases in her institution are submitted for clonality testing by PCR. Last year the EuroClonality Group published guidelines on the correct interpretation of results obtained with the EuroClonality/BIOMED-2 PCRs (Langerak AW, et al. *Leukemia*. 2012;26:2159–2171). Dr. Groenen and her coauthors wrote, "As clonality testing is not a quantitative assay, but rather concerns recognition of molecular patterns, guidelines for reliable interpretation and reporting are mandatory."

At the Association for Molecular Pathology 2012 annual meeting, Dr. Groenen spoke on the development of the EuroClonality/BIOMED-2 multiplex PCR kit and the recently published guidelines for its use. She was joined for a discussion of challenging cases by Rita Braziel, MD, professor and director of hematopathology in the Department of Pathology at Oregon Health and Science University, and James R. Cook, MD, molecular hematopathology section head in the Robert J. Tomsich Pathology and Laboratory Medicine Institute, Cleveland Clinic.

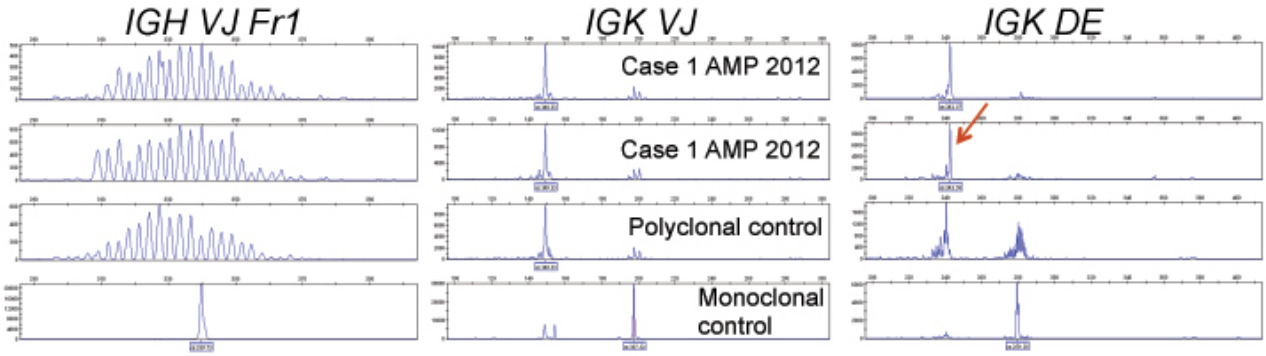
"For the vast majority of B-cell lymphoma cases that we diagnose, we are not going to do molecular clonality testing," Dr. Braziel said in an interview. "We have flow cytometry, kappa and lambda analysis by in situ hybridization, and other immunophenotypic profiles. We will almost always do molecular clonality analysis in new peripheral T-cell lymphomas."

Molecular clonality testing is utilized more for T-cell lymphoma, Dr. Cook agrees. He and colleagues do it more often for T-cell lymphomas because there is no flow method to assess clonality. "For B-cell lymphoma we usually look at clonality by flow cytometry," Dr. Cook said in an interview. "We do PCR [for B-cell lymphoma] only when flow is not available or gives equivocal results." Dr. Cook estimates that T-cell lymphomas make up only about 10 percent of non-Hodgkin lymphomas, and that he uses PCR clonality analysis in perhaps five percent to 10 percent of lymphoma diagnoses. "And we see unusual cases [in our referral practice]," he says. "In a community practice setting you may need it even less often. Most community practices are going to send these cases out rather than do it themselves."

It's no surprise that molecular analysis of clonality by PCR can be difficult, including interpretation of patterns obtained from the EuroClonality/BIOMED-2 multiplex primer sets, since clonality testing usually is performed on difficult cases. "It is one thing to have this test on the market," Dr. Groenen says. "However, the other critical thing is that we want to make sure the PCR kit is used properly." So the EuroClonality Group organizes workshops to teach users to interpret the reagents correctly. The first workshop was held in 2006, only three years after information about the BIOMED-2 primer set was published (van Dongen JJ, et al. *Leukemia*. 2003;17:2257–2317). "We were doing molecular clonality testing for diagnostics," Dr. Groenen says. "Then within a short period a couple

of groups asked whether they could come to our lab for one or two weeks. But that takes so much time. So we started thinking—apparently there is a need for this. That's how the Dutch workshops started." Now workshops are held in Nijmegen annually. "Each year these workshops are full," Dr. Groenen says. "People come from Europe, but also Australia, Asia, and the U.S. So obviously there is still a need."

In 2008 the AMP invited the EuroClonality team to do a daylong workshop on BIOMED-2 testing, sponsored jointly with InVivoScribe. The workshop was repeated in 2010. Last year Dr. Groenen again crossed the Atlantic where she joined Dr. Cook and Dr. Braziel in the workshop on how to apply the new guidelines. Dr. Groenen presented a synopsis of the guidelines, after which the three participants discussed five challenging cases. "In a workshop people can get the impression that interpretation can be so difficult," Dr. Groenen said in an interview. "Because workshops should have educational value, we present cases that people can learn from." They therefore present cases that are difficult from the pathology or molecular biology perspective. "People may forget that the test and the uniform scoring system [guidelines] works well for more than 95 percent of the cases. You can have 10 straightforward cases, but these will not be the cases that are discussed in the workshops."

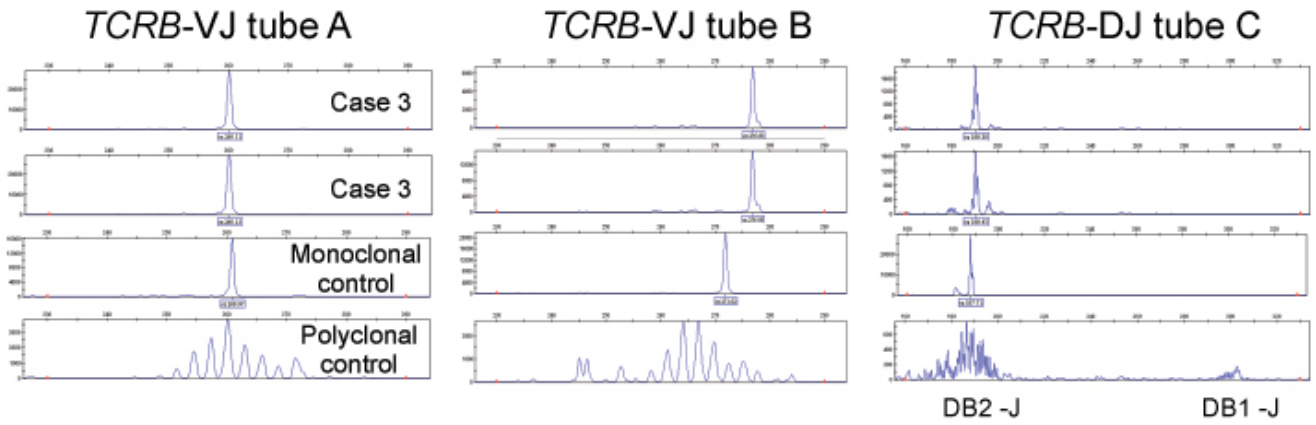


Difficult diagnostic case: follicular lymphoma? Polyclonal IGH-rearrangements and IGK-VJ rearrangements were detected; however, a clonal IGK-DE rearrangement was detected (arrow). The overall molecular interpretation of this case is: Monoclonality detected.

But cases that come to molecular clonality testing may be a bit more problematic than the ordinary run of specimens to start with. "From the pathologist's viewpoint, these cases may already be more difficult," she says. Having a specialized and accurate method can be of help.

"The problem I've been seeing and that is of concern to me," Dr. Braziel says, "is that now with kits available from InVivoScribe with multiplex PCR for antigen receptor clonality, it's very easy technically to do the test. So now lots of people are doing the test who have limited experience in molecular testing and even more limited experience in interpreting the results." She has been doing PCR analysis of immunoglobulin (Ig) and T-cell receptor (TCR) genes, so-called antigen receptor genes, for about 15 years. "Even so, it was a really steep learning curve for us when we started using the IVS BIOMED-2 GeneScan kit. I will still send samples [to Dr. Groenen] for a second opinion." Use of multiplex PCR reaction tubes in the EuroClonality/BIOMED-2 multiplex PCR kit creates complex patterns that require an understanding of the primers to resolve. In Dr. Braziel's section there are six hematopathologists, all of whom sign out clonality tests. "Most of the time we will look at cases together and discuss them," she says.

Where this level of expertise is not available, mistakes can be made. In their consultative practice, Dr. Braziel says, "We have seen cases where misinterpretation of clonality results has led to misinterpretation of the hemepath diagnosis." Helping participants to achieve a more sophisticated understanding of the EuroClonality/BIOMED-2 primer sets and how to interpret them was the major objective of the AMP workshop. "Most people who attended the workshop wanted to increase their level of understanding of the kits and to increase their skills at doing the test and interpreting it," Dr. Braziel says. "My goal in this workshop was not to say, 'You should send your cases to me,' but to help them understand the test better."



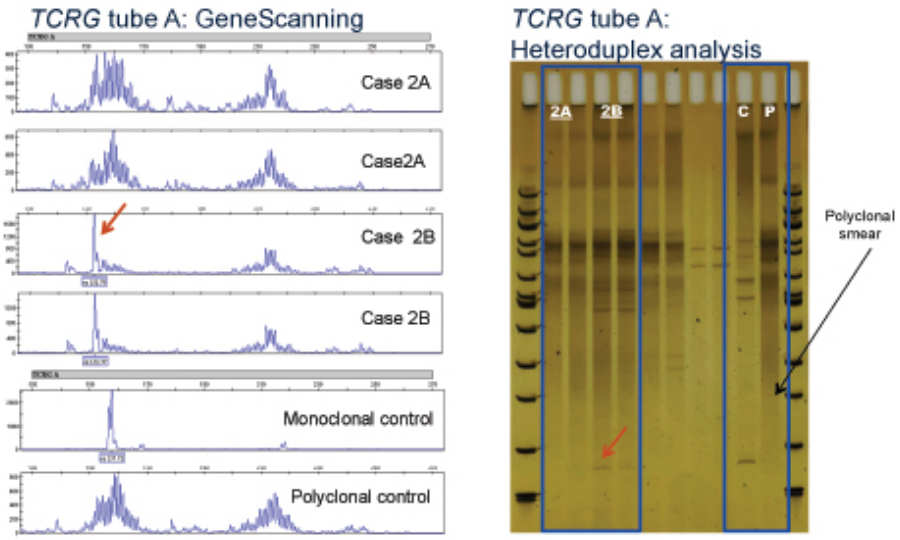
Case 3 showing three clonal TCRB gene rearrangements and a clonal TCRG rearrangement (not shown here) as well. The technical interpretation for all shown PCR tubes is: clonal. The overall molecular interpretation is consistent with the presence of one T-cell clone (monoclonality), with on one allele two clonal TCRB gene rearrangements (VDJ and DB2-J) and on the other allele one clonal TCRB VDJ-rearrangement.

“The BIOMED-2 primers and protocols are used widely in the U.S.,” Dr. Cook says. Numbers in the CAP Molecular Hematologic Oncology Survey bear this out. “Many labs have been using their own in-house tests,” he says. “I think they are being phased out and replaced by InVivoScribe kits. We use the EuroClonality primers through IVS for both B- and T-cell PCR.”

Of course, as Dr. Braziel noted, molecular clonality testing was performed before the EuroClonality/BIOMED-2 primer sets were introduced in 2003. It was done by Southern blotting, which was the gold standard for many years, and even by PCR in some institutions. However, Southern blotting was cumbersome and neither method was sufficiently sensitive.

“When we started doing clonality testing by looking at genes for immunoglobulin heavy chain [IgH],” Dr. Braziel says, “some labs used only one primer set, for framework three. Others used primers for framework one or framework two. People found they had the best results if they used primers for all three framework regions.” By relating this piece of history, Dr. Braziel was making an important point about the composition of the EuroClonality/BIOMED-2 multiplex primer kit—the critical feature that makes it work and challenging to interpret in some situations: Single-primer PCR reactions were abandoned in favor of multiplexed primer combinations for Ig and TCR gene rearrangements. “Using multiplex PCRs has markedly improved this type of testing,” Dr. Braziel says. “It means that most people are using the same primers and it has standardized testing, so that most people will get the same results if they do the test right and interpret it correctly, which most of them do.”

One can get some idea of the need for complex testing in identifying Ig and TCR gene rearrangements by looking at the genetic events that give rise to these large and complex molecules. (A good explanation is provided in the preface to the group’s 2003 *Leukemia* paper.) Basically, during human development, recombination occurs among the genetic regions for three components of the Ig molecule—the V, D, and J regions. (TCRs share the basic structure of Ig molecules and are formed in the same way.) Joining of these three regions in various combinations creates a repertoire of Ig molecules. Because each region consists of a family of genetic variants, the number of possible combinations in the resulting immunoglobulin population in an individual is very large—estimated at 10^{12}. Thus the need for multiplex PCR reactions in the test.



Interpretation of TCRG VJ-rearrangements (tube A), showing polyclonal TCRG rearrangements (case 2A) and a clonal TCRG rearrangement (arrow) in a polyclonal background (case 2B). The rearrangement patterns were detected by both GeneScanning and heteroduplex analysis.

In the 2003 *Leukemia* publication, the kit contained 95 different primers for the Ig/TCR targets in 14 multiplex PCR tubes. How was this amazing feat accomplished? "We made use of family-specific primers," Dr. Groenen explains. Each region of the gene for Igs is composed of many variants, but variants are grouped in families that share sequence homology. In addition, tubes are selected based on complementarity among regions. For instance, Dr. Groenen calls the combination of TRG tube A and TRB tube A "my personal favorite" when there is limited amount of DNA from a diagnostic specimen. "We tested the primers and verified that all family members [for each genetic region] can be identified with the primer set. So the kit works quite well."

In performance studies the primers detected clonality in nearly 100 percent of all cases examined in a survey among members of the EuroClonality Group, including 369 B-cell malignancies belonging to five World Health Organization-defined entities (Evans PA, et al. *Leukemia*. 2007;21:207–214) and 188 T-cell malignancies belonging to five WHO-defined entities (Brüggemann M, et al. *Leukemia*. 2007;21:215–221). The exception was a 79 percent detection rate for anaplastic large cell lymphoma (ALCL), which includes the 20 percent to 25 percent of ALCL that do not have TCR gene rearrangements, the so-called null-ALCL. Brüggemann and colleagues concluded, "Our study indicates that the BIOMED-2 multiplex PCR tubes provide a powerful strategy for clonality assessment in T-cell malignancies assisting the firm diagnosis of T-cell neoplasms." They added, "The detected TCR gene rearrangements can also be used as PCR targets for monitoring of minimal residual disease."

When establishing the guidelines for interpretation, the EuroClonality Group started with the rationale that different immunobiological conditions in diagnostic specimens present with different molecular patterns. For instance, reactive lymphocytes represent a broad immune response, while monoclonality (mono- or biallelic) can represent leukemia or lymphoma. Further, each immunobiological condition will have an expected profile in the PCR reaction. In the two preceding examples, the PCR profiles will be predicted to be (irregular) Gaussian curve/smear and "1 or 2 peaks/bands," respectively. Thus, each case is scored on two parameters:

- technical description per PCR (multiplex) tube.
- overall molecular conclusion of the entire rearrangement profile.

The third important level is the integration of the clonality testing results with morphological, immunophenotypical, and clinical data, usually performed by the pathologist. Examples of Ig/TCR rearrangement patterns are shown in the figures.

Dr. Groenen reported in her AMP talk that the guidelines were tested in a PT/QA study among the 25 members of

the EuroClonality Group. Fifty consecutive cases submitted for Ig/TCR clonality testing at each institution were evaluated, for a total of more than 1,150 cases, representing requests for both B-cell clonality and T-cell clonality. "Centers were able to use the standardizd scoring system to report almost 97 percent of cases," Dr. Groenen said. Only 3.1 percent were "molecular difficult to interpret."

In a separate study, EuroClonality/BIOMED-2 primers were compared with Southern blotting. "We have seen that PCR works as effectively as Southern blotting," Dr. Groenen told CAP TODAY. This is a major advance from comparisons Dr. Braziel and colleagues published in 2001 and 2002 (Arber DA, et al. *J Mol Diagn*. 2001;3:133–140; Bagg DA, et al. *J Mol Diagn*. 2002;4:81–89) and shows the benefit of the multiplex approach.

"Southern blot was a good test, but it was labor-intensive and not that sensitive," Dr. Groenen says. "And it needed fresh frozen material. But that's not the situation in many hospitals. Much material is formalin-fixed and embedded in paraffin [FFPE], for which Southern blot is not applicable. Fixation degrades the DNA. By doing PCR we can do archival cases, and all centers that only have paraffin blocks can do clonality testing on this type of material." Dr. Groenen acknowledges that working with DNA from FFPE can be a problem because of degradation. However, she says, "Many labs that do molecular diagnostics have set up a routine practice for fixation and paraffin embedding, where fixation times are short and standardized."

Says Dr. Cook: "The issue comes down to how large of a fragment of DNA you can amplify out of formalin fixation. For this assay some of the products you are looking for are up to 350 base pairs [bps]." The BIOMED-2 kit includes housekeeping genes of 100 to 600 bps, which allows laboratories to assess how large a fragment of DNA they can amplify out of a particular specimen. "We can usually amplify 400 base pairs out of formalin-fixed paraffin-embedded tissues," Dr. Cook says. "If we get at least 300 base pairs, we consider the sample adequate for testing. If it is 100 but less than 300 base pairs, then we consider it suboptimal." Dr. Cook notes that laboratories need to pay attention to the effect that their fixation protocols have on extraction. "We see different quality of DNA material from different labs," he says of their reference work.

PCR clonality testing is a molecular biology test in the pathology setting, Dr. Groenen notes, but it is different from looking for a *KRAS* mutation to help direct therapy. In cases where molecular clonality testing is invoked, the whole diagnostic workup, and especially interpretation of the clonality test, can be difficult. "It needs good contact between a pathologist who understands what the molecular biologist is doing and molecular biologists like me who understand the pathologists' questions," she says. "What are the situations when the pathologist needs to know whether the specimen is clonal?"

Dr. Braziel says the EuroClonality Group stresses that these results have to be interpreted with experienced hematopathology review of the case. "Sometimes labs [doing PCR clonality testing] don't have an experienced hematopathologist who knows how to interpret the patterns and they run into trouble. Some cases would be very hard to interpret if you're not seeing a fair number. You can read 50 papers, but you really need to do cases," she says.

The test has a degree of subjectivity in how it's interpreted, Dr. Cook says. "It doesn't produce a strict yes or no answer. For some challenging cases [most likely the cases that fall in the three percent 'difficult to interpret cases'] you may not even get agreement among people who see these a lot." He acknowledges that subjectivity is not unique to PCR clonality analysis: "Morphology certainly is subjective. Immunophenotyping is a bit more standardized, but there is still an aspect of subjectivity to everything we do in lymphoma diagnostics."

Dr. Cook sounded a note of caution, one that the other workshop discussants expressed. "It's very important to remember that clonality by itself is not diagnostic," he said. "It has to be interpreted in the context of everything you have in terms of morphology and phenotype." As Dr. Groenen emphasized, "Morphology and immunophenotyping determine the diagnosis. Clonality does not predict progression to lymphoma."

Dr. Groenen also stressed the need to establish reproducibility, saying it's "essential to prevent misinterpretation." Duplicates are strongly recommended for FFPE samples, which can have suboptimal DNA quality, and in cases with low number of lymphocytes, such as those from skin and the gastrointestinal tract. "Having low numbers of

lymphocytes can easily result in overinterpretation of dominant peaks," Dr. Groenen warned.

Dr. Braziel said overinterpretation of an apparent clone is "fairly common" in skin biopsy FFPE blocks. "We've seen a number of cases where clonality testing was done, an apparent T-cell clone was picked up and the skin biopsy was called malignant, positive for cutaneous T-cell lymphoma, when the histologic and immunophenotypic findings did not support a malignant diagnosis. We repeated the clonality studies and found that the apparent clone did not repeat. So we revised the diagnosis to benign," she said, adding, "You have to do skin biopsies in particular in duplicate."

Another case reviewed at the workshop illustrated the problem of documenting clonality in a setting where you have clearly reactive lymphocytes that don't meet the diagnosis of definite lymphoma. "Salivary gland sialoadenitis is a classic example of that," Dr. Braziel said in an interview. "When we started doing Southern blot analysis for B-cell clonality, we found you could detect a B-cell clone in almost all cases with reactive sialoadenitis and Sjogren's syndrome. That's a classic example of a B-cell clone that does not equate with malignancy." She had seen a case like this the day before the CAP TODAY interview. "It had a clear-cut monoclonal peak. We had to make the point again to our house staff and the outside pathologist that you have to have histological and immunologic evidence for malignancy in these cases because you almost always find clonality." Dr. Braziel frequently gets this type of case in consultation and says it can be very challenging to diagnose. "We are not doing these patients any favors by overcalling lymphoma," she cautions. "We will sometimes see two or three biopsies from a patient with reactive lymphoid hyperplasia over multiple years in which we can identify a B-cell clone. These may trend toward frank neoplasia over time, but often there is no significant clinical change, even with extended followup. So overdiagnosis is not good."

Dr. Braziel identified two other pitfalls when interpreting results from PCR clonality testing. "We know where nonspecific bands occur in these tubes. We illustrated some of those in the workshop. They are nonspecific but reproducible. You have to know what sizes they are, where they occur, in which tubes, and you have to be looking for them." She has many times seen people interpret reproducible but nonspecific peaks as clonal.

A second pitfall is the problem of "false-positive" patterns. "You need to be aware of cases where you find a clone that doesn't equate with malignancy," Dr. Braziel said. She cites an example of a patient who had a T-cell/histiocyte-rich diffuse large B-cell lymphoma in which there was both a detectable B-cell clone and an obvious T-cell clone. "If you run both T- and B-cell clonality analysis, you will find that 15 percent to 20 percent of B-cell lymphomas have a T-cell clone and some percentage of T-cell lymphomas will have a B-cell clone," she said. "You just have to put these results together with immunohistochemistry and flow and morphology to make the final diagnosis." She notes that this can be a particular problem for commercial laboratories that only turn out clonality results and don't see the rest of the case. "We get occasional cases like that," Dr. Braziel said. "All you can do is interpret the GeneScan and say there is a T- or B-cell clone and add the caveat that the significance of this clone is not clear and must be interpreted in conjunction with other findings."

Now it's time to recognize the elephant in the room. It's impossible these days to talk about anything in molecular pathology without at least making a nod to "next-gen"—next-generation sequencing. Molecular clonality testing is no exception. At the workshop Dr. Groenen mentioned NGS and gave her opinion: "Clonality assessment by PCR fragment analysis will be around for a few years yet."

In an interview she said she does see a role in the future for next-generation sequencing for analysis of clonality. "We want to know the clone and the sequence, especially maybe for T-cell malignancies. That will be important. However, we currently have defined what clonality is and have set up guidelines for clonality testing by GeneScan and heteroduplex analysis. Pathologists and clinicians understand what a clone means in the context of the pathology and the clinical picture of the patient." When next-gen sequencing is performed, more detailed information will be obtained, she says. "You will see a particular clone and maybe also see subclones. Then it will be extremely important to redefine clonality. We will enter a whole new era. It will be exciting and challenging." Whether (sub)-clones have diagnostic and/or prognostic meaning will have to be worked out, she says.

Dr. Groenen also raised the issue of cost and reimbursement. “These tests have to be paid,” she says. “And the price for next-generation sequencing should be in a comparable range to the current price of molecular testing.”

Of NGS, Dr. Cook says it’s an open question. “There have been studies done using next-gen sequencing to generate this kind of data. So far there are no data in the literature to really compare where that stands.” He predicts that for the next five or so years, PCR is going to be the predominant method for clonality testing. “This is something that can be done on fairly standard equipment in a molecular lab. Lots more labs have equipment for analyzing PCR assays than have next-gen sequencers,” he says. “That will change over time, but we don’t know how rapidly.”

William Check is a writer in Ft. Lauderdale, Fla.