In situ hybridization: more harmony across checklists

Anne Paxton

August 2016—As the use of in situ hybridization (ISH) expands, laboratories employing this form of testing increasingly rely on the CAP Laboratory Accreditation Program checklist for guidance. That is one reason members from three CAP committees started meeting to revise the ISH checklist, says CAP Surgical Pathology Committee member Aleodor Andea, MD, MBA. Another reason: to harmonize and streamline the ISH checklist requirements across three different disciplines.

Members from the Surgical Pathology Committee, the Cytogenetics Resource Committee, and the Molecular Oncology Committee formed an ISH/FISH project team in 2015 to produce the revisions in the 2016 ISH checklists, released in August.

Dr. Andea

"Historically ISH was performed only in cytogenetics labs, and, as a consequence, the cytogenetics checklist contains the most comprehensive section for ISH," says Dr. Andea, an associate professor and director of the dermatopathology molecular diagnostics laboratory at the University of Michigan School of Medicine. "However, in the modern era, anatomic pathology labs that have an adjacent immunohistochemistry lab have implemented FISH and chromogenic ISH tests in their environment." It started mainly with FISH HER2 testing for breast carcinoma, he says, but the ISH menu has quickly expanded to other areas including infectious diseases, soft tissue, solid organs, skin, and cytology, to name just a few. "And then the molecular sections of pathology have also started doing their own FISH in addition to PCR and sequencing."

Thus, three specialized laboratories in pathology—cytogenetics, anatomic pathology, and molecular pathology—are now performing ISH-related tests, each with its own checklist containing ISH sections that have different requirements, Dr. Andea says, and it has been confusing at times for laboratories to choose which checklist to use. "If you already have a histology lab and you want to do ISH, you would probably select the anatomic pathology checklist. However, for a newly established lab performing mostly FISH testing, the choice would be less clear. I was confronted with this myself when, as director of our dermatopathology molecular diagnostic laboratory, I had to select a checklist dealing with ISH for our new FISH test for melanoma. Eventually we used the cytogenetics checklist because it was the most comprehensive, but we could have used any of the other two."

Since the three disciplines are performing more or less the same tests, Dr. Andea says, the project team's goal in the 2016 revisions was to look at the three separate checklists and design one that would fit all of them and be inclusive.

Dr. Klein

The requirements in each of the three checklists were similar but not identical, says Roger D. Klein, MD, JD, a molecular pathologist and member of the Checklists Committee and the ISH/FISH project team. “So the intent from an organizational standpoint was to linguistically harmonize the preexisting requirements for ISH testing to have the same standards for equivalent tests whenever possible.” The committee also sought to standardize the format of the requirements and where they are located, as well as avoid too much redundancy with other checklist requirements, such as those in the common checklist.

This was a challenge, says Dr. Klein, medical director of molecular pathology for the Cleveland Clinic Foundation, because the nature of the testing in the three areas can differ in content and in emphasis or relative volume. “So, for example, in the molecular pathology lab, the ISH testing would typically be related to paraffin-embedded tissue but would exclude IHC, whereas in AP they would tend to focus more on tissue, but some of the requirements would be mixed with IHC, while cytogenetics tends to have a much greater emphasis on congenital testing than either of the other two areas.”

A molecular laboratory is more apt to perform fluorescence ISH testing, while AP would more commonly do chromogenic hybridization, which is performed under bright-field and typically requires a pathologist. For many of these tests, Dr. Klein says, a pathologist must be included, particularly in the solid tumor area.

As a result of the revision process, many checklist requirements are now identical across anatomic pathology, cytogenetics, and molecular oncology. But there are still three separate checklists, so unification is a work in progress. “Some things we have not yet worked out,” Dr. Andea explains. “For example, AP laboratories perform FISH mostly on paraffin-embedded tissue sections, so items in the checklist regarding metaphase analyses of chromosomes do not pertain to them.” On the other hand, “we made progress with many other requirements, for example the requirements regarding validation of ISH probes and establishing normal cutoff values, which were not uniform among the three checklists.”

In the anatomic pathology checklist, an important change was that “‘FISH’ was replaced with ‘ISH’ to reflect that in situ hybridization includes all ISH methods: fluorescence, chromogenic, and silver-enhanced ISH,” Dr. Andea says. In ANP.22956, which addresses ISH probe validation, a new note refers labs to a separate checklist item, ANP.22978, for specific validation requirements for HER2 testing in breast carcinoma.

Also new is ANP.22957 (same as CYG.42900 and MOL.38625), which refers to normal cutoff values for interphase ISH. “This requirement was included originally in the cytogenetics checklist but not in the AP or molecular checklists. Rigorous normal cutoff values are paramount with FISH assays performed on tissue sections, and such tests are often in the purview of AP laboratories. Due to nuclear truncation or overlap, the distribution of observed nuclear probe signal counts in a normal sample is wider than what would be biologically expected. For example, normally you expect to see two dots in a normal nucleus, but you may see less than two dots if the nucleus is truncated by the section or more than two if there is nuclear overlap. Therefore, it is important to determine the range of values that would be acceptable in a normal sample,” Dr. Andea says.

Other new ANP checklist requirements cover new reagent lot ISH probes (ANP.22958), ISH assay performance (ANP.22959), and ISH probe intended target (ANP.22960). The ISH scoring requirement (ANP.22963) is not new but has been revised slightly, Dr. Andea says. “It was originally written specifically for scoring FISH assays in which procedures requiring the evaluation of a minimum number of nuclei are common. However, by replacing FISH with ISH, the requirement applies now also to other chromogenic assays which do not require a set number of nuclei to

be evaluated for interpretation—for example, ISH for EBV or immunoglobulin light chains." This particular issue surfaced during the open comment period, he says. "In response, we changed the requirements to state, 'When applicable, there are written procedures for scoring in situ hybridization results . . .'"
ANP.22964, which deals with ISH controls, is also not new, but it has been re-edited and matches CYG.43200 and MOL.39146.

In the area of retention of photographic or digitized records, the project team chose to rewrite the checklist requirement (ANP.22965). A comment was added that there are no retention requirements for images as long as the slides remain readable.

Other changes the project team considered will require consultation with other committees before they are included in the checklists, to avoid unintended consequences. "For example, ISH is often performed in parallel with IHC for breast predictive markers, and as a result some requirements in the ANP checklist may impact both ISH and IHC," Dr. Andea says. "We did not want to change those without input from subspecialty committees such as IHC and Surgical Pathology."

Additional checklist requirements for automated systems did not make it into the 2016 revisions, he says. "The project team is still working on the best way to implement those. The automated systems are relatively new and significant experience with these systems is lacking, so we want to be careful on how we proceed."

For instance, one automated device tests urine for urothelial carcinoma, using four or five FISH probes that are applied simultaneously, and the results can be generated automatically. "I think more automation lies ahead, and as time goes by, some tests, if not completely automated, may have the interpretation assisted by a computer," Dr. Andea says. "And our dilemma in how to write this checklist item was that obviously there are different requirements when there is a human who will eventually release the results and check them, versus a computer releasing them automatically. The validation that controls a completely automated system, without a human checking the results, will need to be much more rigorous. It is a complex problem, and we were not able to solve that just yet."

In the 2016 checklist revision process for cytogenetics, says project team member Yassmine Akkari, PhD, a member of the Cytogenetics Resource Committee, the intent originally was to revamp the cytogenetics checklist—but in sections. "It was also noted that we have very similar requirements across the three checklists, but they are worded differently. They may mean different things. So we felt the need to take a closer look at each of these requirements to see whether it was possible to standardize them."

Dr. Akkari, who is scientific director of cytogenetics, technical director for molecular pathology, and manager for genetic operations at Legacy Laboratory Services in Portland, Ore., says the project team considered whether any individual items were the same concept written in three different ways across checklists. "If the concept applied to all three disciplines, then we needed to word it the same way. If not, we explained, for clarification, why the concept was different in all three disciplines."

The result of this process in cytogenetics was one new requirement, one deleted requirement, and several revised requirements.

The project team wanted to cater to all the possibilities in the field, Dr. Akkari says. "We wanted the CAP to be consistent in what it is asking—the conceptual requirements on quality, validation, and control. And some of these requirements were written so long ago that the language may not apply anymore."

Dr. Akkari

For example, she says, “One checklist requirement was that every time you set up a FISH experiment or slide or test you need to use an external control. In the past, the way many of the probes were designed, we needed an external control in a lot of situations because that was the only way the lab could tell the probe went on the right target. But these days, most probes are designed to include an internal control, and external controls are not necessary in all cases.” To clarify what could be considered an internal control, several examples are provided.

“It’s a matter of being efficient while being extremely precise and accurate,” Dr. Akkari says.

To hammer out the revisions, project team members from surgical pathology, cytogenetics, and molecular oncology met monthly by phone. “We went item by item to tell each other how we interpret it, and we sometimes realized some of us interpreted the same item differently. Which means this checklist item was not written properly because it’s open to so many interpretations. What are our labs—and more importantly, what are our inspectors—doing with it?”

So the project team members tried their best to reach a common understanding of the checklist and convey it without ambiguity, Dr. Akkari says.

In cytogenetics, slides are stored uniquely, she says. “Our slides are fluorescent where other ones are just staining. But also, we do not do infectious diseases. It is the surgical pathologists and molecular oncologists who deal with that. If a checklist requirement is truly only applicable to infectious disease, then it doesn’t belong in a cytogenetics checklist.”

In Dr. Akkari’s view, ISH is not on the increase but nor is it going away. “Other modalities may be able to achieve similar results as FISH, but complete, accurate results require several modalities, and FISH is one of them. FISH is very good at detecting minimal residual disease, whereas in next-generation sequencing the very low-level mosaicism is still hard to detect. So I see ISH as still needed. It’s still a complementary test to other modalities.”

All in all, the revisions were mainly standardization of language. “Right now we are looking at digitized imaging, whether it’s capture and/or analysis, and we are in the midst of working on that checklist item. It’s not done yet.” It’s an ongoing process that may be continued in the next round of checklist changes, or later, Dr. Akkari says.

From a cytogenetics perspective, the 2016 revisions mean that “we are caught up with the times in terms of probe design and controls and validation. We hope the language of the checklist items is clearer, more succinct, and more standardized across disciplines, she says. “This in turn will increase the quality of patient testing, which is our ultimate goal.”

Joel Moncur, MD, PhD, vice chair of the Molecular Oncology Committee, says the project team discovered there were a number of helpful items in some checklists but not others. “So we focused on bringing the best parts of all three into a unified checklist.” The cytogenetics checklist, for instance, had a requirement that new reagent lots be tested before they’re used clinically. “So we brought that into the other checklists.”

ISH is being used increasingly in molecular oncology, says Dr. Moncur, pathology department chief at Walter Reed National Military Medical Center. “There’s been a general transition from fluorescent ISH to bright-field ISH, which is a lot easier in many instances to use, so it’s become more broadly available to all labs. For those for whom bright-field ISH is a new technique, it’s important to have good guidance for quality, and that’s one of the huge benefits of the checklist items.”

As a result of the project team's review, the main accreditation requirements in molecular ISH were changed significantly, Dr. Moncur says. One area that remains challenging is automated image analysis. "That's a very difficult area to get uniform across all the different checklists because AP has so much automated image analysis for IHC and the standards are a little different."

Six new requirements were added in the molecular pathology checklist, relating to probe validation, interphase cutoff values, new reagent lots, assay performance, intended targets, and temperature-controlled processing systems, while two items were deleted and several requirements were revised. "For example, in molecular we made a new requirement for establishing normal cutoff values for each probe used. The requirement that each lot be checked for acceptable performance was originally only in cytogenetics, then it was added to AP and the molecular checklists," Dr. Moncur says. MOL.39004 addresses the need for written procedures for scoring. "Each probe will have its own criteria for positive or negative or equivocal results if that's applicable, so it's critically important to define these on a probe-by-probe basis as part of the assay validation."

One goal of the revisions in ISH was to drop separate requirements for fluorescence and bright-field ISH. "That's part of what allowed us to streamline and set fewer accreditation requirements by just referring to them all under the term 'ISH,'" Dr. Moncur says. However, the requirement that the laboratory retain photographic or digitized images for all slides was difficult for the project team to develop uniformly.

"That's because, with FISH, you need an archivable image because the fluorescence will fade, whereas the bright-field slide can be archived. In addition, cytogenetic tests have different image storage requirements because this field deals with constitutional disorders, whereas molecular oncology relates to somatic or acquired disorders." So in cytogenetics there was a separate requirement in the area of image retention.

Emerging trends in ISH could affect future rounds of revision, Dr. Moncur says. "There appears to be a trend toward using RNA ISH in addition to DNA ISH. Because RNA is more labile than DNA, preanalytical factors like specimen handling are of greater importance. That will have to be addressed later by the committee, if the trend continues of increasing the number of probes that examine RNA by ISH."

Some of these components of ISH were not stated specifically in any of the checklists, he believes. "Particularly given that more labs are doing these techniques as time goes on, it's very helpful that the standards are more explicitly stated. This encourages labs that are just implementing the techniques to meet the standards used by those with more experience."

While the 2016 ISH revisions were largely generated by the expert opinion of the ISH/FISH project team, Dr. Klein says, "the discussions were informed by not only the committee members but also CAP staff, who field many questions from accredited labs. We circulate the changes on a Listserv and members of all the various committees give them a thorough vetting."

Dr. Klein expects that molecular, cytogenetics, and anatomic pathology labs will not have much difficulty transitioning to the revised ISH checklist requirements. "By and large, most labs are complying with these requirements because they typically overlap with requirements in other areas of the individual checklists. But I think labs will need to scrutinize the particular requirements that have been embedded in the new 2016 checklists, to make sure there are no gaps."
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