Commentary

Cystic Fibrosis Testing Comes of Age

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This issue of *The Journal of Molecular Diagnostics* features three articles on various aspects of molecular genetic testing for mutations in the *CFTR* gene, which are associated with the development of cystic fibrosis (CF). In the last few years, CF has become the most widely tested hereditary disease at the gene level, thanks to the promulgation and steadily increasing acceptance of professional guidelines for universal carrier screening of the reproductive-age population.¹ This impressive test volume has not been achieved without significant technical and genetic counseling challenges, including the tremendous mutational heterogeneity of the *CFTR* gene, controversies over the variability of the clinical phenotype and allele frequencies in different ethnic and racial subsets of the population, and slow uptake or outright resistance from the screening providers, primarily obstetricians. These controversies date from the discovery of the gene in 1989, and there are some who still argue that CF was not the ideal choice for population-wide screening. Factor V Leiden screening, for example, would be more straightforward due to its single mutation analyte, and genetic counseling of test results would be unnecessary. Yet it too is subject to ambiguities based on low penetrance and risky interventions, and general factor V Leiden screening of the healthy population cannot be recommended.² Despite numerous challenges, CF carrier screening has in general been successful: becoming firmly incorporated into routine prenatal care, spurring the development of commercial platforms for multiplex mutation analysis that can now be applied to other complex genetic panels, and, perhaps most importantly, significantly diminishing the number of unwanted CF births, at least in the few outcome studies reported thus far.^{3,4}

The three articles in this issue illustrate that, with almost 8 years of accrued experience behind us, we are now entering what one might call the “mature” phase of CF mutation testing. These studies represent the next phase of fine-tuning and tweaking CF genetic testing approaches, now that routine testing and screening methodologies have been established. The carrier screening program is now widely entrenched in clinical practice,

and the mutation-detection technologies at our disposal have grown ever more sophisticated. Experienced laboratories now have no trouble handling the core screening panel of 23 mutations,⁵ and many use ever-larger “expanded” panels for either diagnostic or (more controversially) screening purposes.⁶ A major initial hurdle, the lack of available positive mutation controls for the alleles in the American College of Medical Genetics (ACMG) core panel, was addressed several years ago by both collecting and culturing patient blood cells and artificially constructing mutation controls using recombinant DNA methods and site-directed mutagenesis. These products have been either deposited in the Coriell mutant cell repository or sold commercially as packaged controls.^{7–10} Additional quality control materials to cover even rarer mutations are now needed due to the expansion of testing panels beyond the ACMG core panel and incorporation of additional mutations into the commercial assay platforms. The paper by Pratt et al¹¹ addresses this need admirably. In a multicenter effort performed under sponsorship of the Centers for Disease Control and Prevention, cell lines and extracted DNA preparations were developed for an additional 15 *CFTR* mutations commonly targeted by these expanded panels. (Some of the existing Coriell samples from the ACMG core panel were re-characterized and further verified as well.)

One might sense an inherent contradiction between the accepted ACMG panel of 23 mutations and this arduous effort to develop control materials that deliberately go beyond it, especially given the controversy surrounding expanded CF carrier screening panels.⁶ But as Pratt et al point out, and should always be remembered, the ACMG panel was devised and recommended only for population-based carrier screening. It was not intended to also serve as the standard for diagnostic testing or newborn screening; for those purposes, laboratories are free to select panels that examine greater or fewer mutations. In practice, of course, considering the biology of the system and the unwanted expense of duplicating equipment and reagents for different test indications, most laboratories consider their carrier-screening panel

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to be also adequate for diagnostic purposes. Yet in other settings, such as newborn screening in California, testing for additional mutations presumably targeted to the particular ethnic mix of the population has become standard.¹² In addition, many laboratories and commercial assay platforms use expanded panels even for routine carrier screening.¹³ Therefore, the availability of the additional, multisite and multiplatform validated positive mutation control materials reported here will be a welcome gift to the CF testing community.

The papers by Schwartz et al¹⁴ and Hantash et al¹⁵ fall under the "fine-tuning" category. As robust as the available multiplex testing platforms are, they are still subject to false results due primarily to interfering complexities of the *CFTR* sequence itself. This was recognized even in the initial ACMG recommendation, in which tests for several presumably benign polymorphisms were included in the core screening panel because of their tendency to interfere with DNA probe hybridization for certain genuine mutations.¹ For example, presence of the polymorphism F508C can interfere with probe hybridization to the normal allele F508, causing a type of allele "drop-out" when using certain assay methods. In practice, this could cause a patient who is compound heterozygous for F508C and Δ F508 to appear as a Δ F508 homozygote. Such a result would be surprising and likely questioned in a carrier-screening scenario, but in a diagnostic setting it could be accepted as a reasonable, yet actually false-positive, result. Fortunately, these are rare events, but it is prudent to be prepared for such incidents, especially in laboratories performing large numbers of tests.

In that regard, the study by Schwartz et al, based on over 1,000,000 patient specimens (!) in a large commercial reference laboratory, is most revealing. From this huge sample, they observed 37 cases with unusual electrophoretogram profiles using a popular, commercially available oligonucleotide ligation assay platform, for an aberrancy rate of <0.01%. Specifically, they observed allele drop-out (or failure to detect one of the targeted mutations); this occurred with a variety of mutations in the screening panel. Based on the subset of cases that were confirmed by DNA sequencing, allele dropout appeared to be caused by polymorphic variants at or under the oligonucleotide ligation sites, and was therefore method-specific. It is reassuring that this laboratory, with their extensive CF testing experience, has yet to observe an interfering polymorphism under a PCR primer hybridization site, which is another known and worrisome cause of apparent allele drop-out. Yet we must concede that such variants undoubtedly exist, if not for the ligation sites of this commercial oligonucleotide ligation assay, then under the primer hybridization sites of other assays. In this regard, it is unlikely that any particular assay platform is more or less prone to such anomalous results than the others, though the specific interfering variants/mutations may be unique to each assay. Therefore, it should be possible to investigate and hopefully rule out such anomalous or unexpected results by re-testing of the patient's DNA on a different assay platform (which may require collaboration with another laboratory), as was done here. To suspect these anomalies, one has to hope that some-

thing about the appearance of the original run will be strange enough, and noticed by the laboratory staff, to prompt such an investigation or at least attention to the polymorphic controls included within the assay itself (such as the F508C example mentioned above). It may also require rather sophisticated communication with the ordering physician, which is not always successful; as described by Schwartz et al, some of these physicians declined the recommendation for further testing.

Another known cause of allele "drop-out" producing a false appearance of homozygosity (either for one of the tested mutations or for the normal allele) is the presence of a large deletion in the opposite allele. Since almost all of the CF testing methods available use PCR amplification as an initial step, presence of a deletion involving one (or more) of the primer hybridization sites will result in amplification failure for that particular exonic product. Since the subsequent steps of most assays involve oligonucleotide probe hybridization or ligation or sequencing, the non-amplified, deleted allele will not be seen; only the opposite allele will be analyzed and will thus appear homozygous. The frequency of this phenomenon in genetic disease testing first came to light in DNA testing for familial cancer mutations, in which deletion carriers, accounting for as many as 10 to 15% of *BRCA* mutations, produced normal DNA sequencing results.¹⁶ The phenomenon seems to be less prevalent in the spectrum of *CFTR* mutations, although still appreciable enough that some have recommended reflex testing for exonic copy number, at least in diagnostic situations where complete gene sequencing has failed to identify both mutant alleles.¹⁷

The case presented in this paper turned out to have not just one, but two *CFTR* deletions, on opposite alleles: a large one of 40 kb that deletes exons 4 through 10 and has been described previously,¹⁸ and a novel, small one of just 20 bp in exon 7. Since exon 7 is spanned by the larger deletion, only an exon 20 deletion was readily observed by the sequencing approach, and appeared as if it were homozygous. That is what prompted further investigation and more precise characterization of both the smaller and larger deletions. Likewise, a test result of apparent homozygosity for any rare mutation, especially in the absence of consanguinity, should prompt reflex testing or further investigation for the presence of a heterozygous deletion on the opposite allele.

Taken together, these three papers demonstrate how the widespread and thoughtful experience with CF mutation testing and screening continues to reveal new insights about the mutational alleles of the *CFTR* gene and further refinements in how best to detect them and assure appropriate quality control while doing so. This is as it should be, and provides encouragement for the maturation in practice of other complex molecular genetic tests. There were some who felt that population CF carrier screening should not be implemented because of the mutational heterogeneity of the *CFTR* gene, just as there were some who proposed a moratorium on genetic testing for familial cancers because the penetrance of those mutations is not 100%. But it is only by embarking on these challenges and expanding the accrued experience

across many patients and centers that we can add to our knowledge of these complex genetic elements and thereby provide the most accurate testing and the most informative clinical interpretations for our referring physicians and patients.

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