

ANALYTIC VALIDITY

8. Is the test qualitative or quantitative?

Testing for an inherited susceptibility to colorectal cancer by DNA sequencing is qualitative. For comprehensive sequencing, results fall into three major categories: (1) deleterious mutation, (2) genetic variant of unknown clinical significance, and (3) no abnormality detected. As an example of how one laboratory reports sequencing results, the following text has been taken from the Myriad Genetic Laboratories web site. They list six categories for the results of “comprehensive” (*MLH1* and *MSH2*) HNPCC testing.

“Positive for a deleterious mutation”: Includes all mutations (nonsense, insertions, deletions) that prematurely terminate (“truncate”) the protein product of *MLH1* at least 10 amino acids from the C-terminus, or the protein product of *MSH2* at least 46 amino acids from the C-terminus (based on documentation of deleterious mutations in *MLH1* and *MSH2*).

In addition, specific missense mutations and non-coding intervening sequence (IVS) mutations are recognized as deleterious on the basis of data derived from linkage analysis of high risk families, functional assays, biochemical evidence and/or demonstration of abnormal mRNA transcript processing.

“Genetic variant, suspected deleterious”: includes genetic variants for which the available evidence indicates a likelihood, but not proof, that the mutation is deleterious. The specific evidence supporting such an interpretation will be summarized for individual variants on each such report.

“Genetic variant, favor polymorphism”: Includes genetic variants for which available evidence indicates that the variant is highly unlikely to contribute substantially to cancer risk. The specific evidence supporting such an interpretation will be summarized for individual variants on each such report.

“Genetic variant of uncertain significance”: Includes all missense mutations, mutations that occur in analyzed intronic regions, and mutations that truncate *MLH1* and *MSH2* distal to amino acid positions 746 and 888, respectively, except as noted above. A genetic variant of uncertain significance in either *MLH1* or *MSH2* is considered to be less likely to be deleterious if it has been observed in one or more individuals with a known deleterious mutation in the same gene.

“No deleterious mutation detected”: Includes non-truncating genetic variants observed at an allele frequency of approximately 1% of a suitable control population (providing that no data suggest clinical significance), as well as all genetic variants for which published data demonstrate absence of substantial clinical significance. Also includes mutations in the protein-coding region that neither alter the amino acid sequence nor are predicted to significantly affect exon splicing, and base pair alterations in non-coding portions of the gene that have been demonstrated to have no deleterious effect on the length or stability of the mRNA transcript.

There may be uncommon genetic abnormalities in *MLH1* and *MSH2* that will not be detected by Colaris™. This analysis, however, is believed to rule out the majority of abnormalities in these genes, which are believed to be responsible for most HNPCC. Data on polymorphic variants are available upon request.

“Specific variant/mutation not identified”: Specific and designated deleterious mutations or variants of uncertain clinical significance are not present in the individual being tested. If one (or rarely two) specific deleterious mutations have been identified in a family member, a negative analysis for the specific mutations(s) indicates that the tested individual is at the general population risk of developing those cancers associated with HNPCC.

If the mutation in the family has already been identified, just that site is examined, and the mutation is reported as either detected or not detected.

9. How often is the test positive when a mutation is present?

Analytic sensitivity is the proportion of positive test results, when a detectable mutation is present. In other words, a laboratory sequencing the *MLH1* and *MSH2* genes would have 100 percent analytic sensitivity, if it was able to detect a mutation in one of these genes every time that mutation was present in a sample. Analytic sensitivity can be expressed as true positives divided by the sum of true positives and false negatives. False negative results can be due to technical errors in the analytic phase (e.g. contamination, expired reagents, or non-specific reactions) or to administrative or clerical errors in the pre-analytic or post-analytic phases. Analytic sensitivity only considers the types of mutations that a laboratory says that it is able to detect. For example, if a laboratory does not test for large deletions or insertions, then proficiency testing challenges involving these types of mutations are not considered to be false negatives.

External proficiency testing is one good source of information to determine analytic sensitivity. Proficiency testing samples contain known sequences that are tested blindly by clinical laboratories. The American College of Medical Genetics (ACMG) co-sponsors a molecular genetics proficiency testing program with the College of American Pathologists (CAP). Currently, HNPCC is not included in the program. Existing proficiency testing for mutations (e.g., *BRCA1*, *BRCA2*) offers only indirect insight into the analytic performance of HNPCC sequencing.

Clinical testing for HNPCC is offered by 13 U.S. laboratories, according to GeneTests. We mailed a questionnaire to these CLIA-approved laboratories about their services. Of the 10 responding, eight are currently providing this service and two are not. Of the eight, six participate in the ACMG/CAP proficiency testing program. Since DNA sequencing methodology for HNPCC genes (*MLH1*, *MSH2*) and for breast cancer genes (*BRCA1*, *BRCA2*) is similar, estimates from the proficiency testing involving the latter may be relevant to the former. One laboratory reports that “failure to detect a genetic variant or mutation in the analyzed regions may result from errors in specimen handling and tracking, amplification and sequencing reactions, or computer-assisted analyses and data review. The rate of such errors is estimated from validation studies to be less than one percent” (Shattuck-Eidens *et al.*, 1997). The ACMG/CAP Molecular Genetics Laboratory Survey reports no errors in evaluation of 143 *BRCA* alleles in 28 laboratories. However, only three specific *BRCA* mutations were challenged in those exercises, and only a few laboratories utilized sequencing.

The European Molecular Genetics Quality Network (EMQN) estimated their overall error rate for 1999 to 2002 for *BRCA* gene analysis to be 2.7 percent (www.emqn.org). However, since laboratories were told what exon to examine, the error rate for ordinary clinical samples may have been underestimated. This information, although indirect, is the most relevant for estimating the analytic performance of HNPCC testing via sequencing. Analytic validity data from the EMQN are likely to be valid for US laboratories as well, based on an earlier comparison of relative performance of the European and ACMG/CAP surveys for cystic fibrosis (CFTR) testing (Palomaki *et al.*, 2003). A formal external proficiency testing program for HNPCC and FAP is available in Australia and New Zealand that utilizes lymphoblast cell lines (**Human Genetics Society of Australia?**). This allows laboratories to perform DNA or RNA analyses for unknown mutations. This program also provides DNA for known mutations in a predictive testing scenario. It may be possible for laboratories in the United States to join this program, but specific information is not available.

Gap in Knowledge: For laboratories offering HNPCC testing in the United States, there are no data available to reliably estimate the analytic sensitivity of sequencing the *MSH1* and *MLH2* genes for mutations causing HNPCC from either external proficiency testing or other sources.

10. How often is the test negative when the mutation is not present?

Analytic specificity is the proportion of negative test results when no detectable mutation is present. In other words, a laboratory sequencing the *MLH1* and *MSH2* genes would have 100 percent analytic specificity, if it reported finding no mutation in one of these genes every time that no mutation was actually present. Analytic specificity can be expressed as the true negatives divided by the sum of the true negatives and false positives. False positive results can be due to technical errors in the analytic phase (e.g. contamination, misinterpretation of a polymorphism as a deleterious mutation, expired reagents, or non-specific reactions) or to administrative or clerical errors in the pre-analytic or post-analytic phases.

Gap in Knowledge: There are no data available to reliably estimate the analytic specificity of sequencing the *MSH1* and *MLH2* genes for mutations causing HNPCC from either external proficiency testing or other sources.

11. Is an internal quality control program defined and externally monitored?

The expectation is that all laboratories offering HNPCC DNA testing will be CLIA certified. Although this is a minimum qualification, it does ensure that the laboratory meets specifications for a high-complexity test and is externally reviewed. A more comprehensive review is required for New York State certification. Of the eight laboratories surveyed for this report that are currently offering HNPCC testing, six report having an internal QC program consisting of the blind insertion of samples of known abnormal and normal sequences.

12. Have repeated measurements been made on specimens?

Repeating DNA measurements in multiple assay runs offers the opportunity for the laboratory to assess consistency of performance. Laboratories performing HNPCC DNA testing for clinical purposes are expected to be regularly running positive and negative controls. All of the eight surveyed laboratories offering such testing, for example, reported regularly running positive and negative controls. Although these data are collected, they are usually not available for review outside of the laboratory.

13. What is the within- and between-laboratory precision?

This question is not applicable to the use of DNA testing for HNPCC, since such testing is qualitative.

14. If appropriate, how is confirmatory testing performed to resolve false positive results in a timely manner?

When a *MSH1* or *MLH2* mutation is found, all eight of the surveyed laboratories routinely perform confirmatory analyses. Seven of the eight laboratories sequence the same polymerase chain reaction product in the reverse direction. All eight sequence the products of a second polymerase chain reaction for purposes of confirmation.

15. What range of patient specimens has been tested?

Once microsatellite instability testing identifies an indication for mutation analysis by DNA sequencing, non-tumor tissue must be used. Non-tumor tissue (usually blood or buccal samples) will have only the inherited mutation, whereas a tumor commonly has additional mutations that have arisen during neoplastic progression. Tumor tissue has additional disadvantages; it is sometimes necrotic, requires disaggregation, and may require special arrangements with surgeons or clinical pathologists to obtain fresh material. Fixed tumor tissue is unsatisfactory for sequencing, due to the chemicals used for fixation. Of the eight reporting laboratories, all ac-

cept blood and three accept buccal swabs. Blood is preferred to buccal swabs, because it more reliably provides the quantity of DNA needed.

16. How often does the test fail to give a usable result?

Failure rates for laboratory testing of satisfactory specimens are not available, but are likely to be very low. Five milliliters of anti-coagulated whole blood shipped overnight is adequate for most DNA sequencing laboratories. Most test failures are not due to methodology, but instead to poor sample quality (e.g. obvious contamination or hemolysis, exposure of sample to extreme temperature or delay in transit).

17. How similar are results obtained in multiple laboratories using the same, or different, technology?

No data are available from HNPPC sequencing laboratories to make this comparison, but the expectation is that laboratory results will have a high degree of concordance. Not all laboratories report mutations using the same system of nomenclature. This can cause problems when a second laboratory is asked to test for specific mutations. den Dunnen and Antonarakis have made recommendations for standardizing human sequence variations (den Dunnen and Antonarakis 2001). For nucleotide numbering, they recommend that: nucleotide +1 is the A of the ATG-translation initiation codon. They also give recommendations for numbering 5'UTR and 3'UTR. In addition, they suggest that sequence variations be described in relation to a reference sequence for which the accession number from a primary sequence database should be mentioned.