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American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories, 2014 edition: technical standards and guidelines for Huntington disease

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Huntington disease is an autosomal-dominant neurodegenerative disease of mid-life onset caused by expansion of a polymorphic trinucleotide (CAG) repeat. Variable penetrance for alleles carrying 36–39 repeats has been noted, but the disease appears fully penetrant when the repeat numbers are >40. An abnormal CAG repeat may expand, contract, or be stably transmitted when passed from parent to child. Assays used to diagnose Huntington disease must be optimized to ensure the accurate and unambiguous quantitation of CAG repeat length. This document provides an overview of Huntington disease and methodological considerations for Huntington disease testing. Examples of laboratory reports are also included.

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Key Words: clinical testing; Huntington disease; polyglutamine; practice guideline; trinucleotide repeat disorder

Disease-specific statements are intended to augment the general American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories. Individual laboratories are responsible for meeting the Clinical Laboratory Improvement Amendments (CLIA)/College of American Pathologists (CAP) quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency, and quality control measures. This 2014 edition of the guideline updates and supersedes the original laboratory guideline on this topic, which was published in 2004[1] and reaffirmed once during the ensuing decade.

BACKGROUND OF HUNTINGTON DISEASE

Gene symbol/protein name

HTT (historically known as IT15)/Huntingtin. The GenBank accession number for the reference sequence is NM_002111. The OMIM gene/locus code is 613004.

OMIM phenotype number

143100.

Brief clinical description

Huntington disease (HD) is a neurodegenerative disease of mid-life onset that produces choreic movements and cognitive decline, often accompanied by psychiatric changes. It affects ~3–5 out of 100,000 individuals. However, the prevalence of HD exceeds 15 per 100,000 in some populations, mostly of western European origin.[2] Juvenile-onset HD occurs in approximately 5% of affected patients, is a rapidly progressive variant, and presents with rigidity, spasticity, and intellectual decline before the age of 20 years. The symptoms result from the selective loss of neurons, most notably in the caudate nucleus and putamen, and there is currently no effective treatment. For more information, see the online Gene Reviews profile at http://www.ncbi.nlm.nih.gov/books/NBK1305/.

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Mode of inheritance

Inheritance is autosomal dominant, with clinical manifestations associated with expansion of a polymorphic trinucleotide (CAG) repeat. Variable penetrance for alleles carrying 36–39 repeats has been noted, but the disease appears fully penetrant when the repeat numbers are ≥40.

An abnormal CAG repeat may expand, contract, or be stably transmitted when passed from parent to child. Although small expansions and contractions are common in maternal and paternal transmission of abnormal alleles, large expansions occur only in paternal transmissions.[3]

Gene description/normal gene product

The *HTT* gene on 4p16.3 spans 170 kb and contains 67 exons. The encoded protein, huntingtin, has 3,144 amino acid residues, has a molecular weight of 350 kDa, and lacks homology to any previous known proteins. HD is caused by an expansion of a highly polymorphic CAG repeat in exon 1 of the *HTT* gene. The expanded CAG repeat is translated into a polyglutamine tract in the huntingtin protein. Structural analysis of the *HTT* gene promoter region is consistent with the gene being a housekeeping gene. Although on a cellular level mutant huntingtin is widely expressed in both neural and non-neural tissue, there is region-specific neuronal loss in the neurons in the caudate and putamen.

Genotype/phenotype association

The inverse relationship between the CAG repeat size (at ≥40 repeats) and age of onset is unequivocal. The relationship is particularly strong for high repeat sizes (>50 repeats). However, the repeat length does not allow precise prediction of the age of onset for any one particular patient.

Mutational mechanism

The abnormal polyglutamine expansion results in protein misfolding, nuclear accumulation and aggregation of HTT fragments, and altered post-translational modifications.[4] Intranuclear inclusions of the truncated mutant protein aggregates have been identified in brains of HD patients. These alterations are ultimately associated with, but not necessarily causative of, cell death.

Listing of mutations

Allelic heterogeneity has not been described in HD.

Ethnic association of HD mutation

All major ethnic groups appear to be susceptible to expansion of the HD CAG repeat.

SPECIAL TESTING CONSIDERATIONS

Clinical sensitivity and specificity

CAG repeat expansion mutations account for >99% of cases of HD. Therefore, tests that effectively detect and measure the CAG repeat region of the *HTT* gene are >99% sensitive. The absence of HD pathology has not been documented in any individual with an HD allele size of ≥40 CAG repeats who died, disease free, after living up to or past the normal life expectancy. Therefore, positive results (at least one allele of 40 CAG repeats or more) are 100% specific. Allele sizes of 26 CAG repeats or less have never been associated with an HD phenotype in the US survey or in any published study. Allele sizes of 27–35 CAG repeats are rare and have not been associated convincingly with an HD phenotype but they may be mutable, as described in the Mutable Normal Alleles section. Allele sizes of 36–39 CAG repeats have been reported in both clinically affected and clinically unaffected individuals. Thus, it is not possible to determine the specificity of a test when one or both of the alleles are in the CAG repeat range of 36–39. CAG repeat expansion is offered as a clinical test.[1]

Diagnostic versus predictive testing

Detection of CAG expansion is used for both confirmatory and predictive testing. Positive results for both confirmatory and predictive testing are considered diagnostic. It is strongly suggested that predictive testing not be offered to individuals until they are at least 18 years old. A formal multidisciplinary predictive testing protocol is offered at many sites for individuals desiring determination of their carrier status.

Prenatal testing

Prenatal testing can be used for prenatal diagnosis in amniotic fluid cells and in chorionic villus samples. Maternal cell contamination studies should be performed on every prenatal sample to confirm the fetal origin of the sample being tested. Evidence of a molecular diagnosis of HD in the family should be obtained before prenatal testing.

GUIDELINES

Definition of normal and mutation category

Normal alleles. Normal alleles are defined as alleles with ≤26 CAG repeats.[5–7] These alleles are not pathologic and segregate as a stable polymorphic repeat in >99% of meiosis (**Figure 1**).

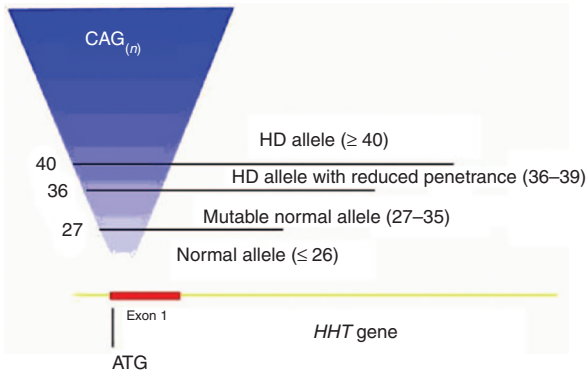

Figure 1 Diagram of *HTT* gene and the location of the polymorphic CAG repeat within exon 1. Boundaries denote CAG repeat length categories and descriptors.

The most common normal allele lengths contain 17 and 19 CAG repeats.[5]

Mutable normal alleles. Mutable normal alleles are defined as alleles with 27–35 CAG repeats, and this repeat range is often referred to as the meiotic instability range, or "intermediate alleles." These alleles have yet to be convincingly associated with an HD phenotype, but they can be meiotically unstable in sperm, and pathologic expansion of paternally derived alleles in this size range has been described.[8,9] There have been no reports of maternally transmitted alleles in this range producing offspring with affected alleles.[10] Approximately 1.5–2% of the general population carry alleles in this size range.[6,11] The likelihood that transmission of an allele in this range will expand into an HD allele is dependent on several factors, including the sex of the transmitting individual, the size of the allele, the molecular configuration of the region surrounding the CAG repeat, and its haplotype.[11,12] This risk may be as high as 6–10% for paternal alleles carrying a CAG repeat of 35.[12]

HD alleles with reduced penetrance. HD alleles with reduced penetrance are defined as alleles with 36–39 CAG repeats. Repeat sizes in this range are often referred to as being in the reduced penetrance range. Alleles in this size range are meiotically unstable and are associated with the HD phenotype in both clinically and neuropathologically documented cases.[7] However, in rare cases, alleles in this range have been found in elderly asymptomatic individuals.[12,13] Although the limited number of documented cases has precluded the development of empirical penetrance risks for alleles in this range, some estimates of risk can be ascertained from an examination of individual case reports.[13,14]

HD alleles with full penetrance. HD alleles with full penetrance are defined as alleles with ≥40 CAG repeats. Although there are no reports of the absence of HD pathology in any individual with ≥40 CAG repeats, a few clinically asymptomatic carriers with 40 and 41 CAG repeats have been reported.[14] The largest expanded HD allele detected to date carries ~250 CAG repeats in a patient with juvenile-onset disease.[15]

Mosaicism. Mosaicism attributable to both mitotic and meiotic instability has been described in brain and sperm, and it appears to be more pronounced in juvenile-onset cases of HD associated with larger CAG expansions.[16] However, the degree of mosaicism is not significant enough to compromise the interpretation of CAG repeat lengths determined from DNA extracted from peripheral blood lymphocytes.

Methodological considerations

Individual US laboratories offering molecular diagnostic testing for HD should be in compliance with all federal and state regulations relevant to clinical laboratory operations. This includes meeting all CLIA/CAP quality control requirements. In addition, all US laboratories offering clinical testing for HD should be active participants in semi-annual HD proficiency testing challenges. All methodological applications should also be in compliance with the Standards and Guidelines for Clinical Genetics Laboratories developed by the ACMG.[17] Non-US laboratories should be similarly compliant with their individual countries' statutory regulations governing oversight of clinical laboratories.

PCR methods. Several sets of primers, polymerase chain reaction (PCR) conditions, and amplicon separation and detection techniques have been published.[18–22] Regardless of the particular PCR-based strategy selected, it is important for assay conditions and post-PCR analyses to be optimized to ensure the accurate and unambiguous quantitation of repeat length (**Figure 2a**,**b**). It should be noted that CAG sizing anomalies have been observed in comparative post-PCR analyses utilizing agarose, capillary, and denaturing polyacrylamide gel electrophoretic methods.[23–25] As such, accurate quantitation of patient amplicon sizes should be empirically determined by comparison with appropriate external or internal standards. These could include, but are not limited to, size standard, cloned reference standards, and appropriate normal and abnormal patient controls whose sizes have been independently verified. Because it is the length of the polymorphic CAG repeat *alone* that is associated with the HD phenotype, patient genotyping based on the use of a *single* primer pair that amplifies both the CAG and the adjacent CCG repeat[22] is discouraged. The CCG repeat, which lies 3′ of the HD CAG repeat, has been shown to be polymorphic; as a result, it may lead to diagnostic inaccuracies for both normal and HD allele sizing.

Appropriate controls that include a range of CAG sizes should be utilized for each analysis. It is the responsibility of the laboratory to empirically determine the detection limits for their assays. Although the upper limit of detection is not known, alleles carrying ~115 CAG repeats have been amplified by PCR-based methods,[26] whereas alleles carrying more than ~125 CAG repeats appear to be refractory to reproducible amplification.[15]

Polymorphisms surrounding or within the CAG tract have been identified and have a collective frequency of >1% in patients referred for HD testing.[11,12,27–31] These nucleotide substitutions generally can be categorized into two groups: those that modify primer-annealing sites and those that result in the loss of interruption between the CAG and CCG tracts. In the first category, nucleotide changes can result in the misinterpretation of genotyping data due to an allele-specific amplification failure associated with primer mis-annealing. As such, in certain clinical circumstances "apparent homozygosity" for two normal alleles identified with standard PCR methods should be interpreted with caution, and the use of alternative primer pairs, triplet repeat primed PCR, or other methodologies (Southern blot or sequencing) should be used to resolve any ambiguous results.[27–31] In the second category, rare A-to-G substitutions in the intervening 12-bp segment between the CAG and CCG tracts (CAG CAG CCG CCG) can result in increased meiotic instability of the tract as well as a miscalculation of

uninterrupted CAG repeat length based on the conventional calculation formula.[12,30]

Southern blot. If the method routinely used by the laboratory is not validated to detect large CAG expansions, then Southern blotting should be used for the identification of expansions associated with juvenile-onset HD in cases of apparently homozygous normal genotypes.[32]

Triplet-repeat primed PCR (TP PCR). The *HD* $CAG_{(n)}$ repeat region is amplified by PCR using a fluorescently labeled forward primer, located upstream of the $CAG_{(n)}$ region, and a chimeric reverse primer located partially within the $CAG_{(n)}$ region (**Figure 3a** and primer sequences **Figure 3b**). The chimeric reverse primer hybridizes to multiple locations within the $CAG_{(n)}$ repeat region, creating PCR products of varying sizes. Reactions are separated by capillary electrophoresis. TP PCR provides a characteristic ladder on the fluorescence trace, enabling the rapid identification of large pathogenic repeats that cannot be amplified using flanking primers. Because the 5′ end of the reverse chimeric primer exactly matches the sequence 3′ of the $CAG_{(n)}$ region, this product is preferentially amplified. The true alleles are therefore distinguished as the highest peaks; stutter peaks extending from the smaller allele and terminating with the larger allele represent products from the reverse primer hybridizing within the $CAG_{(n)}$ region. When an allele is too large to be amplified by this assay, a stutter peak of decreasing amplitude extends to the end of the electropherogram (see **Figure 4a–d** for examples). Results are evaluated for the presence of this distinct stutter pattern extending into the expanded allele range.[33] Positive control (large repeat), negative control (normal size repeat), and no template control should be included on each run. Post-PCR detection of the alleles can be achieved by fragment length analysis.

Although TP PCR can be used as a primary method, it can be used instead of Southern blot for the identification of large expansions associated with juvenile-onset HD and is useful for the confirmation of "homozygous normal" genotypes.

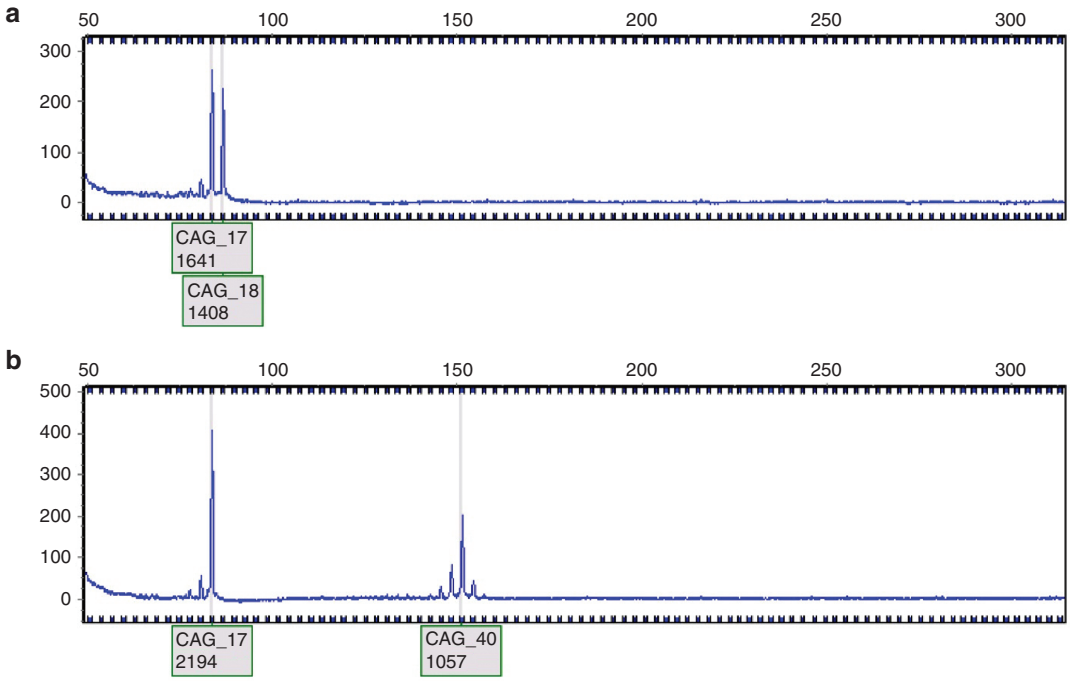

Figure 2 Polymerase chain reaction (PCR) results with different allele sizes. (**a**) PCR results with 17 and 18 CAG repeats. (**b**) PCR results with 17 and 40 CAG repeats.

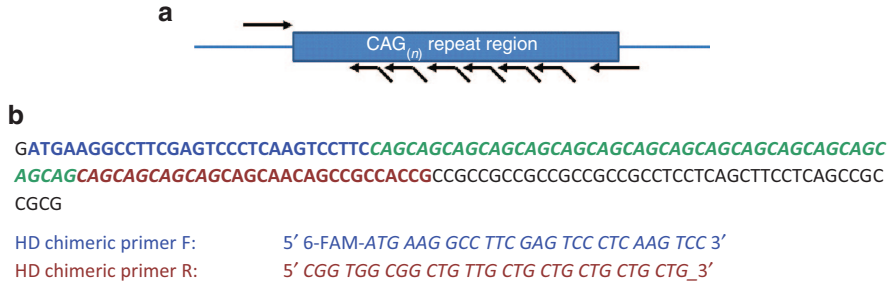

Figure 3 Schematic representation of Huntington disease (HD) chimeric assay. (**a**) HD chimeric assay primers. (**b**) HD chimeric primer sequences.

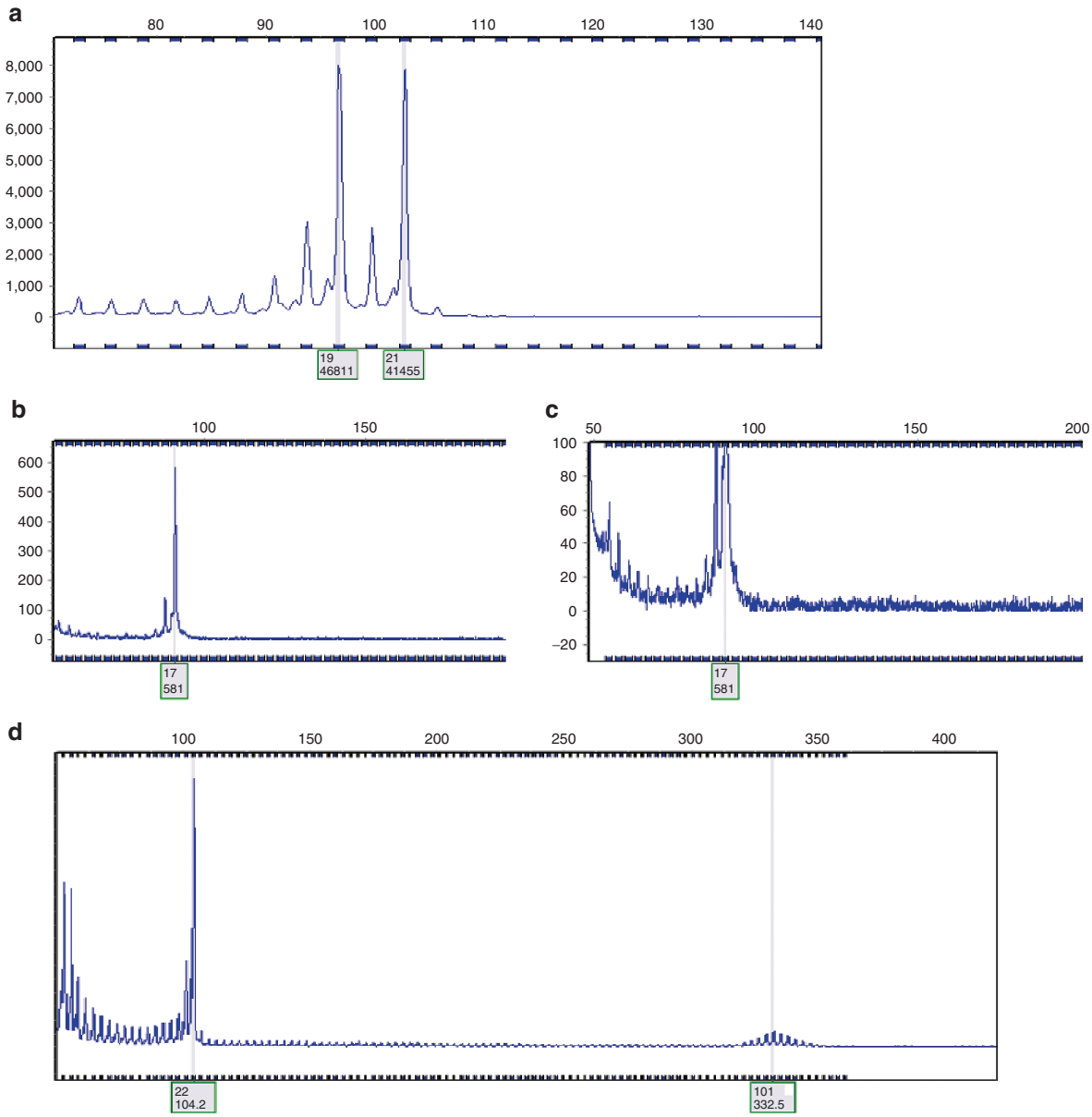

Figure 4 Triplet-repeat primed polymerase chain reaction (PCR) results with different allele sizes. (**a**) Triplet-repeat primed PCR results with two normal alleles (19 and 21 CAG repeats). There is a stark decrease in fluorescence after the larger allele peak, and then a flat line to the end of the electropherogram. (**b**) Triplet-repeat primed PCR results with 17/17 homozygous. (**c**) 17/17 homozygous zoomed in to show the flat line after the 17 allele peak. (**d**) Triplet-repeat primed PCR results with a normal allele of 22 CAG repeats and a pathogenic allele of 101 CAG repeats. Note the stuttering continues from the smaller to the larger allele and then decreases to a flat line after the 101 CAG repeat.

It is recommended that all laboratories consider the use of either Southern blot or TP PCR or establish a formal referral arrangement with a facility that offers this testing for those cases requiring genotypic resolution.

Interpretations

Since 1998, it has been the intent of the CAP/ACMG Biochemical and Molecular Genetics Resource Committee to standardize the accuracy of CAG repeat quantitation. Based on the analysis of previous CAP/ACMG proficiency testing survey results for HD, the acceptable range for sizing CAG repeats was revised in 2012.[34] Acknowledging the technical limitations of size analysis, the ACMG supports the following acceptable ranges for HD clinical testing and as grading criteria for the CAP/ACMG proficiency testing survey: consensus size ±2 repeats for alleles with less than 50 repeats; consensus size ±3 repeats for alleles with 50–75 repeats; and consensus size ±4 repeats for alleles with >75 repeats.

Reporting clinical results. Elements considered essential to the reporting of clinical test results are described in detail in ACMG's Standards and Guidelines for Clinical Genetics Laboratories. The following additional elements should also be included in the reporting of an HD genotype.

The methodology used to assign the genotype. If PCR methodology was used, then a description of the primer

pair(s) should be included as well as the method of amplicon separation and detection. Furthermore, each report should contain a statement regarding PCR sensitivity for the detection of large expansions. If a Southern blot was required, then the restriction enzyme(s) and probe(s) should be identified. Each report must state the CAG repeat length categories and descriptors currently utilized in clinical practice, and each reportable genotype should be classified and interpreted using these categorical definitions.

CAG repeat number. Each report must include the CAG repeat numbers of both alleles with the precision of sizing fulfilling the criteria recommended by the CAP/ACMG Biochemical and Molecular Genetics Resource Committee. For large alleles determined by PCR, Southern blot, and/or TP PCR, qualifying terms such as "approximately" or "estimated" can be used but should not be written because these could create unnecessary interpretive ambiguity. All positive results should state that genetic counseling is indicated and that testing is available for other at-risk family members.

The following points regarding alternative diagnoses may be included:

- HD phenocopies represent ~3% of patients referred for molecular confirmation of a clinical diagnosis of HD,[35,36] and several other loci (HDL1 and HDL2) have been identified.[37,38] As such, *HTT* gene expansion in these patients will be negative.[39] Furthermore, the considerable clinical overlap between HD and dentatorubral-pallidoluysian atrophy may warrant a recommendation of *ATN1* gene testing in those patients referred with a clinical diagnosis of HD who subsequently test negative for the expansion.[40]
- Comments on the significance of mutable normal alleles and HD alleles with reduced penetrance should not be overinterpreted in the absence of specific and well-documented clinical and/or family histories. Laboratories should be familiar with the published data regarding these CAG repeat length descriptors and should be able to articulate an interpretation that balances these data with the specific patient/pedigree information provided to the diagnostic laboratory at the time of testing.[11–14,34,40,41]

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DISCLOSURE

The authors direct laboratories that offer clinical molecular genetic testing for Huntington disease.

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