American College of Medical Genetics and Genomics (ACMG)

TECHNICAL STANDARDS FOR CLINICAL GENETICS LABORATORIES
(2021 Revision)

(For a General Overview of these Technical Standards, including Purpose and Disclaimer, see **Section A**)

G: CLINICAL MOLECULAR GENETICS

These Standards and Guidelines specifically refer to the use of molecular techniques to examine heritable changes in the human genome.

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G1 Specimens and Records

G1.1 In addition to numerical accession files and alphabetical patient listings, members of the same family should be linked together (via an assigned a unique code or other electronic method). Note: This requirement only applies when more than one member of a family is being tested. (See Section G16.1 for maintaining confidentiality in reporting of results.) Use of a family identifier for internal tracking purposes does not violate the confidentiality of individual family members. Disclosure of individual results to other family members is a violation of HIPAA.

G1.2 For required patient information, see Intake Information in Section C2.3.1

G1.3 For specimen labeling, see Specimen Containers in Section C2.1.

G1.4 A judgment about specimen quality should be made at intake. Any problems related to specimen collection (tubes, anticoagulants, transport solutions, labeling, etc.) or quality (lysis, clotting, etc.) must be noted. Appropriate individuals from the referring facility should be contacted regarding any unacceptable sample.

G1.5 A uniform variant nomenclature has been described (den Dunnen et al., 2016) and is periodically updated and available at http://varnomen.hgvs.org/. This nomenclature scheme should be followed, especially for naming of complex variants. For those variants with historic names already in widespread use, such as those for factor V Leiden and the commonly tested cystic fibrosis mutations, both the systematic and historic designations should be given to avoid confusion, both between laboratories and in communication with clinicians, until a time that the systematic nomenclature has been adopted exclusively. Suggestions for possible descriptions for complex variants are available (http://varnomen.hgvs.org/). Clinical reports should describe the level at which the mutation is being described e.g. "g" for genomic sequence, "c" for coding DNA sequence, "p" for protein, etc. (http://varnomen.hgvs.org/).

G2 General Quality Control

G2.1 See Section C6.

In addition, for molecular testing, quality of reagents can be evaluated prior to introduction into testing or at test outcome. However, any reagent which is used at points in a protocol that would lead to complete specimen loss or destruction (e.g., DNA preparation) must be tested prior to introduction. In-house testing can be deferred or delegated to manufacturers' quality control testing, where appropriate. Critical reagents are determined at the discretion of the laboratory director.

G3 Sample Preparation

G3.1 DNA preparation must be performed by validated protocols. Complete references should be included in standard operating procedure manuals.

G3.2 Southern analysis calls for DNA of higher quantity and quality than that required for PCR. The requirements for DNA preparations used for PCR analysis are less rigorous than for Southern analysis. However, appropriate controls must be used in the analysis to ensure that the DNA is a suitable template for DNA amplification.

G3.3 Excess sample material (isolated DNA) should be stored at a temperature no higher than 0-5° C. To ensure long-term stability, the DNA should be stored frozen.

G3.4 RNA preparation for preanalytic collection, stabilization, transport, and storage conditions are critical for obtaining accurate analytic test results. RNA is notorious for rapidly degrading if special precautions are not taken to preserve it. RNA collection, transport and storage conditions can be different by genes and assays, so it must be performed by validated protocols.

G4 Probe/Primer/Locus Documentation

The sequence of all loci used for analysis in the laboratory need to be well documented according to a current genome build (http://www.ncbi.nlm.nih.gov/ or http://www.ensembl.org) or by publication in the peer-reviewed scientific literature. Probe sequences should be subjected to a BLAST search to identify other homologous genomic sequences or common single nucleotide polymorphisms (SNP) sites which could interfere with hybridization of the probe/primer to the target sequence (http://www.ncbi.nlm.nih.gov/BLAST/). This documentation must be maintained in up-to-date laboratory records.

In addition, the following information should be included: genome location, linkage data, literature references, cloning vector, cloning site, size of insert, enzyme used for the detection of the RFLP, the sizes of the alleles and any constant bands, the allele frequencies in each racial or ethnic group (if known), new mutation rate (if known), probe preparation, hybridization conditions and wash conditions. For oligonucleotide probes or primers, documentation must include specific sequences. For primers, PCR conditions and the size of the expected amplicons should be included. There must be internal documentation that the probe/primer used is consistent with the above data (i.e., a photograph indicating that the size of the insert isolated from the vector is the correct size or that the conditions used by the laboratory produce the appropriate result).

G5 Assay Validation

G5.1 Each laboratory must determine the clinical and analytic validity of the technique chosen for analysis of each gene. Validation with well characterized samples is critical. Where available, performance characteristics should be compared with an existing "gold standard" assay. In the absence of "gold standards" for comparison of results of new assays, the splitting of samples with another laboratory with an established clinical assay may be considered. Documentation of validation results must be available for review. For further detail, see Test Validation in Section C10.

G5.2 The laboratory must document clinical validity through its own or other published studies.

G6 Southern Analysis

G6.1 Restriction Digestion and Electrophoresis

G6.1.2 Quality control of restriction digests must be done by one of the following methods:
a) Run a test gel prior to electrophoresis. If incomplete, redigest the specimen. Laboratory personnel must know how to recognize a partial digest and a degraded specimen.
b) Assess the completeness of digestion after running the analytical gel. Evaluate the analytical gel by visually comparing size markers or the patterns of all DNA samples on the gel, including controls, for consistency of satellite bands as well as high and low molecular weight bands.

G6.1.3 Each test must include human DNA control(s) with a documented genotype at the locus tested.

G6.1.4 All gels run for Southern analysis should include size markers to assist in the sizing of the alleles and therefore interpretation of the results.

G6.2 Membrane Preparation

G6.2.1 Prior to transfer, the gel run for Southern analysis must be photographed to provide a hard copy documentation of the gel.

G6.2.2 The method of transfer must be documented in the laboratory manual with appropriate references. Efficiency of transfer must be validated and documented either at time of transfer or at the end of the assay.

G6.3 Hybridization

G6.3.1 Hybridizations must be carried out by validated procedures and documented with appropriate references.

G6.3.2 Verification that proper hybridization occurred can be confirmed by evaluating the controls included in the assay.

G6.3.3 For new probes, a previously used Southern blot membrane, if available, containing DNA cut with the appropriate enzyme (or a control DNA of known genotype), can be used for further quality control of

hybridization.

G6.3.4 The laboratory must retain a representation of the primary data (gel, film, autoradiograph, etc.) demonstrating the reported hybridization pattern. Further suggestions for documentation can be found in the Clinical and Laboratory Standards Institute document MM1 A3, Molecular Methods for Clinical Genetics and Oncology Testing (2012).

G7 General Guidelines for PCR-Based Methodologies

More specific method-based guidelines can be found in sections covering specific methodologies and in the Clinical and Laboratory Standards Institute document MM1 A3, Molecular Methods for Clinical Genetics and Oncology Testing (2012).

G7.1 Avoiding False Positive Results Caused By PCR Contamination

In a clinical molecular diagnostic setting, preventing the contamination of specimens by other nucleic acid targets is a significant challenge. The major sources of contaminants are PCR products, plasmids, phage, or DNA. Specific work practices must be in place to prevent the contamination of specimens since it has the potential to alter a patient's results.

G7.1.1 ***Laboratory Design***

An ideal laboratory design would include physically distinct areas for reagent preparation, sample preparation, amplification and PCR product detection. At a minimum a pre-PCR and post-PCR area is required. The pre-PCR area requires that strict guidelines be in place to prevent contamination of the workspace. When possible, the workflow should be designed to be unidirectional from pre to post-PCR areas and to minimize traffic from post-PCR to pre-PCR areas. PCR workstations are useful for preventing contamination from other areas in the lab. The workstation area can be UV-treated and cleaned more easily than an open lab area.

G7.1.2 ***Laboratory Practices***

G7.1.2.1 ***Protective Clothing***

Protective clothing dedicated to the pre-PCR area (e.g., lab coats, gloves and booties), can be used to prevent the transfer of PCR products to the technologists' clothing, hands and feet.

G7.1.2.2 ***Pipettes***

Pipettes should be dedicated to either to pre or post-PCR areas. Positive displacement pipettes or hydrophobic filtered tips should be used to prevent contamination from aerosols.

G7.1.2.3 ***Reagents and Solutions***

Dedicated reagents, equipment and supplies for sample preparation and amplification should be present in the pre-PCR area.

To decrease the chance of contamination, reagents should be aliquoted into small volumes. This will minimize the manipulation of reagents by repeated opening of the tubes. In the event that an aliquot of reagent is contaminated, only that aliquot would need to be discarded, sparing the laboratory the expense of discarding the entire lot of reagent. The assembly of PCR reagents into master mixes also decreases the

chance of contamination.

G7.1.2.4 ***Controls***

A 'no DNA' negative control should be included in each assay to monitor for contamination. The solution replacing the DNA in the PCR reaction should be a reagent used in sample preparation such as the buffer used to rehydrate DNA. Another practice is to bring a blank sample through the DNA isolation procedure and use the resulting sample for the no template control. This allows all reagents used in DNA isolation and PCR to be assessed for contamination.

G7.1.2.5 ***Preventing Contamination of the pre-PCR area***

Preventive cleaning of the pre-PCR work area (e.g., bench tops, floors, racks, and pipettes) can be accomplished by periodically wiping nonmetallic surfaces with freshly prepared 10% bleach followed by removal of the bleach with ethanol. Bleach causes oxidative damage to nucleic acid and prevents it from being reamplified in subsequent PCR reactions. In addition, contaminating DNA can be inactivated with UV irradiation.

G7.2 Target Gene and Primer Documentation: See also Section G4.

The target gene, the RefSeq transcript of interest, the primer sequences and the rationale of the design should be well documented. The target gene should be investigated, as much as possible, using the scientific literature and available databases to assure appropriate primer design. Relevant information includes chromosomal position, pseudogenes, polymorphisms, mutation spectrum and frequencies of mutations in the disorder and population differences in sequence variations. Information should be reviewed on an ongoing basis.

G7.3 PCR Assay Validation (See Section G5.1 for a more extensive discussion regarding assay validation).

G7.3.1 ***Amplification***

G7.3.1.1 All reaction conditions (reagents and thermocycling parameters) must be established for each test system. Reaction conditions must provide the desired degree of PCR product specificity that assures accurate test results. A thorough exploration of reaction conditions helps to identify critical parameters in the assay. These critical parameters should be well documented. PCR thermal cyclers with temperature gradient capability are particularly useful for understanding how the PCR is affected by temperature. Optimization of PCR reactions is especially important in allele-specific techniques.

G7.3.1.2 When amplification involves a sequence of *variable* length, the impact of differential amplification should be evaluated. Whenever possible, the size limit of detection should be determined. This evaluation is especially important when using PCR to amplify the polymorphic alleles associated with nucleotide repeat diseases (e.g., fragile X syndrome, Huntington disease, myotonic dystrophy). Differential amplification should be avoided in quantitative techniques.

G7.3.1.3 Amplicons developed for use in multiplex PCR reactions must be thoroughly assessed for compatibility prior to use in clinical testing. Optimization, as discussed in Section G7.3.1.1, should demonstrate that all amplicons have suitable specificity and are not subject to allele drop out. For further detail, refer to the Clinical and Laboratory Standards Institute document MM17 A, Verification and Validation of Multiplex Nucleic Acid Assays (2008).

G7.3.2 ***PCR Product Detection and Analysis***

G7.3.2.1 A variety of detection systems are employed in diagnostic testing protocols. These include gel and capillary electrophoresis, membrane hybridization, microarrays, particle-based detection (e.g., beads or microspheres), FRET, OLA and real-time amplification, some of which are described in this document. These and other methods are described in detail in the Clinical and Laboratory Standards Institute document MM17 A, Verification and Validation of Multiplex Nucleic Acid Assays (2008). These systems should be validated and well documented. The laboratory must demonstrate that a level of specificity characteristic of the selected detection system has been attained internally and that the level of specificity is adequate for detecting the expected products.

G7.4 Controls and Standards for PCR-based assays

Controls must be included to provide evidence of appropriate amplification and to ensure correct interpretation of results. A 'no DNA' negative control, containing all reaction components except the DNA, must be included in all PCR-based assays. In addition, when PCR is used to detect the presence of a single specific sequence variant, a normal control (negative for the mutation being assayed) and positive control (heterozygous or homozygous for the mutation being assayed) must be included in each assay. For multiplex assays that detect more than 1 mutation, it may be not be practical to run a positive control for each mutation. In this case, it is acceptable to rotate controls. Size standards covering the range of expected results should be included in each assay.

G8 Detection of Single Nucleotide Variants, Small Insertions/Deletions, and Trinucleotide Repeats

G8.1 Forward Allele-Specific Oligonucleotide Hybridization (ASO)

Overview: The ASO method is based upon hybridization of a labeled oligonucleotide probe containing either the reference sequence or known variant sequence to the target.

Interpretation of Results: Comparison of the autoradiograph of the no variant control (normal) filter and the variant filter based upon position is necessary for interpretation of test results. In general, a positive result at a given position only on the no variant filter is interpreted as normal, a positive only on the variant filter is interpreted as homozygous for the variant, and a positive result on both filters is interpreted as heterozygous for the variant. For some tests, a number of filters are necessary to obtain results on all desired variants. Thus, it is important that results from all filters be read prior to interpretation, particularly when two different variants are detected in the same patient.

G8.2 Reverse Dot Blot Hybridization (RDB)

Overview: An alternative approach to ASO is reverse dot-blot (RDB) hybridization. In this method, the roles of the oligonucleotide probe and the target amplified DNA are reversed. Probe pairs, complementary to variant and reference DNA sequences, are bound to nylon membranes in the form of dots or slots. DNA that has been amplified in multiplex reaction(s) and labeled using end-labeled primers or internal incorporation of biotinylated dUTP, is hybridized to the membrane. Although probe design and production of the spotted membranes may be complex, variant detection using this method is convenient, rapid, and robust, and requires no specialized interpretation skills. However, this approach is relatively inflexible and not easily expanded to include additional variants.

Interpretation of Results: The genotype of the patient is easily read from the array of hybridization

signal(s) on each strip. Since the hybridization signal fades over time, the strips should be photocopied, photographed, digitized, or scanned in order to keep a permanent result record for each patient.

G8.3 Amplification Refractory Mutation System (ARMS)

Overview: ARMS is based on the observation that oligonucleotide primers that are complementary to a given DNA sequence except for a mismatch (typically at the 3' OH residue) will not, under appropriate conditions, function successfully as primers in a PCR reaction. For genotyping, paired PCRs are performed for each variant tested. One primer (the common primer) is used in both reactions, while the other is specific for either the variant or reference sequence. In principle, ARMS tests can be developed for any single nucleotide variant or small deletion/insertion.

Interpretation of Results: Individual test results are interpreted by review of the banding pattern in comparison with a molecular weight standard. The disadvantage of assays without paired wild-type reactions is that they do not discriminate between the heterozygous and homozygous states. Therefore, additional testing by another method must be performed to accurately interpret the results. Advantages of the ARMS method are that it is rapid (results can be obtained in one working day), reliable, and does not require expensive instrumentation.

G8.4 Oligonucleotide Ligation Assay (OLA)

Overview: The oligonucleotide ligation assay (OLA) is an approach to detect single nucleotide variants, small deletions, and small insertions. This method consists of PCR amplification of the target sequence followed by hybridization and ligation. Hybridization involves three separate probes, one specific for the normal allele, a competing probe specific for the variant allele, and a common probe that binds to both alleles. The 5’ probe is an allele-specific oligonucleotide (ASO) designed with either the normal or the variant nucleotide(s) at the ultimate 3' end. The 3' probe is a ligation-specific oligonucleotide (LSO) which binds immediately adjacent to the site to be interrogated. This common probe is phosphorylated at the 5' end to enable the ligation reaction. A thermostable DNA ligase is used to ligate either the normal or variant ASO to the LSO. Ligation only occurs in the presence of a perfect match between the ASO, LSO, and amplicon.

One method of allele detection involves the addition of a mobility modifying tail at the 5' end of each ASO, with the tail length differing between the variant and normal alleles. This allows for electrophoretic size separation and, therefore, differentiation between the normal and variant alleles. In this case, the LSO probe contains a fluorescent dye marker at the 3' end to allow detection upon separation. A second method of allele detection involves labeling the 5’ end of the normal and mutant ASO with two different fluorescent dye markers. In this case, the OLA products are the same size but are differentiated by the fluorescence signal detected.

Separation of the OLA products and allele detection requires the use of an automated sequencer capable of multi-fluorescence detection and may be performed in a gel or capillary format. The normal and variant peaks are identified based upon their product size and/or fluorescent tag. A properly designed OLA gives only the appropriate normal or variant product(s).

Interpretation of Results: The data can be analyzed using commercially available software that has been configured with protocol specific parameters, which support the generation of results. The peak heights for heterozygous loci should be half the intensity of the homozygous (normal or variant) peaks. Since

many variants can be analyzed simultaneously in one reaction tube, it is critical that the position of migration for each allele is appropriately confirmed to ensure accurate interpretation of patient results. It is also important that the laboratory sets thresholds for peak height to avoid pitfalls of misinterpretation due to background noise. It is recommended that the laboratory confirm that the multiplex reaction, which includes all alleles to be analyzed, both normal and variant, is robust and reproducible. Automated peak assignment is an attractive feature of some commercially available software and is desirable for quality assurance issues. Visual inspection of the data, however, is recommended.

G8.5 Fluorescence Resonance Energy Transfer

Overview: A fluorescence resonance energy transfer (FRET) assay involves two concurrent reactions in a single well. The primary reaction utilizes two different oligonucleotide probes, one specific for the normal sequence and the other specific for the variant sequence. Both probes hybridize to the target genomic DNA, forming an overlapping structure. This structure is recognized by a proprietary enzyme, resulting in the release of a DNA fragment, which forms the substrate for the secondary reaction. The secondary reaction involves the binding of the released DNA fragment to a FRET cassette containing a fluorescent reporter and quencher molecule. The overlapping structure created by the binding of the released DNA fragment to the cassette is recognized by the same enzyme as the primary reaction. The second structure is cleaved, separating the fluorophore and quencher, generating a detectable fluorescence signal. Mismatch between the variant probe and wildtype target DNA or wildtype probe and variant target DNA in the primary reaction prevents the formation of the overlapping structure and the generation of the subsequent fluorescent signal. By utilizing two different allele-specific (normal and variant) probes in the primary reaction, with each binding to a different FRET cassette with a unique spectral fluorophore, two sequence variants (normal and mutant) at a single site can be detected in the same well.

Interpretation of Results*:* The genotype of the sample is determined using software-generated calculations. The ratio of each fluorescent signal compared to the negative (no DNA) control determines the net signal for each probe. Based on the ratio of the net signals for each sample (wildtype:variant), the genotype is determined to be homozygous wildtype, heterozygous, or homozygous variant for each analyte. Samples that do not fall into the predetermined ranges for each genotype are flagged as equivocal and must be repeated. Samples that generate low counts are flagged as 'low signal' and must be repeated. Results for each sample are reported on an easy to read summary page. Results for each mutation analyzed are available in greater detail in a separate report.

G8.6 Liquid Bead Array

Overview: Liquid bead arrays provide simple and high-throughput analysis of DNA variants with discrete detection of wildtype and variant alleles in a complex genetic assay. Commercially available bead-array platforms are available for the detection of variants associated with many diseases. Bead-array platforms use either universal tags or allele specific capture probes that are covalently immobilized on spectrally distinct microspheres. Because microsphere sets can be distinguished by their spectral addresses, they can be combined, allowing as many as 100 analytes to be measured simultaneously in a single-reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the molecular interaction that has occurred at the microsphere surface. The microspheres, or beads, are dyed internally with one or more fluorophores, the ratio of which can be combined to make multiple bead sets. Capture probes are covalently attached to beads via a terminal amine modification. Bead arrays offer significant advantages over other array technologies in that hybridization occurs rapidly in a single tube, the testing volume scales to a microtiter plate, and unlike glass or membrane microarrays, bead solutions can be quality tested as individual

components.

Interpretation of results: Output files generated during detection are typically processed automatically and made available in a report format through customized software. The software should allow for controlled access to data, patient reports, comments and sample history. Electronic data output is archived into a database format for data integrity, quality control tracking, and result trending and incorporates batch processing of results, highlighting samples with variants and genotype calling. One advantage of customized software is data masking, or the ability of the user to display the genotype for variants determined to be appropriate, such as only those mutations associated with the diseases for which testing has been requested by the ordering clinician.

G8.7 End-point and Real-time PCR Analysis

These specially designed primer systems (such as TaqMan®-based and beacon-based systems) are used in end-point or real-time analysis systems to amplify and detect the mutant and normal alleles using sequence-specific hybridization-based assays. Each laboratory is responsible for establishing the characteristics of the specially designed primers in the detection system used in that laboratory. Results for controls and detection cut-off limits (95% confidence) must be closely monitored to identify inadequate specimens or reaction conditions.

G8.8 Probe-Based Melting Curve Analysis

Overview: There are several real-time PCR instruments. By coupling PCR with fluorescent hybridization probe analysis, these instruments can be used to detect single nucleotide variants. In the most common format, the PCR reaction includes locus-specific primers in addition to a pair of fluorescently labeled oligonucleotide probes (FRET probes). One of the probes is labeled at the 3' end with fluorescein (donor dye) and the second probe is labeled at the 5' end with LC Red 640 or LC 705 (acceptor dye). The 3' end of each probe is blocked with either a dye or a phosphate group to prevent extension during PCR. The position of the probes is selected so they hybridize to the target sequence adjacent to one another, with one of the probes positioned on the variant site. When the probes are in close proximity, the energy emitted by the excitation of fluorescein is transferred to the acceptor dye, which then emits fluorescence at a longer wavelength.

The stability of each probe/target complex as indicated by the melting temperature (Tm), depends on the length, G:C content and sequence order. When a nucleotide mismatch is present, the thermal stability is altered. The change in stability depends on the nucleotides involved in the mismatch, the mismatch position and the sequence context. A melting curve of the hybridization probe fluorescence can be used to detect changes in thermal stability and therefore discriminate single nucleotide variants. During melting curve analysis, the temperature is slowly increased while the fluorescence is monitored. As the probes begin to melt from the target, the fluorescence decreases, since the probes are no longer in close proximity. If a variant is also present, the mismatch with the probe causes the hybrid to melt at a lower temperature. The software plots the negative derivative of the fluorescence with respect to temperature. The generated peaks occur at Tms specific for the wild-type and variant alleles. If additional sequence variation is present in the target, the melting profile may be altered.

For genotyping samples, only one reaction and one set of probes are necessary. Design of PCR primers and hybridization probes follows standard methods. The assay has a large dynamic range, enabling DNA of a wide range of concentrations to be used. A number of assays using this technology have been

published. The assay format can be adapted easily to mutation analysis in a number of systems.

Interpretation of Results: Sample genotype is determined by examining the melting curve for the presence or absence of peaks whose Tm is specific for a wildtype or variant allele. The laboratory should establish acceptable Tm ranges for the wildtype and variant alleles, as the Tm values have inter- and intra-run variability. In addition, it is useful to monitor and establish a range for the DTm (Tm (wild type) – Tm (mutant)). The DTm is less variable than the Tm values and is a more useful value to help identify additional sequence variations. Fluorescent melting curve analysis allows the detection of additional sequence variants within the target sequence. These additional variants are identified through altered melting curve profiles that have peaks whose Tm does not match the wildtype or variant allele. The peak shifts may be subtle (<1°C). Sequence variants are most easily identified by a DTm value that is outside the range for normal and variant alleles. It is recommended that these sequence variants be confirmed by DNA sequencing.

G8.9 High Resolution Melt Curve Analysis (without the use of probes)

Overview: Denaturation of two strands of DNA with heat (melting) is a fundamental property of DNA. High resolution melting analysis (HRMA) can genotype specific variants and also scan for unknown variants. Unknown variants are detected by comparing the positions and shapes of melting curves that are affected by sequence changes. Heterozygous (and sometimes homozygous) variants can be identified after PCR, including insertions, deletions and single base changes. Mutation scanning is most useful when many exons need to be scanned and the benign polymorphism frequency is low. Advantages are quick turn-around time, reduced cost, closed system analysis, and a simpler process when compared to other scanning approaches and sequencing. A disadvantage of scanning is that any sequence differences are not specifically identified and would likely need to be confirmed through sequencing.

The accuracy of scanning is critically dependent on instrumentation with highly controlled temperature transitions and data acquisition. New DNA binding dyes that saturate available PCR products, without inhibiting PCR, while exhibiting minimal redistribution during melting, increases mutation detection. Amplicon length also influences sensitivity with improved detection below 400 bp. Single base insertions and deletions may be somewhat more difficult to detect than substitutions. Other factors such as the type of base substitution or the variant position within the PCR product appear not to affect sensitivity. The effect of amplicon GC content is difficult to dissect from its effect on PCR. The number of melting domains for optimal detection remains controversial. Although some have argued in favor of a single melting domain, others report no decrease in sensitivity with two or more domains. With high resolution instruments, improved DNA binding dyes, and appropriate software, the sensitivity of heterozygote scanning approaches 100%.

Interpretation: Amplicons with variant melting curves should be further studied. Common variants can be genotyped by a variety of similar melting methods. Simultaneous mutation scanning of the entire amplicon and genotyping of common variants with one or more unlabeled probes and can be performed at the same time in the same tube, vastly decreasing the need for re-sequencing in genetic analysis. Amplicons with abnormal melting curves in which a common variant is not confirmed by a genotyping assay are sequenced to identify the mutation.

G8.10 Single Nucleotide Primer Extension

Overview: A multiplex single nucleotide extension is completed by hybridizing region-specific

oligonucleotide primers to the template. These SNE primers hybridize 1 base pair above the variant of interest. Primer length varies by a few nucleotides in order to distinguish variants by fragment length. Only fluorescently-labeled dideoxynucleotides are included in the reaction. The complementary dideoxynucleotides are incorporated at the mutation site by the DNA polymerase. Because the 3'OH is removed from the nucleotide, reactions are terminated after a single nucleotide is added. Excess nucleotides are destroyed enzymatically with shrimp alkaline phosphatase. Products are resolved by capillary electrophoresis. Genotypes are distinguished by fragment length (determined by the primer) and fluorescent label (determined by the ddNTP incorporated).

Interpretation of Results: The data can be analyzed using commercially available software that has been configured with protocol specific parameters, which support the generation of results. Since many variants can be analyzed simultaneously in one reaction tube, it is critical that the position of migration for each normal and variant nucleotide is well defined to ensure accurate interpretation of patient results. The peaks outside of the defined areas (bins) must be interpreted with caution and should be confirmed with a different method. It is also important that the laboratory set thresholds for peak heights and background noise to avoid pitfalls of misinterpretation. It is recommended that the laboratory confirm that the multiplex reaction, which includes all alleles to be analyzed, both normal and variant, is robust and reproducible.

G.8.11 Triplet Repeat Primed PCR
Triplet repeat primed PCR (TP PCR) is used to detect the expansion of trinucleotide repeats. The method uses a locus-specific primer flanking the repeat together with a paired primer amplifying from multiple priming sites within the repeat. Specificity is dictated by the fluorescently labeled, locus-specific primer. TP PCR gives a characteristic ladder on the fluorescence trace enabling the rapid identification of large pathogenic repeats that cannot typically be amplified using flanking primers.

G9 Microsatellite Based Analysis

DNA microsatellite markers (short tandem repeats or STRs) have general utility in a variety of molecular genetic analyses (e.g., genotyping, linkage analysis, parent of origin/uniparental disomy studies, characterization of chromosome rearrangements, microsatellite instability testing in tumors, parentage testing, twin zygosity analysis, bone marrow transplant engraftment monitoring, detection of maternal cell contamination in prenatal samples, etc.). STRs are widely dispersed throughout the genome, are highly polymorphic in the population, and can be analyzed rapidly and inexpensively by multiplex PCR techniques. Also, because they are relatively short, they are amenable for testing in specimens that are scant or partially degraded. For that reason, they are now used universally in forensic DNA identity testing.

G9.1 Attention to safeguards for PCR-based assays as described in Section G7 is required. Particular attention must be given to Section G7.3.1.2 (amplification of variable length sequences) to ensure amplification of the range of sizes possible at the locus.

G9.2 Microsatellite analysis performed on automated capillary electrophoresis (automated sequencing) instruments has become the most popular approach because of its speed and increased sizing accuracy and requires special considerations.

G9.2.1 Because each marker system and instrument may have its own unique variabilities, it is important to establish accurate sizing parameters using appropriate internal or external markers. These can be in the form of reference size standards or human allelic controls (forming an allelic ladder) that have been well characterized. Once the external controls are established, the comparisons can be integrated into the sizing software of modern automated sequencer instruments for seamless readouts. These approaches are the same as would be used for accurate sizing of the trinucleotide repeat expansion in Huntington disease, as described in that section of the Standards and Guidelines (Potter, Spector, & Prior, 2004).

G9.2.2 All markers must be tested to determine optimal PCR sample amounts to be loaded (i.e., amplicon intensity must be within the sensitivity parameters of the detection system).

G9.3 While capillary electrophoresis is the most widely utilized methodology for STR genotyping, other manual approaches such as polyacrylamide sequencing gels and radioisotope detection (^{32}Phosphorous-labeled deoxynucleotides) may be employed. For radioisotope detections, multiple X-ray film exposures are recommended to obtain all possible autoradiographic signals.

G9.3.1 For manual approaches using ^{35}Sulfur or ^{33}Phosphorous-labeled deoxynucleotides, gel drying may be necessary before autoradiography and should be standardized to avoid underdrying or overdrying, both of which may affect interpretation, e.g., through blurry bands or by gel cracking. Individual autoradiographic exposures are necessary.

G9.4 For manual approaches using blotting of polyacrylamide sequencing gels followed by chemiluminescent detection, blotting should be standardized to establish a minimal blotting time as well as times for optimal autoradiographic exposure.

G9.5 Microsatellite data interpretation is similar for each use. However, care should be taken in interpretation due to the appearance of shadow-bands, stutter peaks (a peak generated by strand slippage during DNA synthesis that is one repeat less than the true allele), and variability in gel or capillary migration. Stutter artifact tends to be more noticeable with the smaller STR repeat types (mono- and dinucleotide repeats) than with larger repeat units (tetranucleotide repeats). In general, the highest peak or most intense band in the group or smear will represent the actual genotype; compound heterozygotes with adjacent repeat lengths may be difficult to discern. Conversely, the system must be tested for maximum repeat length detection (within the expected range) so that an apparent homozygote is not falsely genotyped due to PCR failure of a second, much longer allele.

G9.6 For analysis of mixed samples, as in mosaicism, maternal cell contamination, bone marrow transplant engraftment, and tumor analysis, artificial mixing experiments should be conducted to determine the lower limit of sensitivity of detection of a minority genotype, and this parameter should be included in the test reports. A control specimen mixed at this limit of detection should be run with each assay.

G9.7 For identity testing for legal purposes, the proper identification of the individuals being tested must be recorded, including photographs as appropriate, and proper chain-of-custody procedures must be in place.

G9.8 For genotyping tests performed on blood or bone marrow specimens, the laboratory must obtain a history of blood transfusion or bone marrow transplantation at the time of specimen collection.

G9.9 For identity and parentage testing, matching probabilities must be calculated using published tandem repeat allele frequencies for the population in question.

G10 DNA Sequencing Analysis

G10.1 Sanger Sequencing

Overview: Sanger sequencing has long been considered the "gold standard" for the analytic validation of new DNA-based mutation testing. While a large percentage of sequencing-based testing is performed using next generation sequencing (NGS), Sanger sequencing is still widely utilized for single gene testing, for coverage of regions not amenable to NGS, and for confirmation of variants detected by NGS.

For a more thorough discussion of sequencing, please review the Clinical and Laboratory Standards Institute document MM9 A, Nucleic Acid Sequencing Methods in Diagnostic Medicine (2014).

G10.1.1 Although the sequence assay shares elements in common with all other DNA diagnostic assays, there are unique concerns regarding sequencing which should be considered. Issues that arise in DNA sequencing result from the large number of analytical points measured in each particular assay (i.e., the number of bases analyzed) and the relatively small signal strengths that are obtained from any base at any position. The technology for the generation of the sequence information is also generally complicated. Therefore, the sequence information must be verified and controlled at multiple points in the generation and interpretation of the sequencing data.

G10.1.2 False positive results are more likely to occur during DNA sequencing than false negative results. This is because peak mobility shifts, increased background, and peak fronting (a smaller peak that occurs in front of a major peak of the same color) can produce a sequence that differs from the reference sequence at one or more bases. The potential for missing a heterozygous base substitution is also a concern. To increase the sensitivity of heterozygote detection with fluorescent sequencing, the sequencing chemistry and polymerase used should be optimized to produce uniform peak intensities. Sequencing both strands of the DNA is recommended to optimize the sensitivity and specificity of an assay.

G10.1.3 The most widely used method is Sanger dideoxy chain termination, which can be applied in several forms. Fluorescent sequencing reactions can be performed using dye primers or dye-labeled primers or dye terminator chemistries and one of several polymerases. Data collection uses an imaging system and appropriate software.

Automated fluorescent sequencing can be performed using an automated sequencing instrument equipped with electrophoresis apparatus and data collection software.

Capillary gel electrophoresis for sequencing is the most common detection system and has advantages over the older gel-based systems.

G10.1.4. All previous guidelines for sample collection and DNA preparation apply. The use of a commercially available DNA preparation kit is recommended to provide consistency in sample concentrations and DNA quality. However, validated laboratory-developed methods are also acceptable.

G10.1.5. The upper limit of accurately readable DNA sequence based on chemistry and instrument capability should be determined by the laboratory. This must be used to establish the maximum length of DNA that can be sequenced in a single run.

G10.1.6 The quantity of the DNA must be sufficient to generate adequate PCR product. This can be determined by meeting an expectation of PCR efficiency (e.g., an agarose gel separation of an aliquot of the PCR can be compared to a standard).

G10.1.7. The PCR product should be analyzed by gel and purified prior to the sequence reaction to ensure the highest quality of results.

G10.1.8. The overall quality of the sequence reactions must be monitored. The concern is that poor quality electropherograms containing artifacts such as "stops," compressions, or "Ns" will be difficult to analyze and will result in incorrect interpretation of the sequencing data. Every effort should be made to minimize these artifacts. Routine sequence analysis of the opposite strand of DNA can minimize the chance of incorrect interpretation of sequencing data due to the presence of artifacts. The use of a different sequencing chemistry or polymerase may resolve specific regions, since artifacts may not occur in identical spots under alternate conditions. A comparison of each test with a known standard (e.g., GenBank) is required, including judgment of peak height. However, caution should be exercised, since not all sequences in GenBank are correct. Objective measurement of the base calls by statistically generated quality factors, known as a quality score, should be reviewed and evaluated by the laboratory to assess the sequence quality.

G10.1.9 Manual re-reading of areas where the software has had difficulty should be performed with caution. The chromatograms of both the forward and reverse strands should be evaluated and the consensus compared to the standard sequence.

G10.1.10 Sequence analysis software is needed to compare data of the reference sequence and patient sample in both forward and reverse directions.

G10.1.11 Verification of sequence data using data obtained from sequencing the opposite strand and/or a second sequencing reaction is required. Some mutations may be missed if sequencing is performed in only one direction. For direct sequencing, a second PCR amplicon should be used for repeat sequence analysis.

G10.1.12 Differences from the reference sequence that are not known benign polymorphic variants should be documented and reported according to the ACMG Recommendations for Standards for Interpretation of Sequence Variations (Richards et al., 2015).

G10.1.13 The report should note the exact base change and location by nucleotide position as referenced in GenBank (and the corresponding position change in the protein using standard nomenclature

(http://varnomen.hgvs.org/).

G10.1.14 Reports in which no mutations are detected by sequence analysis should indicate that the sensitivity of the test is <100%. If sequencing was confined to the coding region of the gene, the possibility of mutations in the promoter or intragenic regions not covered by the test should be clearly stated. Sequencing will not detect large gene deletions or duplications. In addition, a mutation in a different gene that contributes to the disease, as well as misdiagnosis of the proband, constitute other explanations for a negative result. Disclaimers should also acknowledge the possibility of a polymorphism in a primer that inhibits amplification of one allele, causing a false negative result.

G10.1.15 ***Validation***
Each laboratory must validate the technique for each gene to be analyzed. Validation with known mutations as well as normal samples is required. Results of validation studies for each gene analyzed must be available for review. For details regarding Ultra-Rare Disorders, see Section G19.1.

G10.2 Next Generation Sequencing
See ACMG clinical laboratory standards for next-generation sequencing (Rehder et al., 2021) for details.

G10.3 Pyrosequencing
Pyrosequencing is a DNA sequencing technique that can be used for quantitative purposes. It is based on the detection of released pyrophosphate (PPi) during DNA synthesis. Inorganic PPi is released as a result of nucleotide incorporation by polymerase. The released PPi is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase and generate light. Because the added nucleotide is known, the sequence of the template can be determined.

Homopolymeric regions and complex indels mutation regions should be carefully designed. Positive control (known mosaic ratio), mutation negative control and no template control should be included for each run. Mutation detection limit should be specified based on validation. It should be noted that the size of region for pyrosequencing is much smaller than regular sequencing and confirmatory testing in rare instances can be required for difficult regions.

G11 Detecting large insertions and deletions

G11.1 Dosage analysis
Overview: There are several screening methods for the detection of point mutations, such as single-stranded conformation polymorphism, heteroduplex analysis, denaturing gradient gel electrophoresis, and chemical cleavage. These are powerful tools for the identification of small sequence changes, but fail to detect heterozygous deletions or duplications of exons, genes or chromosomes. There are many genetic disorders where the primary defect is either due to allelic deletions (Duchenne muscular dystrophy, spinal muscular atrophy, alpha thalassemia, growth hormone deficiency, familial hypercholesterolemia, etc.) or duplications (Charcot-Marie-Tooth, etc.). Strictly speaking, Klinefelter Syndrome and Down Syndrome are not examples of allelic duplication but chromosomal non-disjunction due to errors in meiosis. Furthermore, for the determination of the carrier state, for disorders such as Duchenne muscular dystrophy and spinal muscular atrophy, the accurate determination of heterozygous deletions is essential.

G11.1.2 ***Southern Blot Dosage***

In order to perform gene dosage from Southern blots, one determines whether the restriction fragment of interest exhibits no reduction (normal two copies) or 50% reduction (heterozygous state) in the hybridization intensity of the restriction fragments bands of interest. In the case of a genomic duplication the restriction bands should double in intensity. To further increase the accuracy of the dosage analysis, the autoradiographic bands should be scanned with a densitometer. Although dosage from Southern blots can provide an assessment of gene copy number, there are technical limitations. Dosage analysis of Southern blots requires optimal conditions; very good quality blots are necessary, with even transfer and hybridization, and low background. Rather than directly comparing single bands, band ratios are calculated as a means of decreasing the error caused by differences in the amount of DNA in each lane. The normal control ratio is established by comparing a potential band lacking in the patient against a band present in the patient (which serves as an internal control) in an unaffected control. When this ratio in the patient is approximately half the control ratio, this indicates that the patient has a single copy of the restriction fragment. Depending on the specific restriction fragments of interest and the DNA probe, one may be extremely limited as to what bands are used in the dosage determinations. Bands greater than 10 kb and less than 0.5 kb typically result in weaker intensities and are not always adequate for scanning purposes. Lastly, the difference between one or two copies is relatively straightforward to detect but differences between two and three copies, or sometimes three or four copies, in the case of a duplication or co-migrating restriction fragments can be very difficult.

G11.2 MLPA

Multiplex ligation-dependent probe amplification (MLPA) is now a standard technology in the molecular genetics laboratory to detect copy number changes in targeted genes. MLPA is based on size-separation of the amplification products, after probe hybridization, ligation and amplification.

Hybridization: Two sequence-specific oligonucleotide probes (one short and one long) are hybridized to genomic DNA at the regions of interest.
The two oligonucleotide probes hybridize adjacently in a head to tail fashion at each hybridization site. Each probe is tagged with common sequence tails complementary to a universal forward and reverse primer.

After overnight hybridization, a ligation reaction is performed to join the short and long oligonucleotide probes when both are hybridized to the sequence specific genomic template. Unhybridized oligonucleotide probes will not be ligated.

Amplification: After hybridization, the ligated probes are amplified simultaneously using the single universal primer set complementary to the probes' sequence tags. Since the probes are designed with differing lengths, the resulting amplified products are size-separated by capillary electrophoresis. Up to 40 different probes and internal control probes can be combined in one reaction. PCR products are analyzed quantitatively for probe ligation, comparing the targeted hybridization regions to control regions. If the hybridization site is deleted, no or half amount of hybridization takes place at that allele. If the hybridization site is duplicated, one and a half to two times as many oligonucleotide probes will hybridize to the genomic DNA.

Visualization: The peak heights (according to MRC-Holland) are quantified and examined for normal (1X), deleted (0X or 0.5X) or duplicated (1.5X or 2X) dosage. Deletions of genomic DNA within the probe recognition sequences are apparent by a 35-50% reduction in relative peak area of the amplified product.

Duplications are apparent by an increase in relative peak area.

Controls: Internal controls covering different chromosome regions are included with the reagents. Known deletion and duplication controls should be included in each run, if possible, to verify assay performance. External normal copy controls may be used with each run to perform statistical analyses.

Analysis: Raw data from the sequencer can be examined for the presence or absence of designated probe using GeneMapper (Applied Biosystems). GeneMapper does not perform copy number analysis. There are two packages available to perform copy number analysis; Coffalyser (MRC Holland) and GeneMarker (Softgenetics). Coffalyser is an excel macro to assist copy number calculation using Excel. GeneMarker is an automated program created specifically to perform copy number calculation on raw fragment data. This analysis algorithm normalizes peak height (fluorescence intensity) using exponential fit with either the chromosome control probes (built into the probe mix) or the entire population (all fragments from each sample in each run). After fitting the normalized data to a regression model, data can be presented in either an MLPA ratio or a MLPA T-test distribution.

Limitations: One limitation of this assay is the interference of mutations/polymorphisms affecting the probe ligation which could result in potential false-positive results. Therefore, a deletion of a single exon requires confirmation by sequence analysis of the region to rule out interference by a nucleotide variant, by family studies or by other independent method.

G11.3 Exon-targeted Array CGH

Array CGH (aCGH) has been widely used to analyze the copy number variations in clinical laboratories. When large insert clones such as bacterial artificial chromosomes (BACs) are used as the probe for aCGH, the maximum resolution can reach ~40-50kb (Snijders et al., 2001). The use of oligonucleotide probes (oligos) or sequence-defined PCR products for aCGH allows the resolution to be theoretically increased to a level solely based on the size of the genomic region covered by these probes (Lucito et al., 2000; Mantripragada, Buckley, Jarbo, Menzel, & Dumanski, 2003). The sufficient resolution and scalability of oligonucleotide probes allow the implement of exon-targeted aCGH which could address the copy number changes at a single exon level. Compared to other molecular methodologies that can be used to analyze copy number variations, exon-targeted aCGH can simultaneously interrogate multiple exons, multiple genes or genomic regions, in addition, by using tiling probes, the boundary of deletions/insertions can be delineated accurately.

Except for certain genes such as DMD in which deletions/duplications account for a majority of the mutational spectrum, it is recommended that the exon-targeted aCGH should not be used as the first-tier testing to investigate the genetic changes. It is prudent that this technology be used in combination with sequencing-based technologies for clinical diagnostic purposes.

Design of Exon Array CGH: while whole genome exon-targeted CGH array is available and has the potential to uncover a full spectrum of genetic variations when combined with whole exome sequencing, currently, custom-designed, gene specific exon-targeted aCGH is commonly used in clinical laboratories. Several aspects need to be considered when designing a custom exon-targeted aCGH:

1. The array should contain a set of normalization probes for which a normal ratio is established for each analysis. The normalization probes could be the negative spots that do not hybridize to human DNA and the positive saturated spots.
2. Only unique oligonucleotide sequence will perform well, therefore it is necessary to avoid possible cross-hybridization and repetitive sequence elements in the design. Exons with long stretch of repetitive sequences may not be amenable for this technology.
3. **Optimal length of oligonucleotide probes:** It is important to evaluate the minimal length of an array probe that gives maximal performance. The short 25mer oligonucleotides seem to produce highest noise level per probe and 70mer probes produce maximum signal-to-noise ratio (Lucito et al., 2000; Zhao et al., 2004). Generally, longer oligonucleotides yield better hybridization specificity; however, oligonucleotides longer than 85bp become increasingly impure (Selzer et al., 2005). Currently, the most common length of probes ranges from 50 to 75 nucleotides.
4. **Density of probes:** The density of probes on the array depends on the array format and the size of targeted regions. Different software are available for the automatic *in silico* design of probes and desired probe density.
5. It is recommended to have appropriate backbone coverage across the genome so that large copy number changes (clinically significant incidental findings) in the non-targeted region will not be missed. A further investigation using chromosome microarray is appropriate.
6. Due to the length of probes commonly used, it is not recommended to investigate the copy number changes for genes with pseudogenes using exon-targeted aCGH.

Analysis: The patient and control DNA samples are differentially labeled with Cy3 and Cy5 and hybridized to the array. Signal levels from patient and matched control DNA are measured after hybridization and plotted on the log2 scale by analysis software. Copy number neutral regions will appear on the "zero" axis. Regions with signals deviated from the "zero" axis are considered a gain or loss. The threshold for calling a signal deviation is based on the statistical calculation of the average noise level of an array and the filter set-up of analysis software. None of the current aCGH platforms can make a definite call for gain or loss based on the signal level change of a single oligonucleotide probe. Generally, validation data can define for decision of how many consecutive probes are necessary for reliable call; however, 5 or more consecutive probes are considered necessary for a reliable call. However, when exons consistently show a copy number gain or loss on repeated analysis, even the number of probes within that region are less than 5, a further investigation by alternative technologies is warranted.

It is possible that the targeted exonic regions are located within a large CNV region, when a gain or loss involving the entire gene or even larger regions are detected, it is advisable to check CNV databases before reporting a positive finding.

The sensitivity of detecting mosaicism using exon-targeted aCGH has not been documented; however,

data is available for chromosomal aCGH array.

Quality control: while different manufacturers have platform-specific cutoff values for the hybridization quality, there is no uniformed consensus regarding the cutoff for the signal/noise ratio. Each laboratory should develop their own standard for rejecting an assay based on validation studies.

Confirmation of results: while the hybridization artifacts involving 5 or more consecutive probes are unlikely for well-designed arrays, it is generally recommended, especially during assay validation, to confirm the array results by alternative technologies such as quantitative PCR or sequencing. Sequencing the deletion junction region not only confirms the deletion, but also can provide information regarding the genetic architecture of the junction region.

G12 Methylation-Specific PCR (MS PCR)

The methylation status of CpG dinucleotides within a particular DNA segment can be assessed by Methylation-Specific PCR (MS PCR). DNA is first treated with sodium bisulfite which converts unmethylated but not methylated cytosine residues to uracil residues (CG>UG), thereby changing the underlying sequence of the DNA. The treated DNA can then be amplified using primers specific to the methylated or unmethylated alleles.

Primer design: Primers used after bisulfite modification should be designed to be specific for regions originally containing cytosine residues to distinguish methylated from unmethylated DNA after bisulfite treatment. Because the unmethylated sequence specific primer will only contain three different nucleotides (A, T, G), longer primers (24-30 base pairs) are necessary increase the specificity. To prevent a false positive or false negative result, primers should also be designed to only amplify DNA sequence after a successful bisulfite reaction.

Controls: A methylated and unmethylated control sample should be included in each assay.

Detection methods: Post-PCR detection of the presence of unmethylated and methylated alleles can be achieved by several different methods including direct visualization by agarose gel, sequencing, melting curve analysis, fragment length analysis, and real time PCR utilizing specific fluorescence reporter probes etc.

G13 cDNA Synthesis

G13.1 Source of Samples

G13.1.1 The starting material for this assay is RNA obtained from any tissue by standard methodology (see Section G3 for DNA/RNA preparation). Total RNA is usually the material of choice. When working with RNA, care should be taken to avoid contamination of reagents, lab equipment and disposables with RNases. Methods for RNA isolation may use strong denaturants such as guanidinium hydrochloride or guanidinium thiocyanate to denature endogenous RNases. Gloved hands, new plasticware, barrier tips, and DEPC-treated glassware should be used to minimize contamination with RNases. Shipping and storage condition of samples can influence the stability of RNA. Bone marrow and blood should not be

frozen. They should be transported to the lab on ice when temperature is high. Solid tissues should be snap frozen and transported on dry ice. RNA stabilizers can be used prior to RNA isolation.

G13.1.2 The source tissue must be of sufficient quality to provide enough RNA for downstream testing. In addition, the tissue type must express the mRNA of interest in sufficient quantity for accurate and sensitive analysis. Isolated RNA can be used for qualitative or quantitative expression analysis.

G13.2 The usual safeguards against contamination by PCR products should be used (see Section G7.1).

G13.3 RT-PCR Amplification from RNA (Reverse-transcription PCR)

G13.3.1 When RNA is the starting material, cDNA is first synthesized from the RNA using oligo dT, random hexamer, or gene-specific primers.

G13.3.2 A second round of PCR using a nested primer pair is required to amplify target mRNA transcripts. Same general precaution of handing PCR reactions applies when using cDNA as the template. Tubes containing cDNA product should be opened one at a time to prevent potential tube-to-tube contamination. No-template control (NTC) needs to be included throughout the entire process including RNA isolation, cDNA synthesis and subsequent PCR reaction.

G13.3.3. When the assay is to measure the level of expression, it is recommended to test an internal control gene simultaneously. A report of relative expression level (target gene transcripts/internal control gene transcripts x 100%) is preferred. Sometimes, the expression level may necessary to be normalized to a "standard sample" to facilitate inter-laboratory result comparison. It is also recommended that each PCR reaction should be run in duplicate or triplicate using cDNA template generated from independent cDNA synthesis reactions.

G13.3.4 When a mutation is detected using cDNA as PCR and sequencing template, it is recommended to confirm the mutation using cDNA generated from different reactions to avoid artifactual mutations introduced during reverse transcription process.

G13.3.5 RT-PCR controls should include controls for positive, normal, amplifiability, and negative (no DNA) controls. A normal control for the specific region of the gene to be analyzed should be included in each assay.

G13.3.6 Amplification by RT-PCR followed by electrophoresis may reveal gross rearrangements such as gene deletions (complete or partial), insertions, splice mutations and possibly duplications.

G13.3.7 Differences between the two alleles in terms of transcription efficiency or RNA stability can influence results. A genomic DNA control segment with a previously identified heterozygous sequence in the gene must be PCR-amplified in parallel to confirm that both alleles have been amplified in each patient sample.

G13.3.8 The quality of RNA should be documented by either gel analysis or by amplification of a housekeeping gene to ensure that it is an appropriate starting template.

G14 Additional Methods

Many methods are available for detection of changes in the genome. Some of these methods have been covered in disease specific ACMG standards and guidelines or by other agencies.

G15 Interpretation of Data

G15.1 All results must be read by two individuals (identified in records) independently, one of whom must be the director, laboratory technical supervisor or other qualified individual.

G15.2 All file materials relating to individual and/or family studies should be cross-referenced for accessibility.

G15.3 All questionable or inconsistent data must be resolved by either repeating the assay or using an alternative method. The use of positive controls for specific mutations can be helpful in certain situations. The possibility of mistaken parentage (paternity or maternity), maternal cell contamination, sample mix-up, co-mingling of specimens, allele drop-out, and bioinformatics bugs should be considered when results are not consistent with the family history or phenotype.

G15.4 For PCR-based assays, care must be taken to assess the possibility of differential allele amplification.

G16 Records of Molecular Testing

G16.1 Scoring sheets must contain the following information (if applicable):

- Specimen numbers
- Locus names tested (probe name and locus identification)
- Test system used (Southern, PCR, etc.)
- Mutation detection system (RFLP, ASO, etc.)
- Enzymes used for RFLP analysis, lot numbers and expiration date
- Alleles detected
- Results
- Master mix lot numbers including all components (e.g., polymerase, deoxynucleotides, magnesium chloride and buffer)
- Pathogenic sequence changes, benign variants and variants of unknown clinical significance detected in sequencing assays
- Properly labeled photograph with molecular weight standards
- Lot number of standards
- Version and name of software being used for analysis (if applicable) including components of bioinformatics pipeline
- Area for technologist and laboratory director to initial after analysis

All results should be entered and recorded in a laboratory database

G16.2 All results must be recorded on written forms which are retained and kept in the patient file, the family file and/or with the photographs or autoradiographs or in an electronic database.

G17 Molecular Genetics Reports

G17.1 A report should be issued only to the ordering physician or send-out laboratory. In states where direct to consumer tests are prohibited, the laboratory should not give results directly to patients. There are additional state and federal laws which govern the release of reports and/or testing records directly to the patient/family which need to be followed. See Records in Section C5 for issues regarding record dissemination. In general, the report should include the following:

- Collection date
- Date (and time, if applicable) of receipt in the laboratory
- Specimen type
- Name of individual
- Gender (if applicable)
- Date of birth
- Ethnicity/race where appropriate
- Laboratory identification number
- Family/kindred number, if applicable
- Date of report
- Reason for testing
- Disease locus tested
- Test performed, methodology, genes or specific mutations tested
- Notation of any deviation from the laboratory's standard practice
- Limitations of the assay, including reportable range and gene/locus coverage
- The genotype and/or haplotype established for the individual
- A statement interpreting the data (interpretation should be understandable to a non-geneticist professional), including clinical implications which may include a specific diagnosis or disease risk, follow-up test recommendations, and genetic counseling indications
- Summary of evidence upon which the clinical interpretation is based, including references
- Recommendations
- Documentation if a preliminary report has been issued
- Signature of the laboratory director or technical supervisor or other authorized individual above his/her printed name.
- A means to contact the laboratory director or designee
- The following disclaimer for tests that are not approved by the FDA: This test was developed and its performance characteristics determined by the [Name of DNA Laboratory]. It has not been cleared or approved by the Food and Drug Administration. FDA approval is not required for clinical use of the test, and therefore validation was done as required under the requirements of the Clinical Laboratory Improvement Act of 1988.
- References

G17.2 Any report should ensure the confidentiality of the other family members whose studies were used to provide information to the proband. Except in the case of minors and their parents or legal guardians, a patient's test results or other medical information should not be disclosed to the patient's family members without appropriate written authorization from the patient.

G18 Prenatal Testing

G18.1 Samples

Many genetic analyses are amenable to prenatal diagnosis using both direct and cultured cells from amniotic fluid (AF) and chorionic villi (CVS). However, in some cases one of these particular specimen types may be more appropriate. For each prenatal genetic test, the laboratory should determine the appropriate prenatal specimen and specify the amount of material required for testing. The laboratory should provide these requirements and appropriate instructions to referring centers and professionals. It is important that fetal cells be maintained in culture and that backup flasks maintained until the molecular analysis is completed and reported. It is recommended that the mutation status of one or both parents, as appropriate, be tested prior to testing of fetal specimens, preferably within the same laboratory. To the extent possible, laboratories should have a follow-up program in place to monitor the accuracy of their prenatal testing.

G18.2 Sample Processing

As with other genetic tests, prenatal testing must be performed with the utmost level of caution to ensure accuracy of the predicted result. Laboratories should have procedures in place to assure accurate sample handling. In addition, there are specific time considerations associated with prenatal testing. Laboratories should make every effort to expedite results and have an accurate estimate of turnaround time for prenatal specimens available. If there is sufficient material and whenever possible, prenatal testing can be performed in duplicate using DNA extracted from two separate specimens.

G18.3 Maternal Cell Contamination

The contamination of both direct and cultured cells from AF and CVS with maternal cells is well documented and therefore represents a potential source of error in prenatal diagnosis. Prenatal samples should be examined in parallel with a maternal sample to rule out error due to maternal cell contamination (MCC). Laboratories should understand how their testing methods are affected by the presence and the amount of MCC. For example, prenatal detection of a deletion using PCR, as is the case in testing for DMD and SMA, is expected to be more sensitive to maternal contamination, since a normal maternal allele could mask the deletion. A prenatal test using an allele-specific PCR reaction to detect a paternal *RhD* gene in the fetus of a RhD-negative mother is much less sensitive to maternal contamination.

For example, Chamberlain, Gibbs, Ranier, Nguyen, and Caskey (1988) explored potential problems with maternal contamination in a multiplex PCR test for deletions in the dystrophin gene by mixing DNA from a partially deleted sample and a non-deleted sample. This study demonstrated that 3-5% contamination could be tolerated if the amplification cycles were limited to 25. In contrast, Hessner et al. (1997) used similar mixing experiments to determine the impact of maternal contamination on prenatal testing for paternally inherited alloalleles using allele-specific PCR. In this situation, where the fetus is being tested

for an allele that the mother does not have, the paternal alloallele could still be detected with more than 90% contamination. These two examples illustrate how the effects of MCC depend on the specific test and the method being used.

Laboratories should perform similar studies, when possible, and in the absence of this information should seek to confirm the test results from contaminated samples. The results may be confirmed from an alternate sample, if it is available. This may include a cultured sample prepared from original direct sample or an independent culture. If necessary, the obstetrician should be contacted about the possibility of an additional amniotic fluid sample.

The laboratory should have procedures in place to assess the presence and level of maternal cell contamination. These methods should detect, at a minimum, the level of contamination that would affect the test results. A combination of several polymorphic STR or VNTR loci is recommended for ruling out MCC. Batanian, Ledbetter, and Fenwick (1998) showed that two VNTR loci could be used to rule out MCC in 30/30 cases. However, some of these cases required a paternal sample to complete the testing for MCC. As a paternal sample may not be available, the laboratory should be able to complete the testing for MCC without the paternal sample. Therefore it is likely the laboratory will need at least 3 loci to resolve all cases. If a paternal sample is used, the laboratory should be aware that the MCC studies might identify mistaken paternity.

There are a number of marker systems suitable for MCC analysis. Many multiplex kits are commercially available that enable a number markers to be analyzed in one PCR reaction. These markers systems are also used to detect chimerism in hematopoetic stem cell transplant patients. A list of the marker systems being used in engraftment testing laboratories can be found in the Monitoring Engraftment Survey distributed by the CAP proficiency testing program. The validation of MCC assays should include sensitivity studies to determine if the appropriate levels of MCC can be detected.

G19 Testing for Ultra-Rare Disorders

G19.1. Definition

A rare disorder, as defined by the Orphan Drug Act of 1983, affects populations smaller than 200,000 individuals in the USA. An ultra-rare disorder, as defined by common usage, is a disease occurring in less than 2,000 individuals in the USA.

G19.2 Clinical Validation of Testing

For laboratories planning to offer testing for URD on a single gene basis, the issue of clinical validation is an important topic to address. All of the available literature should be interrogated to determine the sensitivity and specificity of the test to be offered. Care should be taken when considering monogenic disorders that may be caused by genetic heterogeneity, if there are phenocopies of the disorder that are not due to the gene being tested, and if information regarding the clinical presentation associated with a variant in the gene is minimal.

G19.3 Analytical Validation

In general, testing for URD requires variant analysis of specific genes. Testing is utilized to interrogate the entire gene, as, for most families in whom an URD is segregating, the causative variant(s) are private

to that family. It is, therefore, not possible to perform test validation for each variant that might occur within the specific gene.

In addition, given the paucity of cases/samples in the context of URD, it is problematic for a laboratory to follow best laboratory practices such as performing testing on DNA samples with known genotypes and then on a series of blinded samples before launching the clinical test. Particularly, if a laboratory is experienced in the methodology to be used for testing, testing for the URD may be offered clinically after the assay performance has been verified on a number of normal control samples.

For custom variant analysis, in which a sequencing-based test is set up to confirm a variant identified in a research laboratory, the test can be considered to be "self-validating." A sample known to contain the targeted variant can act as its own positive control and allows for the analytical validation of the test for that particular variant. A positive control therefore always exists for the custom variant analysis tests except in those instances in which a research finding cannot be confirmed. Custom variant testing can also be used to test for a variant that was clinically identified in a relative of the individual; the relative's sample would then act as the positive control. Custom variant analysis should only be performed by laboratories highly experienced in performing targeted testing.

G19.4 QC Standards and QA Programs

Depending on the testing methodology utilized by a laboratory, the requirement for positive and/or negative controls will vary.

In the special situation of custom PCR-based testing for a known variant, if the variant was identified in an outside laboratory, the laboratory performing the clinical assay should make every effort to obtain a positive control and must attach a disclaimer to negative reports if one was not available. A control is recommended in order to confirm the accuracy of the reported variant, to test the sensitivity of the detection method to be used, and to confirm that the primers can amplify the variant allele in that family.

Proficiency testing (PT) for ultra-rare disorders is unique in that locus-specific PT is impractical due to: 1) the very small numbers of analyses of any given gene that may be performed by a particular laboratory each year; 2) the large number of genes that may be analyzed by a laboratory; and 3) the fact that PT for the identification of specific variants in any given gene is not broadly representative due to the private nature of most variants in genes associated with ultra-rare disorders. Therefore, PT in the ultra-rare disorders laboratory should be designed to test the proficiency of the laboratory's use of the methods for variant detection, as well as its ability to detect the different classes of variants for which services are offered. For example, if the laboratory uses sequencing, restriction digestion, heteroduplex analysis, and quantitative PCR to identify and/or confirm variants, the PT program for the laboratory should require that 1-3 blinded samples for each methodology should be tested semiannually.

G19.5 Test Interpretation

Interpretation of sequence variants should follow the American College of Medical Genetics and Genomics/Association for Molecular Pathology recommendations for the interpretation of sequence variants (Richards S et al., 2015). The criteria for test interpretation associated with URD are the same for all molecular genetics tests, and should follow the CAP guidelines (www.cap.org). Reporting and documentation should follow the recommendations provided elsewhere in this document (see Section G15-G17).

G19.6 URD Custom Analysis

For purposes of variant confirmation, it is recommended that the test be performed on a new sample obtained from the patient and sent directly to the clinical laboratory, i.e., not a sample that was received and processed by the research laboratory that originally identified the variant. Extracted genomic DNA may also be accepted as long as the DNA was extracted in a CLIA/CAP accredited laboratory. For analytical validation, it is important for the clinical laboratory to confirm the variant in the original proband prior to performing any testing on additional family members, and particularly prior to any prenatal testing. In the event of limited time, it is acceptable to perform the test on the proband (for confirmation purposes) and the additional family member/prenatal sample at the same time. If the variant finding cannot be confirmed by the clinical laboratory on the original proband, this needs to be stated in the patient's report and no reporting can be made of any additional family members/prenatal samples until the discrepancy in the proband's result has been clarified. In some instances the original proband is deceased and variant confirmation cannot be performed on a new sample. In such instances the following options can be pursued:

- Family members who are obligate carriers of the variant can be used to establish the clinical variant confirmation in that family.
- If no family member can be identified in whom variant confirmation can be performed, then variant testing on at-risk family members is performed in the absence of formal variant confirmation. If the variant is found to be present in any family member being tested, the familial variant for that family is confirmed and can be reported. However, if the family member being tested is found not to carry the variant, it is very important to state in the report that a positive control from that family was not available for the variant being tested. The disclaimer should emphasize that the results being reported are dependent on the accuracy of the information received from the research laboratory.
- At the very minimum, in the absence of a new positive control specimen from the family, it is important to obtain a DNA aliquot from the research laboratory to serve as a positive control for a custom test.

This permits technical validation of the primers and conditions for detection of that variant, although it does not rule out possible misidentification of that original research specimen.

G20 Appendix: Methods No Longer Widely Used

G20.1 Denaturing Gradient Gel Electrophoresis (DGGE) Assays

G20.1.1 ***Overview***

Strand length and conformation determine relative electrophoretic mobility of double stranded DNA in a polyacrylamide gel. Several techniques use this characteristic as a method of identifying DNA sequence abnormalities without prior knowledge of the precise location or nature of the sequence change. DGGE makes use of the conformational changes associated with DNA double strand melting as a method for detection of sequence variations. Under DGGE conditions a double stranded DNA sequence is electrophoresed through a gradient of denaturant at an elevated temperature. The mobility of the DNA is affected by the melting behavior of the sequence as it progresses through the increasing denaturant concentration. It is possible in this manner to differentiate between the mobility of two sequences which

differ by as little as a single base.

DGGE uses PCR to generate copies of gene or cDNA segments of several hundred basepairs in length. Each of these is denatured and allowed to renature under conditions that promote heteroduplex formation between the normal sequence strand and the strand with a possible mutation (most patients are assumed to be heterozygous for any unknown mutation). The heteroduplexed fragments are then separated by electrophoresis in polyacrylamide gels containing denaturants that facilitate the melting of the DNA duplexes at unique positions in the gradient. Fragments containing sequence variations will generate multiple bands, while homozygous normal (or homozygous abnormal) fragments will generate only a single band.

The sensitivity of DGGE can reach 100% when sufficient knowledge and experience with the methodology and the gene of interest are available. Variations of basic DGGE such as two-dimensional DGGE have been developed and may provide increased sensitivity. In the event that a large deletion resulting in the heterozygous loss of one or more amplicons is present, an incorrect interpretation of wild type sequence may occur. This disadvantage is shared with all mutation detection techniques. Knowledge of the distribution of mutation types in the gene of interest will permit evaluation of the sensitivity of DGGE for each gene of interest.

The high detection rate of DGGE is dependent on correct design of the assay. Several factors outlined below are of importance in the design and performance of DGGE.

G20.1.2 ***PCR Fragment Design***
All sequences to be analyzed by DGGE should be amplified by PCR using protocols optimized for the amplicon in question. The specificity of the PCR reaction should be such that a single amplicon is seen on a stained gel.

Each amplicon should be designed using available software or empiric analysis to produce a single melting domain throughout the region to be assessed. The primers used in the amplification step should be designed to include a 5˜ clamp sufficient to stabilize the melting domain of the test DNA sequence.

G20.1.3 ***Sample Preparation***
DNA samples should be prepared and stored using established protocols (see DNA Preparation, Section G3).

G20.1.4 Amplification of target sequences should be performed using all standard PCR precautions (see PCR, Section G7).

G20.1.5 Samples should be heated and allowed to renature prior to loading to permit heteroduplex formation. Time and temperature should be standardized.

G20.1.6 Samples should be heated and allowed to renature prior to loading to permit heteroduplex formation. Time and temperature should be standardized.

If a potential homozygous mutant condition is being analyzed, it may be appropriate to mix a known normal control and test sample to force heteroduplex formation.

G20.1.7 ***Gel Electrophoresis***
Appropriate denaturing gradient conditions should be established based on calculated melting profile and empiric results observed with positive controls.

A set of positive controls should include (whenever possible) samples containing mutations distributed throughout the region to be analyzed.

Equipment used to form the gradients in the gels and to run the gels under temperature controlled conditions should be standardized within each laboratory. Any change in equipment will require a revalidation of the assay.

Samples to be run on the same gel should be denatured, renatured, and loaded on the gel at the same time.

G20.1.8 ***Controls***
A positive control sample should be analyzed simultaneously to provide a measure of the adequacy of the heteroduplex formation and the gel running conditions. A negative (normal) control sample can be used to aid in sizing of the observed bands.

It is not necessary to run a sample of every known mutation in each gel. A single mutation control is sufficient to document the reproducibility of the system.

G20.1.9 ***Data Analysis***
Gels should be stained (or visualized based on labeled DNA) in a manner adequate to detect the entire banding pattern created.

Heteroduplexes are often present in smaller amounts than the homoduplex forms and may produce a lighter signal.

Samples on the gels should be identified by an unambiguous method clearly identifying positive and negative controls.

Documentation of gel results by photography or other image storage system is necessary.

Computerized image analysis may be helpful in identification of recurring mutations.

The presence of putative mutations identified by DGGE must be confirmed by sequencing.

G20.1.10 ***Validation***
Each laboratory must validate the technique for each sequence to be analyzed. Validation with known mutations as well as normal samples is required. Results of validation studies for each gene analyzed must be available for review.

G20.2 Single-Strand Conformation Polymorphism (SSCP) Assays
G20.2.1 ***Overview***

Single-strand conformation polymorphism (SSCP) analysis is a method for detecting mutations and sequence polymorphisms in genes. SSCP is generally performed by denaturing PCR products and electrophoresing under nondenaturing conditions. The technique relies on the fact that single-strand DNA under certain conditions has defined secondary structure. The electrophoretic mobility of folded single-strand DNA molecules depends on both length and conformation. Mutations can alter the mobility of one or both single strands. Direct sequencing is performed after SSCP analysis to ascertain the nature of the sequence changes.

The sensitivity of SSCP is not 100%. Sensitivity depends on the size and sequence of the segment as well as the gel matrix utilized, the temperature, and the concentration of glycerol in the loading buffer. At present, there is no reliable way to predict the sensitivity of novel mutation detection, which typically varies from 50-90%. For segments of a given size under a given set of conditions, the sensitivity depends on the mobility of the wild type sequence relative to the distribution of mobilities of all the possible single base changes.

Each laboratory must determine its own sensitivity and specificity for each gene analyzed.

In order to increase the expected sensitivity of SSCP, two to four different conditions are sometimes employed. However, use of multiple conditions defeats the major advantage of the technique, speed.

Hybrids of SSCP and other methods have been developed in order to increase sensitivity. Three of these methods have the advantage of detecting virtually all mutations, as judged by blinded analysis. Dideoxyfingerprinting (ddF) is best for segments of 300 bp or less, bi-directional dideoxyfingerprinting (Bi-ddF) is best for segments of 300-600 bp, and restriction endonuclease fingerprinting is best for segments of 800-2000 bp.

When performing SSCP, attention to safeguards for PCR-based assays as described in Section G7 is required. Particular attention should be given to Section G7.3.1.2 (amplification of variable length sequences) to ensure amplification of the range of sizes possible at the locus, and Section G7.4 (appropriate controls) for correct interpretation of results. Additional considerations include:

G20.2.2 ***Assay Design***

When **screening for unknown mutations**, DNA fragments between 150 and 300 bp are typically used. Larger fragments can be used if it is known that the specific mutation/polymorphism of interest produces an abnormal SSCP pattern in that DNA segment.

G20.2.3 ***Polyacrylamide Gel Electrophoresis***

Gels should be run for a sufficient length of time (dependent on fragment length) to detect possible mobility shifts. In order to reduce the risk of missing mutations, samples may be run under two electrophoretic conditions that may differ in length of time, temperature, buffer concentration, crosslinking ratio, crosslinking reagents, and presence or absence of glycerol.

It is preferable to standardize electrophoretic conditions for as many different mutations as possible. This can be done by using more than one control mutation (see below).

SSCP requires a stable, uniform temperature throughout the gel. Unstable cooling (as occurs with cooling

fans) may produce unreliable results.

G20.2.4 ***Controls***
A double-stranded DNA control should be run alongside single-stranded fragments to allow identification of both fragments.

Some mobility shifts are observed only with double-stranded fragments.

Optimal denaturation of double-stranded fragments should involve a dilution of the PCR product. This will necessitate use of a sensitive detection method (fluorescence, radioactivity, or silver staining).

The PCR product from at least one normal control should be included on every SSCP gel.

The PCR product from at least one control sample containing a mutation should be included on each SSCP gel in order to ensure that the electrophoresis conditions are optimal for detection of at least one mutation. Inclusion of more than one control mutation is advisable to improve the accuracy and standardization of the assay. If screening for several known mutations in a DNA fragment, use of control samples for each is desirable to ensure that the sequence alteration produces an abnormal SSCP band under the conditions used.

G20.2.5 ***Visualization of Results***
For manual approaches to SSCP using 32P-labeled or 33P-labeled deoxynucleotides, multiple X-ray film exposures are recommended to visualize all signals. Some abnormal SSCP bands may be faint, requiring longer exposures than normal bands.

For SSCP by automated fluorescent analysis, internal size markers help prevent artifactual lane shifting from influencing mobility shift data. It may be necessary to adjust the volume of sample loaded to achieve detection.

G20.2.6 ***Interpretation of Results***
All samples showing a mobility shift should be sequenced to determine the nature of the sequence change. It is possible for different sequence variations to produce similar SSCP results.

G20.2.7 ***Validation***
Each laboratory must validate the technique for each gene to be analyzed. Validation with known mutations as well as normal samples is required. Results of validation studies for each gene analyzed must be available for review.

G20.3 Protein Truncation Tests for Mutation Detection
G20.3.1 ***Overview***
The protein truncation assay uses RNA (or DNA in the case of large exons) to produce a PCR amplified modified cDNA. The cDNA is then placed in a linked transcription/translation system to produce a protein product that can be analyzed by gel electrophoresis to identify abnormally sized products. Protein truncation analysis can be used to search for possible mutations in a gene of interest. Knowledge of the proportion of previously identified mutations known to result in a truncated protein product must be available before use of this methodology in a clinical setting can be considered.

This assay system is very complex and each gene analyzed will present a unique set of challenges. Therefore, extensive experience with each gene is required before application of the assay to clinical use.

G20.3.2 ***Source of Samples***
The starting material for this assay is DNA or RNA obtained from any tissue by standard methodology (see Section G3 for DNA/RNA preparation). RNA is the material of choice unless one or more large exons provide a useful target for analysis.

The source tissue must be of sufficient quality to provide high molecular weight DNA or RNA. In addition, the tissue type must express the mRNA of interest in sufficient quantity for accurate and sensitive analysis.

G20.3.4 ***PCR Amplification of DNA or cDNA (see Section G13 for cDNA synthesis)***
The usual safeguards against contamination by PCR products should be used (see Section G7.1).

The 5´ primer is designed to introduce a bacteriophage promoter sequence and a mammalian translation initiation sequence (Kozak sequence) into the PCR product. It is not necessary to include a stop codon in the 3´ primer since absence of a stop codon does not appear to influence the translation efficiency of PCR products failing to reach the natural stop codon.

Although PCR products of at least 5 kb can be translated, it is recommended that multiple overlapping segments be amplified, each less than 2 kb with a minimum overlap of 200-300 bases. This minimizes the risk of missing mutations that are close to the primer sequences.

Each PCR reaction should be run in duplicate or triplicate to avoid false identification of artifactual mutations arising through amplification of chance polymerase errors leading to production of truncated polypeptides.

A normal control for the specific region of the gene to be analyzed must be included in each assay.

G20.3.5 ***RT-PCR Amplification from RNA (Reverse-Transcription PCR)***
When RNA is the starting material, cDNA is first synthesized from the RNA using oligo (dT), random hexamer primers, or mRNA-specific primers.

A second round of PCR using a nested primer pair may be necessary to amplify low abundance mRNA transcripts. PCR controls including a water blank must also be re-amplified to permit detection of low-level contamination.

Amplification by RT-PCR followed by electrophoresis may reveal gross rearrangements such as gene deletions (complete or partial), duplications, insertions or splice mutations without need for the protein truncation assay.

Differences between the two alleles in terms of transcription efficiency or RNA stability can influence results. A genomic DNA control segment with a previously identified heterozygous sequence in the gene must be PCR-amplified in parallel to confirm that both alleles have been amplified in each patient sample.

The quality of RNA should be documented by either gel analysis or by amplification of a housekeeping gene to ensure that it is an appropriate starting template.

G20.3.6 ***Coupled Transcription and Translation***
After amplification, the unpurified PCR product is added to the mixed components of a reticulocyte lysate system which enable transcription and translation to be accomplished.

It may be necessary to optimize potassium salt concentration to overcome inappropriate translation termination.

G20.3.7 ***SDS-PAGE Electrophoresis***
Translation products are separated by discontinuous SDS-PAGE. Commercially available protein markers are usually used as molecular size standards. If the protein product of interest is very large, special standards may be required.

A normal control must be run with each batch of test samples. Previously prepared (known product size) controls may be used as an external size indicator, but a simultaneously transcribed/translated control is also required.

G20.3.8 ***Interpretation***
A mutation is indicated by the presence of a novel band of lower-than-normal molecular weight representing a truncated peptide. If the band representing the full-length polypeptide is present in the same sample, it can serve as an internal control.

"Background" bands are often observed. Some of these are artifact, resulting from translation from internal AUG codons downstream from the authentic start codon or erroneous translation termination due to a non-optimized "in vitro" system. Other background bands present may represent proteins in the reticulocyte lysate or alternatively-spliced products from the gene of interest. Again, comparison of bands with those from a known normal control assayed simultaneously is essential.

The presence of a truncated polypeptide is suggestive of an underlying genomic mutation. In most cases, the length of the truncated polypeptide (determined by using the protein markers as standards) can be used to localize the putative mutation. If the polypeptide is truncated due to a large deletion, the deletion site can be determined by restriction endonuclease mapping.

The analytical specificity and sensitivity of the protein truncation assay is not known. It is essential to verify the presence of each mutation by either sequencing genomic DNA or sequencing cDNA followed by analysis of genomic DNA using RFLP or ASO methodologies.

G20.3.9 ***Validation***
Each laboratory must validate the technique for each gene to be analyzed. Validation with known mutations as well as normal samples is required. Results of validation studies for each gene analyzed must be available for review.

G20.4 Heteroduplex Assays

G20.4.1 ***Overview***

Heteroduplexed double-stranded DNA molecules result from the annealing of complementary DNA strands containing base mismatch(es) due to a mutation or polymorphism in one of the strands. Regions of interest can be amplified, denatured, and allowed to reanneal to facilitate heteroduplex formation. Mutations or polymorphisms can be detected by differential migration of heteroduplexes vs. homoduplexes on acrylamide gels, presumably due to sequence-dependent conformational changes in double-stranded DNA. Sequence changes as little as a single-base substitution may be detected by heteroduplex analysis, depending on factors such as the type of base mismatch, the size of the PCR product, and the distance of the mismatch from the ends of the fragment. Gel matrices developed for heteroduplex analysis are available commercially (MDE) or have been described in the literature (CSGE), and isotopic or non-isotopic detection systems can be used. Heteroduplex analysis is a relatively simple technique to perform and has been applied successfully for numerous genetic disorders. Detection rates of approximately 80-90% have been reported for small DNA fragments (<300 bp), which is comparable to that of SSCP.

G20.4.2 ***PCR Fragment Design***

PCR product sizes of approximately 150-300 bp are ideal for screening unknown mutations by heteroduplex analysis. Larger fragments can be used to detect specific mutations or polymorphisms once it has been established that a heteroduplex band can be consistently detected under standardized conditions.

The location of the mutation/polymorphism of interest should be at least 40-50 bases from the ends of the DNA fragments. Thus, PCR primers in flanking intron sequences should be at 40-50 bases from the intron-exon junctions.

G20.4.3 ***Sample Preparation***

The preparation and storage of DNA samples should be performed according to standard protocols (see DNA Preparation, Section G3).

PCR amplification of the regions of interest should be carried out according to all standard precautions (see PCR, Section G7). It is critical that each amplicon produce a clean, single band for use in heteroduplex analysis.

Samples should be heat denatured and allowed to reanneal to facilitate heteroduplex formation. The time and temperature for denaturation and annealing should be standardized.

In case of potential homozygous mutations, PCR products from wild type controls should be mixed, denatured and reannealed with the test samples to force the formation of heteroduplexes.

G20.4.4 ***Gel Electrophoresis***

The composition of the gel matrix to be used for heteroduplex analysis, the thickness of the gel, the length and time of the run, and the electrophoresis equipment should be standardized within each laboratory. Samples to be analyzed on the same gel should be denatured, reannealed and loaded on the gel run to

validate the results for each gel.

G20.4.5 ***Data Analysis***
Heteroduplex gels should be visualized by staining or by autoradiography, depending on the detection system employed, to detect the entire banding pattern required for mutation detection. The detection system used to detect the heteroduplex bands (e.g., the specific staining protocol) should be standardized in each laboratory.

Heteroduplex bands are usually seen at a lighter intensity because they comprise a stoichiometrically smaller amount of the total DNA sample.

Results should be scored unambiguously by comparison with the positive and negative controls. All putative positive results detected by heteroduplex analysis should be confirmed by sequencing to identify the mutation or polymorphism involved.

G20.4.6 ***Validation***
The heteroduplex analysis technique should be validated by each laboratory where this assay is to be performed. Validation should be carried out using sequence variations (which should exhibit detectable and in many cases characteristic heteroduplex banding patterns for specific sequence variations), as well as normal control samples. For each gene analyzed by heteroduplex analysis, validation test results should be available for review.

G20.5 Linkage Analysis
Linkage analysis should employ software in wide general use. It should be used only by individuals with a working knowledge of the specifics of each package in use.

The laboratory must keep an up-to-date reference list documenting linkage relationships (i.e., location relative to locus in question, recombination fractions and/or ˜ values at 95% confidence intervals) for each disorder analyzed by indirect linkage methods. The laboratory must have documented linkage relationships for all in-house generated probes prior to use in a clinical setting (see Section G4).

In order for linkage analysis involving probes with significant recombination distances from the locus in question to be reported, the analysis must contain data from two informative flanking markers. If this is not possible, the reason must be stated so as to indicate that every effort was made to provide such. For linkage analyses involving probes with negligible recombination distances from the locus in question, it is only necessary to use one highly informative marker. For each disease specific system in use, the number of informative markers to be used is dependent upon the informativeness of each marker, the disease specific recombination frequency and the availability of markers.

G20.6 Denaturing High Performance Liquid Chromatography (dHPLC)
G20.6.1 ***Overview***
Denaturing high performance liquid chromatography (dHPLC) can be used for rapid, automated, and high-throughput mutation detection based on principles similar to those for heteroduplex analysis. Recent advances in the development of this technology have led to the introduction of automated instruments. The software is useful in both predictions of the optimum run conditions based on the DNA sequence and analysis of the results in distinguishing homoduplexes and heteroduplexes. This technology is particularly

suited for detection of point mutations, small deletions and insertions. It has also been applied for analysis of fragment size differences and for sensitive detection of sequence differences in minor cell populations such as tumors. The basic principle is that DNA is negatively charged, the column cartridge is neutral, and a positively charged binding ion--triethylammonium acetate (TEAA)--links the two. Heterozygous mutations are detected through differential binding of homo- and heteroduplexes to the column. Analysis is performed at a temperature sufficient to partially denature heteroduplexes. The melted heteroduplexes are resolved from the corresponding homoduplexes by HPLC. Denaturation leads to a reduced double-stranded PCR fragment. Single-stranded fragments elute earlier than double-stranded fragments due to the reduced negative charge. Thus, heteroduplexes elute prior to homoduplexes.

Sensitivity depends upon the size and sequence of the PCR fragment, in particular the melting profile, as well as the conditions of analysis, including temperature and buffer concentration. At present, there is no reliable way to predict the sensitivity of detection for novel mutations, which have been reported in various genes to exceed well over 90%. Nevertheless, diagnostic laboratories must validate the sensitivity of this detection method for each gene test developed. For each PCR fragment under a given set of assay conditions, the sensitivity depends on the elution profile of the wild-type homoduplex sequence relative to the heteroduplex with the sequence alteration. In order to increase the sensitivity of dHPLC, two or three different temperatures may be employed.

G20.6.2 ***PCR Fragment Design***
PCR fragment design is critical to the success of dHPLC analysis. dHPLC can be used for fragments up to 600 bp; however, generally optimum separation is achieved with fragments of 200 to 400 bp. For PCR fragment design of regions of large size, it is recommended that overlapping sets of primers be used. It is suggested that the overlap region be a minimum of 50 bp. Prior to ordering oligonucleotide primers, the melting profile of the PCR fragment should be analyzed using the software of the instrument. If there are more than two melting temperatures of the sequence, it may be useful to break the fragment into smaller fragments in order to achieve a more accurate analysis. In some cases it may be necessary to use GC clamps, and in other cases it may not be possible to achieve optimum design based on problematic sequences. It is possible to design well defined small multiplex PCR reactions to analyze by dHPLC, but care must be taken in resolving the different PCR fragments, based on size variation, yet having consistent melting profiles, allowing the same optimized analysis conditions.

G20.6.3 ***Sample Preparation***
DNA preparation is critical to the success of this assay. Some methods, such as certain column preparations, interfere with the binding to the cartridge and cannot be used. It is critical that the laboratory use a DNA preparation protocol that does not damage the cartridge. Therefore it is strongly recommended that each laboratory consult with the manufacturer for recommended DNA preparation kits, of which many exist.

PCR products are pipetted in 96-well plates and loaded on the instrument. Sample mixing is critical to resolve homozygous mutation carriers and for analysis of males for X-linked conditions. For individuals who are heterozygous for a sequence alteration, heating to 95°C and slowly cooling produces a mixture of heteroduplexes and homoduplexes. However, for detection of homozygotes, the PCR product from the patient is mixed with a comparable amount of wild-type PCR product in order to obtain heteroduplexes.

G20.6.4 ***Chromatography***

dHPLC should be performed under optimized conditions to detect possible heteroduplexes. In order to reduce the risk of missing mutations, samples should be analyzed under optimized melting temperatures, which may be multiple, and may also require adjustment in buffer concentrations. The use of dHPLC-grade water or an equal grade is critical for this analysis system to operate efficiently. Any change in water source will require re-standardization of the column. In addition, it is important that the column be standardized at routine intervals (at least weekly) in order to assess reproducibility and quality of performance. The column should be monitored closely for number of analyses and replaced appropriately as recommended by the manufacturer. The software keeps track of column usage, which is a valuable quality control measure for diagnostic laboratories. It is important to recognize that the reproducibility of profiles is highly dependent upon the column and the number of runs. When columns are changed and when the number of runs on a column is high (>2,000), profiles may also change. Therefore it is important to run mutation standards at regular intervals in order to determine test reproducibility. It is important that diagnostic laboratories monitor columns for reproducibility of results, and change columns when mutation-detection is compromised. This should be done at the discretion of the technical director.

G20.6.5 ***Controls***

Both wild-type and positive mutation controls, including heterozygous samples (and homozygous samples when applicable, depending upon the test) must be analyzed alongside test specimens. In particular, it is critical that the wild-type fragment is used for the basis of all comparisons. However, it is impossible when scanning large genes for unknown mutations to be able to validate each sequence variation prior to introduction of this method of analysis. Therefore, one mutation in each fragment of interest is sufficient. In cases where the laboratory is unable to obtain mutations for all fragments to be analyzed either because they do not exist or are not available, the laboratory must develop the conditions for analysis of that fragment using the same high standards as all other fragments analyzed. When a positive control for a particular DNA segment cannot be obtained, it is critical that the laboratory use multiple analysis conditions in order to optimize detection of an unknown mutation. It is noteworthy here that each mutation in a given PCR fragment will have a characteristic elution profile of its heteroduplex. If a pattern variation is identified, the laboratory should confirm the variant by sequence analysis.

G20.6.6 ***Visualization of Results***

The observation of heteroduplex peaks in a chromatogram indicates the presence of a sequence variant, while samples without base mismatches resolve as homoduplexes. Heteroduplex peaks elute earlier than homoduplexes, and can be observed as separate peaks or as shoulders on the leading edge of homoduplex peaks. The manner in which a heteroduplex peak resolves is influenced by the specific nucleotide mismatch present and the melting characteristics of the surrounding bases. Elution profiles that differ from the wild-type or reference DNA indicate the presence of sequence alterations in the form of base substitutions, deletions, or insertions. One cannot predict the type of mutation (i.e., deletion, insertion, nonsense, etc.) from the heteroduplex pattern. The software of the instrument allows real-time visualization of results. Software allows overlay of the patient specimen and the wild-type fragment for aided visual comparison. The software also automatically scores the profile for the presence of a heteroduplex. This automatic scoring must be confirmed by visual observation. Similarly, it is recommended that all "negative" profiles also be confirmed visually. The homoduplex wild-type pattern is typically one peak, but may be two peaks, depending upon the melting profile. It is desirable to optimize the fragment design to have a single peak in order to more readily distinguish wild-type patterns from heterozygous mutant. In addition, it is recommended that each patient specimen that shows a positive result be documented as a hard-copy printout and inserted in the laboratory record. Currently, mutation

profiles are not recorded by the instrument's software in order to enable future comparisons via "pattern recognition." Therefore these mutation heteroduplex profiles always require manual observation. Future development trends may resolve this issue.

The instrument can utilize an ultraviolet detection system or a fluorescent detection system. However, at present only one fluorescent dye can be detected during a single analysis. The rationale for using fluorescence is to achieve more sensitive detection for minor populations or use in single cell PCR. Future trends will be to include a four-dye system in order to allow multiplex analysis of heteroduplexes.

Instrument maintenance is required at routine specified intervals and must be performed and documented.

G20.6.7 ***Interpretation of Results***

All samples identified as heteroduplexes by dHPLC analysis must be sequenced in both directions to confirm and determine the nature of the sequence change. Each sequence change within a DNA fragment is predicted to have a unique heteroduplex pattern. It is recommended that a pattern file be established for quick identification of specific sequence changes. However, pattern recognition alone is not considered sufficient for diagnostic purposes, particularly when scanning genes for unknown mutations. In the case of a recurring mutation within a well characterized DNA fragment such as a targeted mutation test, pattern recognition alone may be sufficient for mutation identification. However, sufficient validation is required by the laboratory prior to introduction of such tests.

For samples in which no heteroduplex is identified in any PCR fragment tested, the report must state the sensitivity of this technique. The laboratory must then determine whether another method should be employed to supplement detection rate, such as sequence analysis, or whether to stop testing.

G20.6.8 ***Validation***

Each laboratory must validate this technique for each sequence to be analyzed. Validation with known mutations as well as wild-type controls is required. Results of validation studies must be documented and available for review.

G20.7 PCR Dosage

The determination of gene dosage can often be improved by using the polymerase chain reaction. Since the extension product of each primer serves as a template for the other primer, each cycle essentially doubles the amount of the DNA product produced in the previous PCR cycle. This results in the exponential accumulation of the specific fragment, up to several million-fold in a few hours. However, to obtain quantitative results, the PCR products must be estimated during the exponential phase of the amplification process, for it is during the exponential phase where the amount of amplified products is proportional to the abundance of starting DNA. This occurs when the primers, nucleotides and Taq polymerase are in a large excess over that of the template concentration. After the completion of an adequate number of cycles (25-30) to visualize the PCR products on an ethidium-bromide-stained gel, the PCR reaction is no longer in the exponential quantitative range. Therefore the gene dosage-PCR is accomplished by amplifying the genomic DNA at lower cycle numbers (before visualization by ethidium bromide), and either using fluorescently primers for automated DNA fragment analysis or running the products out on a gel, Southern transferring the products and hybridizing the amplicons with a radiolabeled probe. Linearity should be well maintained within 15-20 cycles.

Similarly to the dosage determination by Southern blotting, one determines the gene copy number by PCR using dosage ratios. One amplifies a target which is present at the normal copy number in the patient which serves as an internal control. The internal control is co-amplified with the target of interest and serves as a check for several factors: differences in initial template concentrations between different samples, sample-to-sample variations in the PCR and the extent of any DNA degradation. Thus, rather than directly comparing single PCR amplicons, ratios are calculated. The PCR bands can be scanned with a densitometer or peak height ratios can be determined by automated fragment analysis.

The determination of gene dosage via the polymerase chain reaction has several advantages. The amplification of specific targets reduces the background problems which are often present on Southern blots. Furthermore the PCR assay requires less DNA, can be performed more rapidly than Southern analysis, and is both cost and labor effective. However to reliably quantitate the amount of DNA, the range of concentrations of template and the number of amplification cycles must be determined such that they stay within the exponential phase of the PCR. It is critical that samples are assayed within the exponential phase of the PCR reaction, before the plateau phase when the amplification efficiency begins to decrease and the relative concentration of amplicons begin to vary. It is also important to choose an internal control which amplifies equivalently with the target of interest. Ideally the normal dosage control ratio (space need to be deleted) should be approximately 1. Lastly, the internal standard should be different enough in size to be easily resolved from the PCR product of interest.

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