American College of Medical Genetics

STANDARDS AND GUIDELINES FOR CLINICAL GENETICS LABORATORIES

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G	**CLINICAL MOLECULAR GENETICS** These Standards and Guidelines specifically refer to the use of molecular techniques to examine heritable changes in the human genome.
G1	**Specimens and Records**
G1.1	In addition to numerical accession files and alphabetical patient listings, each family studied is assigned a unique code (preferably numeric). Note: This requirement only applies when more than one member of a family is being tested. (See G16.1 for maintaining confidentiality in reporting of results.) Use of a family number does not necessarily 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 C2.4.
G1.3	For specimen labeling, see 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 mutation nomenclature has been described (den Dunnen & Antonarakis Hum Genet 109(1):121-124, 2001.) and should be considered, especially for use with newly discovered mutations. While very useful in genetic and genomic research, this nomenclature will be unfamiliar to many clinical molecular geneticists, and so it is not recommended at this time as a replacement for conventional mutation designations already in widespread use, such as those for factor V Leiden and the commonly tested cystic fibrosis mutations. For those situations in which the conventional or colloquial mutation designations have become fixed and universal in laboratory reports and in the software of commercial testing platforms, it would introduce an unacceptable level of confusion, both between laboratories and in communication with clinicians, for the formal nomenclature to be adopted exclusively. In addition, not all types of mutations (e.g., complex mutations) are covered by these recommendations. Suggestions for possible descriptions for complex mutations are available (den Dunnen JT and Antonarakis SE. Hum Mutat. 2000; 15(1):7-12). Clinical reports should describe the level at which the mutation is being described e.g. "g" for genomic sequence, "c" for cDNA sequence, "p" for protein, etc. (http://www.hgvs.org/mutnomen/)
G2	General Quality Control

G2.1	See C4.3. 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	DNA 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.
G4	Probe/Primer/Locus Documentation All loci used for analysis in the laboratory need to be well documented by NCBI (http://www.ncbi.nlm.nih.gov/ and http://www.ensembl.org/index.html) or by publication in the peer-reviewed scientific literature. Probe sequences should be subjected to a BLAST search to identify other homologous genomic sequences which could interfere with hybridization of the probe to the target sequence (http://www.ncbi.nlm.nih.gov/BLAST/). This documentation must be maintained in an up-to-date laboratory book. 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 sheets also 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 analytic validity (sensitivity, specificity, reproducibility)

	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 (see section C8 and section CF2.11.1 in the Technical Standards and Guidelines for CFTR Mutation Testing).
G5.2	The laboratory must document clinical validity through its own or other published studies.
G6	Southern Analysis
G6.1	Restriction Digestion and Electrophoresis
G6.1.1	Restriction endonuclease digestion of prepared DNA for Southern analysis must be done according to a standardized protocol documented in the laboratory manual.
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	Proper hybridization 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 CLSI document MM1A, Vol.26, No. 27 (2006).
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-A2, Molecular Diagnostic Methods for Genetic Diseases; Approved Guideline—Second Edition (2006).
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 source of contaminants are amplified targets such as PCR products, plasmids or phage. 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 three 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 or barrier 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 template control should be included in each assay to detect 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. In addition, contaminating DNA can be inactivated with UV irradiation.
G7.2	Primer Documentation: Also see Section G4. The target gene, the primer sequences and the rationale of the design should be well documented. The target gene should be characterized, as much as possible, using the scientific literature and available databases to assure appropriate primer design. Relevant information includes map position, pseudogenes, polymorphisms, types 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, Huntinton 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 G.7.3.1.1, 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, Verification and Validation of Multiplex Nucleic Acid Assays (MM17-P, Vol 27 No. 21).
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, Verification and Validation of Multiplex Nucleic Acid Assays (MM17-P, Vol 27 No. 21). 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, a normal control (negative for the mutation being assayed) and positive control (heterozygyous 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 practial 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 base pair changes and small insertions/deletions
G8.1	Forward Allele-Specific Oligonucleotide (ASO) CF 3.2.3.1 **Overview**: The ASO method is based upon hybridization of a labeled oligonucleotide probe containing either wild-type sequence or known mutant sequence to the target, patient DNA. Generally, PCR products from multiplex PCR reactions of patient DNAs are manually or robotically spotted onto replicate filters (dot blots) and then hybridized to labeled ASOs under specific conditions. Design of the multiplex PCR conditions, ASOs, hybridization and wash conditions, and detection is complex. An advantage of this method is that mutations can be readily added to an already existing panel. There are a number of issues that must be considered in the development of this test platform. **Design and Labeling of ASO Probes**: ASOs for the normal and mutant sequence pair should be derived from the same DNA strand. Since G:T and G:A mismatches are less destabilizing during hybridization reactions, it is important to avoid a G:T or G:A mismatch between the mutant oligonucleotide and the normal template. ASO probes are labeled for radioactive or chemiluminescent detection. If radioactively labeled, the laboratory

	determines the need for purification and quantification prior to use. **Multiplex PCR Amplification**: Various parameters can be employed which allow the use of one PCR program for a combination of primer sets. One method is touchdown annealing cycling. Others may depend on primer design. **Dot-Blot Membranes**: To prepare replicate filters, the use of a robotic system or a multichannel pipetting device is recommended to ensure that the same patient PCR product is placed at the same position on each filter. This is critical to the interpretation of the results of this assay. **Hybridization**: For radioactively labeled probes, it is recommended that an optimized and constant number of counts per minute, per milliliter (cpm/ml) be consistently used from run to run in order to obtain consistent quality of results. In addition, it is recommended that a non-labeled competitive probe be included at an increased molar concentration (about 10- to 20-fold higher) in order to eliminate non-specific signal (i.e., increased signal to noise ratio). The optimum conditions for hybridization must be determined by the laboratory. Optimal pooling strategies for combining probes should be determined by the laboratory if pooling is performed. Calculation of melting temperature (Tm) for each oligonucleotide is insufficient to predict the correct conditions for hybridization, which must be empirically determined. **Interpretation of Results**: Comparison of the autoradiograph of the wild-type filter and the mutant filter based upon position is necessary for interpretation of test results. In general, a positive result at a given position only on the wild-type filter is interpreted as normal, a positive only on the mutant filter is interpreted as homozygous for the mutation, and a positive on both filters is interpreted as heterozygous for the mutation. For some tests, a number of filters are necessary to obtain results on all desired mutations. Thus, it is important that results from all filters be read prior to interpretation, particularly when two different mutations are detected in the same patient, such as in diagnostic testing. A grid placed over the filters is recommended for location of exact position, particularly when the analysis is performed in a 96-well format. It is also recommended that at least two (or more) individuals read the results and concur prior to reporting.
G8.2	Reverse Dot Blot Hybridization (RDB) CF 3.2.3.2 **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 mutant and normal 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. This procedure is very amenable to high throughput analysis of high mutation spectrum genes. Although probe design and production of the spotted membranes may be complex, mutation detection using this method is non-radioactive, convenient, rapid, robust and requires no specialized interpretation skills. This technology, while robust, is relatively inflexible and not easily expanded to include additional mutations. **Oligonucleotide Probe Design**: Probes are conjugated at the 5′ end by an amino linker group, added by an aminophosphoramidite during synthesis, for subsequent covalent linkage

	to the carboxyl group of the activated nylon membrane. Length of the allele-specific primer and base composition must be optimized so that the final optimal hybridization and washing conditions for all detected alleles are identical. Probes lengths 15 to 17 nucleotides with 30% to 50% guanine-cytosine (GC) content are adequate to discriminate point mutations. Otherwise, the same guidelines apply as for probe preparation for forward ASO hybridization. However, despite these general rules, probe design for adequate detection may also involve trial and error. **Strip Layout, Manufacture and Quality Control**: Covalent linkage of the amino-modified oligonucleotide to the membrane-bound activated carboxyl group increases the sensitivity of the assay relative to previous enzymatic probe tailing methods. Each oligonucleotide solution should contain a dye such as phenol red to allow for visual inspection of the spotted membranes. The arrangement of oligonucleotides on the strip is a matter of personal preference; wild type and mutant probes can be spotted in separate rows or groups, or interspersed among each other. RDB strips can beproduced manually (Cai SP, Wall J, Kan YW, Chehab FF. Reverse dot blot probes for the screening of β-thalassemia mutations in Asians and American Blacks. Hum Mutat 1994;3:59-63). Alternatively, this process is amenable to the robotic production of large strip lots that can then be stored at room temperature until use. Each lot of strips should be compared to a previous lot to verify consistency with respect to each allele detected in the assay as well as a negative (no DNA) control. For in-house developed strip production, it is often necessary to adjust the amount of new lots of probe that is applied to the strips in order to optimize hybridization signal. **Multiplex PCR Amplification**: All general guidelines for multiplex PCR amplification apply to RDB detection. Semi-nested PCR may increase the hybridization signal for some mutations. It is useful to design the primers so that each product differs by at least 10 bp in length so that robustness of amplification can be visualized on a check gel prior to hybridization. The choice of probe labeling depends on the detection system; primers are biotinylated at the 5′ terminus for subsequent strepavidin-horseradish peroxidase detection. **Controls**: While the laboratory may determine that it is not feasible to include each positive assay control in each run due to batch size limitations, QC on a new lot of RDB should include testing for each mutation. At a minimum, during routine testing, it is recommended that each run include at least one positive assay control and that all positive controls be tested on a rotating basis. The number of positive controls can also be minimized by using genomic or synthetic compound heterozygotes. **Hybridization, Detection and Interpretation**: Hybridization and detection are straightforward and require minimal labor. Care should be taken to protect light sensitive reagents. The genotype of the patient is easily read from the array of hybridization signal on each strip. Individual test results should be read by two reviewers who concur prior to reporting. 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) CF 3.2.3.3 and CLSI document MM1-A2, Vol. 26, No. 27 (2006) **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 as primers in a PCR reaction. For genotyping, paired PCRs are performed for each mutation tested. One primer (common primer) is used in both reactions, while the other is either specific for the mutant or wild-type sequence. In principle, ARMS tests can be developed for any single base pair change or small deletions/insertions. Achieving acceptable specificity is dependent on primer selection and concentration. Use of longer primers (e.g., 30 vs. 20 bp) and inclusion of control reactions have been reported to improve specificity. Primers and conditions for multiplex reactions must be selected so that the relative yields of PCR products are balanced and the PCR products can be adequately resolved with gel electrophoresis. Laboratory developed primer sets must be validated to ensure desired performance characteristics, and new reagent lots should be compared to a previous lot to ensure consistency in performance and robustness. Although the manufacturer performs a level of performance evaluation on these reagents, the laboratory must also complete an internal validation to assess proficiency prior to use on patient samples. **Controls**: Internal control reactions are not required if mutant and wild-type ARMS reactions are combined in the same test. However, for screening purposes, multiplexing mutant ARMS reactions without paired wild-type reactions can result in significant cost savings. Internal controls (additional control primers that amplify unrelated sequences) can be included in each multiplex reaction to ensure that DNA samples will generate at least one PCR product in each tube and reduce the likelihood of false negative results. Negative and positive control samples must be run with each assay but the laboratory may determine that it is not feasible to include all mutation controls in each run due to batch size limitations. Pooled positive DNA control samples can be utilized to allow efficient inclusion of the most common mutation controls in each run. Remaining positive controls can be tested on a rotating basis. **Visualization and Interpretation of Results**: PCR products are separated by gel electrophoresis and visualized by ethidium bromide staining (or other DNA specific stain) and UV transillumination. 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 mutant state. 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), reportedly reliable, and does not require expensive instrumentation.
G8.4	G8.4 Oligonucleotide Ligation Assay (OLA) CF 3.2.3.4 and CLSI document MM1-A2, Vol. 26, No. 27 (2006) **Overview:** The oligonucleotide ligation assay (OLA) is a novel approach to detect point mutations, small deletions and small insertions. This method consists of PCR amplification of the target sequence followed by hybridization and ligation. Hybridization involves 3 probes, one specific for the normal allele, a competing probe specific for the mutant 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 mutant 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 mutant 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 mutant and normal alleles. This allows for electrophoretic size separation and therefore differentiation between the normal and mutant 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 mutant peaks are identified based upon their product size and/or fluorescent tag. A properly designed OLA gives only the appropriate normal or mutant product(s). Protocols constructed with general purpose reagents (GPRs) have been described for CF and a CF genotyping assay IVD kit is commercially available (CF 3.2.3.4). **Controls:** If practical for the laboratory, it is desirable to include all positive controls in each assay. However, for tests with several mutations it may not be feasible to include numerous positive controls in each assay run. Minimally, a normal control, a positive control, and a negative or "no DNA" control should be included in each run. Additional positive controls should be rotated among assay runs. **Visualization and Interpretation of Results:** Fluorescent labeled OLA products are separated by high resolution electrophoresis, usually capillary electrophoresis. 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 will be half the intensity of the homozygous (normal or mutant) peaks. Since many mutations 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 set 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 mutant, 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 (FRET) CF 3.2.3.6 and CLSI document MM1-A2, Vol. 26, No. 27 (2006) **Overview**: The fluorescence resonance energy transfer (FRET) assay involves two concurrent reactions in a single well on a 96-well plate. The primary reaction utilizes two different oligonucleotide probes, one specific for the normal sequence and the other specific for the mutant 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 mutant probe and wild-type target DNA or wild-type probe and mutant 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 mutant) probes in the primary reaction, with each binding to a different FRET cassette with a unique spectral fluorophore, 2 sequence variants (normal and mutant) at a single site can be detected in the same well. Disease-specific ASR platforms run on a microfluidics card utilizing the FRET assay are commercially available. This format enables the user to run multiplex FRET assays. The heat-stable card contains 8 raised samples lanes (1 lane per sample) with each lane subdivided into 48 separate reaction chambers. This allows for a single pipetting step of reagents into up to 48 different reactions. PCR amplification of the target DNA is performed using a limited number of cycles. The amplified DNA is transferred to the card, which contains dried down oligonucleotide probes and FRET cassettes in each chamber. After the addition of enzyme, the cards are sealed using a scoring device, and incubated. After the incubation is complete, the fluorescence generated from each sample is read by a fluorescent plate reader that can accommodate a 96-well format and is equipped with the appropriate filters. **Controls**: Due to the nature of the assay, it is not practical to run genomic DNA positive controls for each mutation analyzed using this assay. However, it may be possible to run several positive controls for each run. At a minimum, a normal (wild-type), heterozygous mutant, and negative (no DNA) control should be included in each run. Positive controls could be rotated among each assay run. Failure of any control to give the expected result invalidates that particular run and the assay must be repeated. **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 (wild type: mutant), the genotype is determined to be homozygous wild-type, heterozygous, or homozygous mutant 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 (See also CF 3.2.3.5). **Overview**: Liquid bead arrays provide simple and high-throughput analysis of DNA polymorphisms with discrete detection of wild-type and mutant alleles in a complex genetic assay . Commercially available bead-array platforms are available for the detection of mutations 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. **Multiplex PCR Amplification**: All general guidelines for multiplex PCR amplification apply to liquid bead array-based detection. All commercial products use a single multiplex PCR with proprietary primers designed to accommodate the hybridization and detection system being used. Since liquid bead arrays work well with various front-end chemistries, including oligonucleotide ligation, allele-specific single base extension, ASO hybridization and allele-specific primer extension (ASPE), the detection chemistry of the particular detection format can be incorporated into the PCR and/or subsequent amplification modification steps. **Hybridization and detection**: One commercial platform uses biotin-modified PCR products that are hybridized to allele-specific capture probes on different beads. Another uses allele-specific primer extension of the PCR product such that "universal tags" are incorporated into the product for allele discrimination. The biotinylated PCR product or extended PCR product is then hybridized to either capture probes or "universal anti-tags," respectively, which are covalently bound to the beads. Both platforms use a reporter fluorophore, streptavidin-phycoerythrin, in or before the hybridization reaction. After hybridization, the modified amplicon is bound to a reporter substrate and transferred directly to a detection instrument without post-hybridization purification. The sample genotype is assigned by comparing the relative hybridization signal between the wild-type and mutant alleles. The generation of electronic data facilitates the development of automated analysis software and database archiving. The reaction is analyzed for bead identity and associated hybridization signal intensity. Lasers interrogate hybridized microspheres individually as they pass, single file, in a rapidly flowing stream. Thousands of microspheres are interrogated per second, resulting in an analysis system capable of analyzing and reporting up to 100 different hybridization reactions in a single well of a 96-well plate in just a few seconds. **Visualization and interpretation of results**: Output files generated during detection are automatically processed 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 mutations and genotype calling. One advantage of customized

	software is data masking, or the ability of the user to display the genotype for mutations determined to be appropriate, such as only those mutations associated with the diseases for which testing has been requested by the ordering physician. **Quality Control (QC) and Controls**: It may not be feasible to include a genomic DNA (gDNA) for each positive assay control in each run due to reagent cost and batch size limitations. QC on a new lot of beads should include gDNA-based testing for each mutation. However, at a minimum, during routine testing, it is recommended that each run include at least one positive assay control and that all positive controls be tested on a rotating basis. The use of either genomic or synthetic compound heterozygotes can also maximize the number of positive controls while limiting the number of reaction wells used. The last sample in each batch should be a no-template control, to assess for reagent contamination by previous or current amplicons. The ratio of wild type to mutant signal, adjusted for background for each control, should fall into previously set ranges that maximize the signal to noise ratio and the no-template controls should fall below an arbitrary pre-set detection limit.
G.8.7	**End-point and Real-time PCR Analysis** These specially designed primer systems (such as TaqMan(r)-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	**Melting Curve Analysis Using FRET Hybridization Probes (See also MM1-A2 Vol 26 (27) (2006)).** **Overview**: There are several real-time PCR instruments. By coupling PCR with fluorescent hybridization probe analysis, these instruments can be used to detect mutations, particularly single-base mutations. 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 mutation 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 base mismatch is present, the thermal stability is altered. The change in stability depends on the bases 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 base mutations. 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 mutation 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 mutant alleles. If an additional sequence variation is present in the target, the melting profile is 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. Some systems (Taqman(r)) use only single labeled probes. This system uses a single internal oligonucleotide probe bearing a 5' reporter fluorophore (e.g., 6-carboxy-fluorescein) and a 3' quencher fluorophore (e.g., 6-carboxy-tetra-methyl-rhodamine). During the extension phase the TaqMan(r) probe is hydrolyzed by the nuclease activity of the Taq polymerase, resulting in separation of the reporter and quencher fluorochromes and consequently in an increase in fluorescence. In this technology, the number of PCR cycles necessary to detect a signal above the threshold is called the cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the assay. The change in the amount of signal corresponds to the increase in fluorescence intensity when the plateau phase is reached. Using standards or calibrators with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample. **Controls**: Controls should be included to ensure the capability of differentiating homozygous normal, heterozygous carrier and homozygous mutant patterns. At a minimum this requires a heterozygous control and a negative control. When available, genomic controls are preferred over synthetic controls. Failure of any control to give a result with the correct genotype invalidates the assay and requires that the assay be repeated. **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 wild-type or mutant allele.The laboratory should establish acceptable Tm ranges for the wild-type and mutant 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 themselves and is a more useful value to help identify additional sequence variations. Fluorescent melting curve analysis allows the detection of additional sequence variations in the target sequence. These additional variations are identified by altered melting curve profiles that have peaks whose Tm does not match the wild-type or mutant allele. The peak shifts may be subtle (<1oC). Sequence variations are most easily identified by a DTm value that is outside the range for normal and mutant alleles. It is recommended that sequence variants be confirmed by DNA sequencing.
G8.9	Denaturing High Performance Liquid Chromatography (dHPLC)

G8.9.1	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.
G8.9.2	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.
G8.9.3	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 homozyotes, the PCR product from the patient is mixed with a comparable amount of wild-type PCR product in order to obtain heteroduplexes.
G8.9.4	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 (>2000), 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.
G8.9.5	Controls Both wild-type and positive mutation controls, including heterozygous samples (and homozygous samples when applicable, depending upon the test) must be analyzed along side 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.
G8.9.6	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. Heterodupex peaks elute earlier than homodupexes, 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.
G8.9.7	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. 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.
G8.9.8	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.
G8.10	High Resolution Melting **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 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. Disadvantages of scanning are that any sequence differences are not specifically identified and that mixing with wild type is required to detect all homozygous or hemizygous variants. 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 bps. 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%. **Design:** Amplicon length is typically less than 400 bps. As with all primer design, known SNPs should be avoided or masked within the primer region. To reduce numbers of amplicons that need further investigation, known polymorphisms with the intronic regions should be avoided. Specificity can be increased, while decreasing sequencing burden, by confirming common polymorphisms with small amplicon melting, unlabeled probes or snapback primers using the same instrumentation and dyes. Some homozygotes and hemizygotes (for X-linked diseases when analyzing samples from males) require mixing with “normal” DNA to create artificial heterozygotes. **Controls:** Mutation detection by melting is dependent on comparison to “normal” sequences: therefore, normal or wild-type controls are crucial. A mutation positive control

	for each amplicon is not feasible, particularly for large genes with multiple amplicons. Instead, rotating controls with mutations in different amplicons is a reasonable alternative. This is similar to rotating positive mutation controls for genotyping assays with multiple mutations. For X-linked or homozygous detection, DNAs from the test sample and control (normal) can be amplified together (mixed pre-PCR) or mixed post PCR. An unmixed sample should be included. For best sensitivity and specificity, DNA from the controls and unknown samples should be isolated by the same method, resuspended in the same buffer, and used in PCR at the same concentration. **Visualization:** After PCR, the fluorescent melting curve is acquired during a temperature ramp of 0.1-0.3°C, although some instruments may require slower ramps to obtain adequate resolution and data density. Fluorescence data is best analyzed based on the shape of the melting curve; specifically, differences in the melting curve shape easily identify heterozygotes. Shape differences in melting curves are conveniently displayed by superimposing normalized curves and plotting the fluorescence differences between samples and normal (wild-type) controls. From these difference plots, curves deviating from the normal (wild-type) are easily visualized. Automated clustering programs can help to visualize variants, although computer-assisted calling is not a replacement for human judgment. Tightness of the clusters can be used as a QC measure. Different heterozygotes usually produce different melting curves that also clearly vary from homozygous variants. However, definitive identification requires genotyping or sequencing to identify the sequence change. **Interpretation:** Amplicons with variant melting curves are further studied. Common variants can be genotyped by a variety of similar melting methods (i.e. small amplicon melting, unlabeled probes or snapback primers). 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.
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.). Because they are so widely dispersed throughout the genome, are highly polymorphic in the population, and can be analyzed rapidly and inexpensively by multiplex PCR techniques, STRs have largely supplanted Southern blot-based RFLP markers for most applications of identity testing and linkage analysis by DNA polymorphism analysis. 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. 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. In addition, a number of technical aspects unique to the use of these markers must be considered:

G9.1	For manual approaches to microsatellite analysis using polyacrylamide sequencing gels and radioisotope detection (^{32}phosphorous-labeled deoxynucleotides), multiple X-ray film exposures are recommended to obtain all possible autoradiographic signals.
G9.2	For manual approaches using ^{35}sulfur or ^{33}phosphorous-labeled deoxynucleotides:
G9.2.1	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.
G9.2.2	Individual autoradiographic exposures are necessary.
G9.3	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.
G9.4	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.
G9.4.1	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 *et al*. 2004).
G9.4.2	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).
G9.5	Microsatellite data interpretation is similar for each use. However, care should be taken in interpretation due to the appearance of shadow-bands, stutter peaks, 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.

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 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. For futher discussion see: Potter NT, Spector EB, Prior TW. 2004. Technical standards and guidelines for Huntington disease testing. *Genet. Med*. 6:61-65.
G10	**DNA Sequencing Analysis**
G10.1	Overview DNA sequencing is considered the "gold standard" for the analytic validation of new DNA-based mutation testing. In addition, since it is capable of the exact determination of every base within a gene including the promoter and splice sites, it can be used to determine the genotype of an individual. It is often the method of choice for genes with a large number of unique mutations specific to individual families. Clinically, DNA sequencing technology should be applied when the contributing gene is well characterized as follows: a) The full and complete sequence is available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html or http://www.ensembl.org/index.html). b) A curated database is available for the identification and location of pathogenic mutations as well as benign sequence variants. c) The presence or absence of any pseudogene sequences complicating interpretation has been established.\ For a more thorough discussion of sequencing, please review the CLSI document MM9-A: Nucleic Acid Sequencing Methods in Diagnostic Medicine, Approved Guideline, 2004.
G10.2	Sequence Standards
G10.2.1	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.
G10.2.2	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.
G10.3	Methodologies
G10.3.1	The most widely used method is Sanger dideoxy chain termination, which can be applied in several forms.
G10.3.2	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.
G10.3.3	Automated fluorescent sequencing can be performed using an automated sequencing instrument equipped with electrophoresis apparatus and data collection software.
G10.3.4	Capillary gel electrophoresis for sequencing is the most common detection system and has advantages over the older gel-based systems.
G10.4	DNA Preparation
G10.4.1	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.
G10.5	PCR Amplification
G10.5.1	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.
G10.5.2	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).
G10.5.3	The PCR product should be analyzed by gel and purified prior to the sequence reaction to ensure the highest quality of results.
G10.6	Primary Base Calling
G10.6.1	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. Currently available criteria include the number of positions at which computer base calling is not possible. 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.
G10.6.2	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.
G10.6.3	Sequence analysis software is needed to compare data of the wild type and patient sample in both forward and reverse directions.
G10.7	Comparison of Sequence Data with a "Within Run" Standard
G10.7.1	The comparison with a standard of a high quality sequence from the same run is also needed to identify base differences.
G10.7.2	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.
G10.8	Interpretation and Data Reporting
G10.8.1	Base differences are correlated with the known gene structure and other relevant data, and the likely effect of the base change on the gene is predicted. The laboratory must follow the *ACMG Recommendations for Standards for Interpretation of Sequence Variations* (Genet in Med 2000; 2(5):302-303).
G10.8.2	The report should note the exact base change and location by nucleotide position as referenced in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) and the corresponding position change in the protein using standard nomenclature (http://www.hgvs.org/mutnomen/).
G10.8.3	For small deletions and insertions or nonsense mutations resulting in a predicted protein truncation, the term "pathogenic mutation" is appropriate.
G10.8.4	For missense alterations, one must consider whether these represent benign or pathogenic sequence variants. For each genetic disease, the laboratory should first refer to polymorphism and pathogenic mutation databases for the specific gene. If the base alteration has not been previously described, the nature and significance of the change may be unclear and should be stated as such in the report. For resolution, family studies and population based studies are appropriate.
G10.8.5	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.
G10.9	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. For details please refer to section 5 of the ACMG Standards and Guidelines for Molecular Genetic Testing for Ultra-Rare Disorders (2006 edition), (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/URD.htm).
G11	**Detecting large insertions and deletions**
G11.1.1	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, Klinefelter syndrome, Down syndrome, etc.). 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 accurate 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.
G11.1.3	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 millionfold 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 should be approximately 1. Lastly, the internal standard should be different enough in size to be easily resolved from the PCR product of interest.
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 and

	ligation. **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 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 areas 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 to verify assay performance. An external normal copy control may be used with each run to perform statistical analyses. **Analysis**: Raw data from the sequencer can be examined 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 marco 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 interference of mutations/polymorphisms very close to the probe ligation site resulting 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 an independent method.

G12	G11.1.1
G12.1	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.
G12.2	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 G4).
G12.3	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.
G12.4	For linkage analyses involving probes with negligible recombination distances from the locus in question, it is only necessary to use one highly informative marker.
G12.5	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.
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 DNA/RNA preparation section). 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. Specimen temperature is an important consideration. Storage and shipping 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 wet ice. 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 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.
G13.2	The usual safeguards against contamination by PCR products should be used (see 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 (nested-PCR) using a nested primer pair is required to amplify low abundance mRNA transcripts. This method offers additional sensitivity as well as the added specificity. However it brings the potential for serious contamination problems. In the second round of PCR, tubes containing first-round PCR product should be opened one at a time to prevent potential tube-to-tube contamination. PCR controls including a negative (no DNA) control must also be reamplified to permit detection of low-level contamination.
G13.3.3	The 5′ primer must be 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.
G13.3.4	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.
G13.3.5	Each PCR reaction should be run in duplicate or triplicate to avoid false identification of artifactual mutations arising through amplification of chance polymerase errors.
G13.3.6	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.
G13.3.7	Amplification by RT-PCR followed by electrophoresis may reveal gross rearrangements such as gene deletions (complete or partial), insertions, splice mutations and possibly duplications.
G13.3.8	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.
G13.3.9	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 paternity, maternal cell contamination, sample mix-up, co-mingling of specimens and allele drop-out should be considered when results are not consistent with the family history or phenotype.

G15.4	For PCR assays, care must be taken to assess the possibility of differential amplification.
G16	**Records of Molecular Testing**
G16.1	Scoring sheets must contain the following information (if applicable): a) specimen numbers b) locus names tested (probe name and locus identification) c) test system used (Southern, PCR, etc.) d) mutation detection system (RFLP, ASO, etc.) e) enzymes used for RFLP analysis, lot numbers and expiration date f) alleles detected g) results h) master mix lot numbers including all components (e.g., polymerase, deoxnucleotides, magnesium chloride and buffer) i) pathogenic sequence changes, benign variants and variants of unknown clinical significance detected in sequencing assays j) properly labeled photograph with molecular weight standards k) lot number of standards l) version and name of software being used for analysis (if applicable) m) area for technologist and laboratory director to initial after analysis n) 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. See C3 for issues regarding record dissemination. In general, the report should include the following: a) collection date b) date (and time, if applicable) of receipt in the laboratory

c) specimen type

d) name of individual

e) gender (if applicable)

f) date of birth

g) ethnicity/race where appropriate

h) laboratory identification number

i) family/kindred number, if applicable

j) date of report

k) reason for testing

l) disease locus tested

m) test performed, methodology, mutations tested

n) notation of any deviation from the laboratory's standard practice

o) limitations of the assay

p) the genotype and/or haplotype established for the individual

q) a statement interpreting the data (interpretation should be understandable to a non-geneticist professional), including clinical implications, follow-up test recommendations, genetic counseling indications

r) recommendations

s) documentation if a preliminary report has been issued

t) signature of the laboratory director or technical supervisor or other authorized individual above his/her printed name.

u) a means to contact the laboratory director or designee

v) The following disclaimer for tests that are not approved by the FDA: This test was developed and its performance characteristics determined by the XXXX DNA Laboratory. It has not been cleared or approved by the Food and Drug Adminstration. 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.

w) references

	x) linkage studies should include a pedigree with the genotype information.
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. 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 et al. (*Nucleic Acid Res* 1988;16:11141-11156) 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. (*Am J Obstet Gynecol* 1997;Feb;176(2):327-33) 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 et al. (*Genet Testing* 1998;2:347-350) 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	Appendix: Methods no longer widely used
G19.1	Denaturing Gradient Gel Electrophoresis (DGGE) Assays
G19.1.1	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.
G19.1.2	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.
G19.1.3	Sample Preparation DNA samples should be prepared and stored using established protocols (see DNA preparation section, G3).
G19.1.4	Amplification of target sequences should be performed using all standard PCR precautions (see PCR section, G7).
G19.1.5	Samples should be heated and allowed to renature prior to loading to permit heteroduplex formation. Time and temperature should be standardized.
G19.1.6	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.
G19.1.7	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 re-validation of the assay. Samples to be run on the same gel should be denatured, renatured, and loaded on the gel at the same time.
G19.1.8	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.
G19.1.9	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.
G19.1.10	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.
G19.2	Single-Strand Conformation Polymorphism (SSCP) Assays
G19.2.1	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 electrophorescing 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 G7.4.1.4 (changing of electrophoretic mobility) for correct interpretation of results. Additional considerations include:
G19.2.2	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.
G19.2.3	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.
G19.2.4	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.
G19.2.5	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.
G19.2.6	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.
G19.2.7	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.
G19.3	Protein Truncation Tests for Mutation Detection
G19.3.1	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.
G19.3.2	Source of Samples The starting material for this assay is DNA or RNA obtained from any tissue by standard methodology (see DNA/RNA preparation section). 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.
G19.3.4	PCR Amplification of DNA or cDNA (see G10.4 for cDNA synthesis) The usual safeguards against contamination by PCR products should be used (see 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.
G19.3.5	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 reamplified 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.
G19.3.6	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.
G19.3.7	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.
G19.3.8	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 artifactual, 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 (see G10.5.2).

	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.
G19.3.9	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.
G19.3.10	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.
G19.4	Heteroduplex Assays
G19.4.1	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.
G19.4.2	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.
G19.4.3	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.
G19.4.4	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.
G19.4.5	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.
G19.4.6	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.