Molecular Genetic Testing:
More Than Diagnosis

MM01-A3—Molecular Methods for Clinical Genetics
and Oncology Testing; Approved Guideline—Third
Edition

Barbara Zehnbauer, PhD, FACMG
Chief, Laboratory Research and Evaluation Branch
Centers for Disease Control and Prevention
Atlanta, Georgia, USA

Polling Question

Have you experienced changes in your molecular
genetics lab test menu in the past 5 years?

☐ Yes

☐ No
Polling Question

Have you used a previous edition of MM01?

- Yes
- No

Polling Question

Are you new to CLSI Molecular Methods documents in general?

- Yes
- No
Objectives of MM01-A3

- Understand the essential quality specifications of the total testing process for accurate molecular genetic testing of disorders with a genetic basis.

- Recognize the variety of genetic variations associated with human disease and the appropriate testing methodologies for their detection.

Purpose of Third Edition

Genotyping can provide indicators of disease:

- Diagnosis
- Prognosis (likely outcome)
- Predisposition
- Progression
- Treatment selection
- Monitor treatment response
- Targets for gene-specific therapies
- Carrier screening (asymptomatic or presymptomatic)
- Prenatal
Scope of Third Edition

Inform laboratories about:
• Appropriate and effective qualities of genetics laboratory operations
• Assay design and performance specifications
• Clinical applications of these strategies
• Common molecular methods included in clinical genetic testing practice

MM01—Why a Third Edition?

• MM01-A2 = Diagnostics for genetic diseases
• Approved June 2006
• Other molecular method guidelines:
  – Infectious diseases
  – Hematopoietic cancers (leukemias and lymphomas)
• No molecular method guidelines for:
  – Other inherited disorders (added to laboratory test menus since 2006)
  – Other cancers (solid tumors)
  – Pharmacogenomics
What’s New in the Third Edition?

• Reorganized sections reflect path of workflow
• Expanded sections:
  – Mutation nomenclature
  – Test validation
  – Genetic variation
  – Prenatal testing
  – Appendixes
• Tables
  – Organized information
  – Easier reference
  – Methods comparisons

Reorganized Sections Reflect Workflow

• Preexamination
  – Family history and clinical data
  – Informed consent
  – Pretest genetic consultation
  – Specimens
    o Types and nucleic acid yield table
    o Accessioning, identification, handling, and processing
• Nucleic acid storage
• Sample retention
• Assay validation
Reorganized Sections Reflect Workflow (cont’d)

• Examination
  – Quality control program
    o Types of controls
    o Process/risk assessment
    o Sources of reference materials
    o Design challenges
    o Monitoring quality
      – Proficiency testing
      – External quality assessment
      – Alternative assessment
  – Minimizing amplification contamination

Reorganized Sections Reflect Workflow (cont’d)

• Postexamination
  – Recording results
  – Reporting results
  – Confidentiality
  – Record retention
  – Duty to recontact
    o Amended reports
    o Technology improvements
  – Genetic consultation
Mutation nomenclature
- Human Genome Organisation and Human Genome Variation Society
- Summary table by mutation type
- DNA and protein level variants
- Mitochondrial (mtDNA) variants
- Pharmacogenetics
- The American College of Medical Genetics and Genomics guidance for variants of unknown significance

Pharmacogenetic Genotypes
- Example: Cytochrome P450 variant allele nomenclature
- Cytochrome P450 2 D 6 * 4 = IVDS3, G-A +1 or 1846 G>A
  - Superfamily
  - Family
  - Subfamily
  - Isoenzyme
  - Allele variant
- Allele Nomenclature Database: [http://www.cypalleles.ki.se](http://www.cypalleles.ki.se)
- May be haplotypes: 100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; 1846G>A; 4180G>C

Test validation
• Medical devices: Classification and labeling
  – *In Vitro* diagnostics device verification or modification
  – Analyte-specific reagent and laboratory-developed tests
  – European Union and European Free Trade Association
• Clinical utility
• Analytical validation
• Clinical validation
• Limitations
• Validation report

Expanded Sections

**Test Validation—Analytical Performance**

• Analytical validation parameters
  – Accuracy
  – Reproducibility
  – Robustness
  – Sensitivity and limit of detection
  – Limit of quantification
  – Specificity
  – Instrumentation/analysis software
  – Data evaluation
### Test Validation—Clinical Performance

- Clinical validation: a randomized control trial is not always practical
  - Intended use and indications for testing
  - Literature review
  - Relevant populations and samples
  - Reference standard, reference range
  - Controls and acceptance criteria
  - Statistical analysis of results
- CLSI documents MM17 and MM19
- Sensitivity, specificity, positive predictive value, negative predictive value

### Expanded Sections

- Genetic Variation
  - Copy number variants (CNVs)
  - Tandem repeats
    - Short tandem repeats, microsatellite instability, loss of heterozygosity, trinucleotide repeats
    - Disorders caused by expansion of nucleotide repeats (see Table 4)
  - Mitochondrial disorders (see Table 5)
  - Drug response: pharmacogenetics overview (see Table 6)
- Epigenetics
- Uniparental disomy (see Table 7)
- Circulating tumor cells and nucleic acids
Prenatal testing for heritable disorders

• Specimen types
• Validation of tests
• Specimen handling
• Information required
• Sample processing
• Maternal cell contamination assessment
• Reporting requirements

Expanded Sections (cont’d)

• Summary of commonly used molecular methods (see Table 8)
• Basic methods
  – Electrophoresis
  – Southern blot hybridization
  – Polymerase chain reaction (PCR), reverse transcription PCR, and real-time PCR
  – Methylation PCR
  – DNA sequencing – (CLSI document MM09 is in revision.)
    o Dye terminator
    o Pyrosequencing
    o Methylation sequencing
    o Next generation sequencing (overview)
Expanded Sections (cont’d)

• Mutation scanning methods
  – High-resolution melting (HRM) analysis
  – Denaturing high-performance liquid chromatography
  – Multiplex ligation-dependent probe amplification (MLPA)
  – Quantitative multiplex PCR
  – Hydrolysis probes for CNVs
  – Screening microarrays (CLSI document MM12 is in revision.)
• Direct mutation detection
  – Allele-specific oligonucleotide, amplification refractory mutation system, restriction fragment length polymorphism, oligonucleotide ligation assay
  – Liquid bead arrays

Tables: Summaries and Comparisons

• Mutations: types, nomenclature, effect
• Specimens: types, applications, nucleic acid yield
• Assay validation parameters
• Nucleotide repeat disorders
• Mitochondrial disorders
• Pharmacogenetic markers
• Uniparental disomy syndromes
• Molecular methods
### Mutation Nomenclature Table

<table>
<thead>
<tr>
<th>Type of Change</th>
<th>AAA CAG ATA AGC</th>
<th>DNA Level</th>
<th>Protein Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense</td>
<td>ACA CAG ATA AGC</td>
<td>c.8A&gt;G</td>
<td>p.Lys3Thr</td>
</tr>
<tr>
<td>Nonsense</td>
<td>TAA</td>
<td>c.7A&gt;T</td>
<td>p.Lys3X</td>
</tr>
<tr>
<td>Frameshift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indels</td>
<td>CTA [AG ATA] AGC</td>
<td>c.11_15delinsTA</td>
<td>p.Gln4_Met5delinsLeu</td>
</tr>
</tbody>
</table>

Abbreviations: DNA, deoxyribonucleic acid; indels, insertions and deletions.

### Examples: Specimen Type/Nucleic Acid Yield

<table>
<thead>
<tr>
<th>Testing</th>
<th>Specimen</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heritable disorders</td>
<td>Whole blood (7 × 10⁶ WBCs)</td>
<td>16–50 mcg</td>
</tr>
<tr>
<td></td>
<td>Buccal swab</td>
<td>0.2–2 mcg</td>
</tr>
<tr>
<td>Oncology</td>
<td>Tissue (5 mg or 0.5 × 10⁶ cells)</td>
<td>DNA: 16–50 mcg RNA: ≈10 mcg</td>
</tr>
<tr>
<td>Prenatal</td>
<td>Amniotic fluid (1–2 × 10⁶ cells)</td>
<td>7 mcg</td>
</tr>
<tr>
<td></td>
<td>Chorionic villi (10 mg)</td>
<td>5–100 mcg</td>
</tr>
</tbody>
</table>

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; WBC, white blood cell.
### Nucleotide Repeat Disorders—Examples

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Gene</th>
<th>Repeat</th>
<th>Mutation Alleles</th>
<th>Position of Repeat</th>
<th>Detection Expansion</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragile X</td>
<td>FMR1</td>
<td>CGG</td>
<td>Pre: 55–200</td>
<td>5’ UTR</td>
<td>PCR, Southern blot</td>
<td>X-linked</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Full: &gt;200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td>HTT</td>
<td>CAG</td>
<td>&gt;40</td>
<td>Exon 1</td>
<td>PCR, Southern blot</td>
<td>AD</td>
</tr>
<tr>
<td>SCA1</td>
<td>ATXN1</td>
<td>CAG</td>
<td>39–91</td>
<td>Exon 8</td>
<td>PCR</td>
<td>AD</td>
</tr>
</tbody>
</table>

Abbreviations: AD, autosomal dominant; HD, Huntington disease; PCR, polymerase chain reaction; UTR, untranslated region.

### Pharmacogenetic Markers—Examples

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Drug</th>
<th>Indications for use</th>
<th>Clinical Utility</th>
<th>Clinical Testing Available</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D6</td>
<td>Mono-oxygenase</td>
<td>Trastuzumab</td>
<td>Breast cancer</td>
<td>Select responders</td>
<td>Yes</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Mono-oxygenase</td>
<td>Warfarin</td>
<td>VTE</td>
<td>Dose selection</td>
<td>Yes</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Glucuronidase</td>
<td>Irinotecan</td>
<td>Colorectal cancer</td>
<td>Dose selection; avoid toxicity</td>
<td>Yes</td>
</tr>
<tr>
<td>EGFR</td>
<td>Receptor tyrosine kinase</td>
<td>Erlotinib, gefitinib</td>
<td>Lung cancer</td>
<td>Treatment selection</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Abbreviation: VTE, venous thromboembolism.
Table 8 summarizes analytical methods.

• Side-by-side comparisons
• Descriptions
• Advantages
• Limitations

### Example

<table>
<thead>
<tr>
<th>Assay</th>
<th>Description</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
</table>
| HRM   | Detection of different melting profiles of double-stranded DNA amplicons depending on sequence | • Highly sensitive for heterozygote detection  
• Does not require postamplification processing  
• Nondestructive, allowing for downstream amplicon analysis  
• Can be high-throughput | • Variants detected must be sequenced to determine exact nature of change  
• Limited sensitivity for detecting homozygotes, large indels, and inversions |
| MLPA  | Multiplex assay; probe hybridization, ligation, PCR, size separation | • Discriminates sequences that differ by only a single nucleotide  
• Up to 45 loci can be assayed in a single tube  
• High-throughput  
• Small amounts of DNA (20 ng) | • Deletions detected by a single probe require independent confirmation |

Abbreviations: DNA, deoxyribonucleic acid; HRM, high-resolution melting; indels, insertions and deletions; MLPA, multiplex ligation-dependent probe amplification.
Example of a Failure Modes and Effects Analysis

• Cystic fibrosis transmembrane conductance regulator (CFTR) multiplex hybridization
  – Failure modes
  – Potential effects
  – Severity
  – Potential cause
  – Frequency of occurrence
  – Controls
  – Detection
  – Risk control measures
  – Risk priority number (RPN)
### Appendix A. Example of a Failure Modes and Effects Analysis

**Product/Process Name:** CFTR multiplex hybridization (laboratory-developed test)

**Prepared by:** FMEA Team

<table>
<thead>
<tr>
<th>Scoring</th>
<th>Severity</th>
<th>Occurrence</th>
<th>Detection</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 – Life-threatening event</td>
<td>5 – Very High (&gt;1/10)</td>
<td>5 – None (&lt;1%)</td>
<td>Critical</td>
<td>&gt;50</td>
</tr>
<tr>
<td>4 – Incorrect genotype calls or similar performance-related problem</td>
<td>4 – High (1/10)</td>
<td>4 – Low (2% to 35%)</td>
<td>Major</td>
<td>18-50</td>
</tr>
<tr>
<td>3 – Laboratory considers failure mode to be significant</td>
<td>3 – Moderate (1/100)</td>
<td>3 – Moderate (35% to 65%)</td>
<td>Minor</td>
<td>&lt;18</td>
</tr>
<tr>
<td>2 – Laboratory notices moderate problem</td>
<td>2 – Low (1/1000)</td>
<td>2 – Likely (65% to 99%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – Laboratory not aware</td>
<td>1 – Remote (&lt;1/100000)</td>
<td>1 – Certain (&gt;99%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Intended Use:** This assay will be used to screen adults for carrier status of the 23 mutations recommended by the American College of Obstetricians and Gynecologists and the American College of Medical Genetics, and to diagnose CF in newborns and children.

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FMEA, failure modes and effects analysis.

### Design/Process Input

<table>
<thead>
<tr>
<th>Specimen Preparation</th>
<th>Potential Failures/Modes</th>
<th>Potential Effects</th>
<th>Potential Cause of Failure</th>
<th>Controls</th>
<th>Proposed</th>
<th>Risk Control Measures</th>
<th>References</th>
<th>Score</th>
<th>Risk</th>
<th>RPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Poor yield</td>
<td>No PCR amplification, no single genotype call(s)</td>
<td>Operator or equipment malfunctions causing equipment failure</td>
<td>3</td>
<td>Add PCR step to control for temperature; add control to test</td>
<td>Add PCR control to control for temperature</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Poor yield</td>
<td>No PCR amplification, no single genotype call(s)</td>
<td>Operator or equipment malfunctions causing equipment failure</td>
<td>3</td>
<td>Add PCR step to control for temperature; add control to test</td>
<td>Add PCR control to control for temperature</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Poor yield</td>
<td>No PCR amplification, no single genotype call(s)</td>
<td>Operator or equipment malfunctions causing equipment failure</td>
<td>3</td>
<td>Add PCR step to control for temperature; add control to test</td>
<td>Add PCR control to control for temperature</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Poor yield</td>
<td>No PCR amplification, no single genotype call(s)</td>
<td>Operator or equipment malfunctions causing equipment failure</td>
<td>3</td>
<td>Add PCR step to control for temperature; add control to test</td>
<td>Add PCR control to control for temperature</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: DNA, deoxyribonucleic acid; RPN, risk priority number.*
Appendix B. Bayesian Calculations

Bayesian Calculation for the Consultand’s Husband

<table>
<thead>
<tr>
<th>Prior probability (based on carrier frequency in the general population)</th>
<th>Husband is a carrier</th>
<th>Husband is not a carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/25</td>
<td>24/25</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conditional probability (given a negative test result and 89% mutation detection rate)</th>
<th>Husband is a carrier</th>
<th>Husband is not a carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>11%</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Joint probability (product of the prior and conditional probability)</th>
<th>Husband is a carrier</th>
<th>Husband is not a carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1/25) × (11/100) = 11/2500</td>
<td>(24/25) × 1 = 2400/2500</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conditional probability (final risk of a given event)</th>
<th>Husband is a carrier</th>
<th>Husband is not a carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>(11/2500) / (11/2500 + 2400/2500) = 0.46% or 1 in 219</td>
<td>(2400/2500) / (2400/2500 + 11/2500) = 99.54%</td>
<td></td>
</tr>
</tbody>
</table>

Appendix C. Comparison of Methods to Detect Small Genetic Variations

<table>
<thead>
<tr>
<th>Platform</th>
<th>K/R A kit</th>
<th>Reported sensitivity</th>
<th>Mutations detected</th>
<th>Mutations reported</th>
<th>Equipment required</th>
<th>Primary advantages</th>
<th>Primary disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanger sequencing</td>
<td>No</td>
<td>20% All, including indels</td>
<td>All, including indels</td>
<td>Thermocycler, capillary electrophoresis system</td>
<td>Considered gold standard assay</td>
<td>Low sensitivity</td>
<td></td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Yes</td>
<td>5% All, including indels</td>
<td>All, including indels</td>
<td>Thermocycler, pyrosequencer</td>
<td>High sensitivity (1% to 5%)</td>
<td>Capital cost of instrument, higher reagent costs</td>
<td></td>
</tr>
<tr>
<td>Reverse line blot</td>
<td>Yes</td>
<td>1% codon 12:6 of 6 possible codons 13:2 of 6 possible codon 61:0 of 9 possible</td>
<td>12:1; G&gt;A, C&gt;T; 12:2; G&gt;A, C&gt;T; 13:1; G&gt;T, 13:2; G&gt;A</td>
<td>Thermocycler, scanner</td>
<td>High sensitivity (&gt;1%)</td>
<td>Only detects a subset of mutations in codons 12, 13, 61</td>
<td></td>
</tr>
</tbody>
</table>
Appendix D. Molecular Technologies No Longer Commonly Used

- Denaturing gradient gel electrophoresis
- Chemical mismatch cleavage
- Ribonuclease A cleavage
- Heteroduplex analysis
- Single-stranded conformational polymorphism
- Linkage analysis

References


References (cont’d)


Other Resources

- 321 References
  - Published articles
  - Many website citations
- Appendixes B and C have additional references
- Section 2.3: Genetic Information
  Nondiscrimination Act & Health Insurance Portability and Accountability Act
Document Development Committee Members

- Kristin G. Monaghan, PhD, FACMG – Co-Chairholder
- Barbara Zehnbauer, PhD, FACMG – Co-Chairholder
- Jessica K. Booker, PhD, FACMG
- Harriet Feilotter, PhD, FCCMG
- Renee M. Howell, PhD
- Jennifer JS Laffin, PhD, FACMG
- Francisco Martinez-Murillo, PhD
- Ronald M. Przygodzki, MD
- Venkatakrishna Shyamala, PhD
- Judy Yu, PhD

Polling Question

Will you use the new MM01 topics sections in your clinical lab practice?

- [ ] Yes
- [ ] No
Suggestions from the Audience

• For which other Molecular Methods topics do you see a need for guidelines?

• What tools could help you implement the MM01 recommendations in your lab practice?

Please enter suggestions into the comment box or e-mail to ikc8@cdc.gov

Questions?
Thank you!