



Molecular laboratory design, QA/QC considerations

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Overview

- ❑ Regulations and Guidelines
- ❑ Molecular Laboratory Design
- ❑ Monitor and Prevent Cross Contamination
- ❑ Assay Validation
- ❑ Quality of Reagents and Controls
- ❑ Use of Controls
- ❑ Specimen Acceptance Criteria
- ❑ Proficiency Testing
- ❑ Standardized Nomenclature

Laboratory Regulatory and Accreditation Guidelines

- ❑ US Food and Drug Administration (FDA):
 - ❑ approves kits and reagents for use in clinical testing
- ❑ Clinical Laboratory Improvement Amendments (CLIA):
 - ❑ Regulations passed by Congress 1988 to establish quality standards for all laboratory testing to ensure the accuracy, reliability and timeliness of patient test results regardless of where the test was performed
- ❑ College of American Pathologists (CAP):
 - ❑ Molecular Pathology checklist
- ❑ State Specific Regulations

Professional Guidelines

- ❑ American College of Medical Genetics (ACMG)
 - ❑ Standards and Guidelines for Clinical Genetics Laboratories
- ❑ Clinical and Laboratory Standards Institute (CLSI)
 - ❑ MM01-A2: Molecular Diagnostic Methods for Genetic Diseases
 - ❑ MM05-A: Nucleic acid amplification assays for molecular hemathpathology
 - ❑ MM09-A: Nucleic acid sequencing methods in diagnostic laboratory medicine
 - ❑ MM13-A: Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods
 - ❑ MM14-A: Proficiency Testing (External Quality Assessment) for Molecular Methods
 - ❑ MM17-A: Verification and Validation of Multiplex Nucleic Acid Assays
 - ❑ MM19-P: Establishing Molecular Testing in Clinical Lab Environments

Contamination

Introduction of unwanted nucleic acids into specimen

- the sensitivity of PCR techniques makes them vulnerable to contamination

Repeated amplification of the same target sequence leads to accumulation of amplification products in the laboratory environment

- ❑ A typical PCR generates as many as 10^9 copies of target sequence
- ❑ Aerosols from pipettes will contain as many as 10^6 amplification products
- ❑ Buildup of aerosolized amplification products will contaminate laboratory reagents, equipment, and ventilation systems

Potential Sources of Contamination

- ❑ Cross contamination between specimens
- ❑ Amplification product contamination
- ❑ Laboratory surfaces
- ❑ Ventilation ducts
- ❑ Reagents/supplies
- ❑ Hair, skin, saliva, and clothes of lab personnel

Setting Up a Molecular Laboratory

- ❑ Mechanical barriers to prevent contamination
- ❑ Spatial separation of pre- and post-amplification work areas
 - Area 1 – Reagent preparation
 - Area 2 – Specimen preparation
 - Area 3 – Amplification/product detection
- ❑ Physically separated and, preferably, at a substantial distance from each other

Unidirectional Flow

- ❑ Both personnel and specimens
- ❑ Amplification product-free to product-rich
- ❑ Remove PPE before leaving one area
- ❑ Avoid or limit reverse direction
- ❑ Reusable supplies in the reverse direction need to be bleached.

Features of the 3 Areas

- Each area has separate sets of equipment and supplies
 - Refrigerator/freezer (manual defrost)
 - Pipettes, tips, tubes, and racks
 - Centrifuge, timers, vortex
 - Lab coat (color-coded), disposable gloves, safety glasses, and other PPE
 - Cleaning supplies
 - Office supplies
 - Ventilation system
- Dead air box with UV light – serves as a clean bench area

Features of the 3 Areas

- Air pressure
 - Reagent Prep and Specimen Prep – Positive
 - Postamplification - Negative
- Reagent Prep – Single entrance, reagents used for amplification should not be exposed to other areas
- Specimen Prep – Specimens should not be exposed to post-amplification work areas

Laboratory Design Example

Mitchell P. S. et al. Nucleic Acid Amplification Methods: Laboratory Design and Operations, 2004, In "Molecular Microbiology: Diagnostic Principles and Practice, edited by D. H. Persing et al" 99. 85-93.

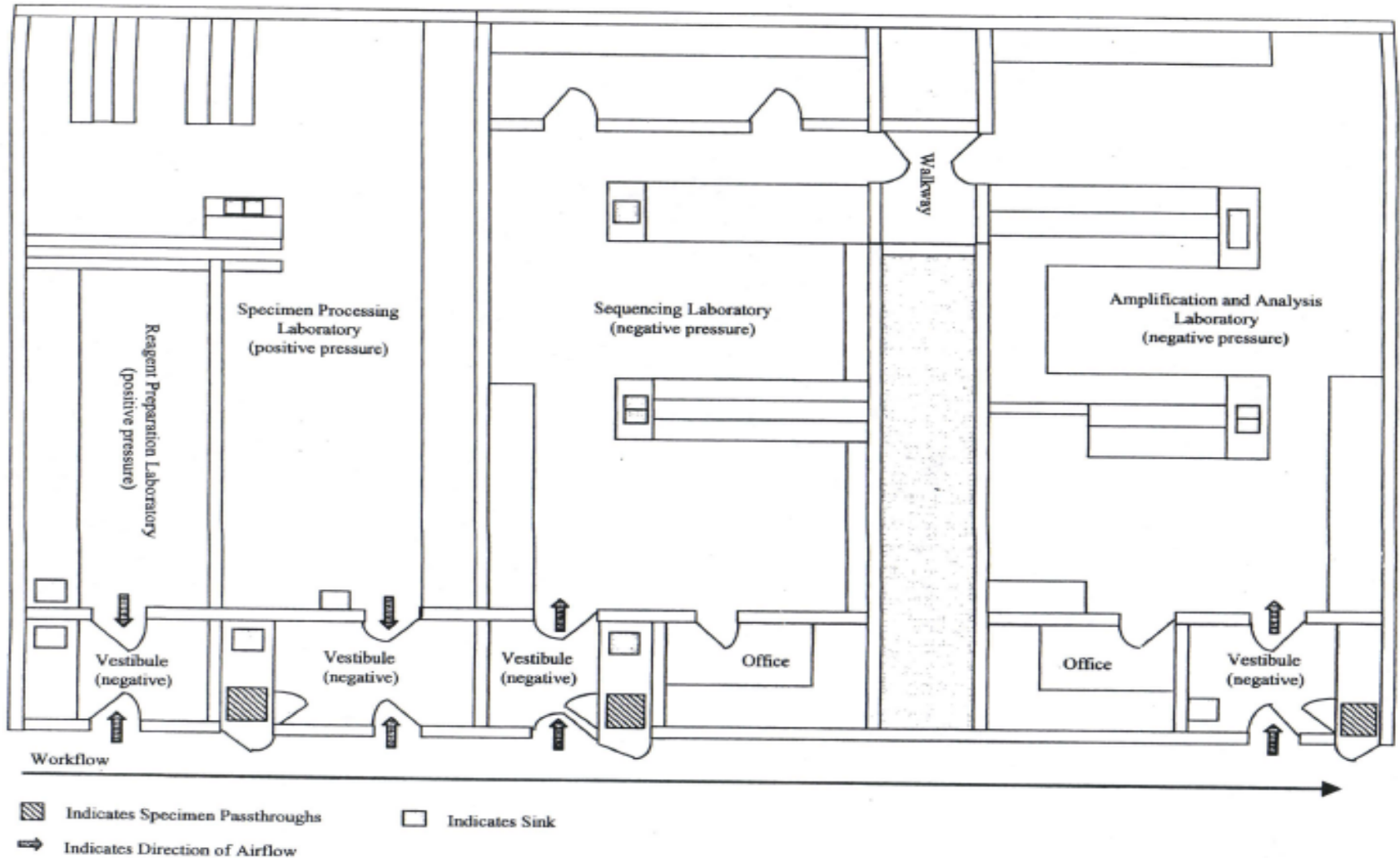


Figure 1. Recommended design for a molecular microbiology facility.

Two Areas Only

- Area 1 – Reagent prep, specimen prep, and target loading – use of laminar-flow hoods
- Area 2 – Amplification/product detection



Alternative to Spatial Separation



- ❑ Class II biological safety cabinet
- ❑ Dedicated areas for each work phase
- ❑ Unidirectional
- ❑ Automated specimen processing station/closed-tube amplification and detection system

Important Details

- Use of positive displacement pipettes and disposable filter tip pipette tips
- Avoid production of aerosols when pipetting
- Use of sterilized single-use plasticware
- Minimizes the risk of amplicon carry-over on clothing, hair and skin
 - Hairnet
 - Dedicated safety glasses
 - Disposable labcoat/gown
 - Gloves
 - Shoe covers



Contamination: Chemical and Enzymatic Barriers

- ❑ Work stations should all be cleaned with 10% sodium hypochlorite solution (bleach), followed by removal of the bleach with ethanol.
- ❑ Ultra-violet light irradiation
 - ❑ UV light induces thymidine dimers and other modifications that render nucleic acid inactive as a template for amplification
- ❑ Enzymatic inactivation with uracil-N-glycosylase
 - ❑ Substitution of uracil (dUTP) for thymine (dTTP) during PCR amplification
 - ❑ New PCR sample reactions pre-treated with Uracil-N-glycosylase (UNG) – contaminating PCR amplicons are degraded leaving only genomic DNA available for PCR

More Important Details

- Use of nuclease free or autoclaved water
- Aliquot oligonucleotides – multiple freeze thaws will cause degradation
- Always include a blank (no template) control to check for contamination
- Wipe test
 - Monthly
 - Detect and localize the contamination
 - Identify the source of the contamination

Decontamination Approaches

- Clean the work area & equipments routinely
 - Clean the PCR workstation at the start and end of each work day/run (UV light, 70% ethanol, fresh 10% sodium hypochlorite, DNA Away)
 - Clean the exterior and interior parts of the pipette
 - Clean the equipment
 - Clean the doorknobs, handle of freezers

Other Considerations

- ❑ Temperature and humidity requirements
- ❑ Exhaust ventilation
- ❑ Water quality
- ❑ Back-up power system

When is a Validation/Verification Study Required?

- ❑ Introduce a new testing system
 - New analyte
 - Analyte previously measured/detected on an alternate system
- ❑ An analyte added to a test system
- ❑ A modification to a test system
- ❑ Applies to
 - Unmodified, FDA-cleared or approved method
 - Modified, FDA-cleared or approved method
 - In-house method
 - Standardize method such as textbook procedure
- ❑ Determine analytic performance of an assay

Assay Validation

- ❑ Accuracy: Verify the method produces the correct results
 - Test reference materials (known positive and negative specimens)
 - Compare test results vs. reference method
 - Compare split sample results
 - Compare results to clinical diagnosis
- ❑ Sample for accuracy study
 - Patient samples with known results
 - QC materials
 - PT materials

Assay Validation (cont.)

- Precision: Measure of the reproducibility
 - Day-to-day variance
 - Run-to-run variance
 - Within-a-run variance
 - Operator variance
- Repeat testing of samples, e.g. known patient or QC samples, over time

Assay Validation (cont.)

- Analytical Sensitivity: Minimum detection limit
 - Quantitate amount of RNA or DNA extracted
 - Control material of known concentration or copy number

Assay Validation (cont.)

- ❑ Analytical Specificity: Detect only the analyte intended to be measured
- ❑ Interfering Substances: Document from product information, literature, or own testing
 - Anticoagulant
 - Specimen type (DBS)
- ❑ Reportable Range: Upper and lower limits of the testing system, presence and absence of mutations
- ❑ Reference Interval: Document the normal values

Conducting a Validation Study

- Planning
 - Determine the number and type of specimens
 - Study duration
 - Establish acceptance criteria
 - Method limitation
 - State the methods to resolve discrepancies
- Testing
- Data Collection and Analysis
- Resolving Discrepancies
 - Sequence amplicon
 - Test sample by another laboratory

Conducting a Validation Study (cont.)

□ Implementation

- Review and Approval by Lab Director
- SOP
- Assure ongoing QA
- PT

Reagents

- ❑ Labeling Reagents:
 - ❑ Content, quantity, concentration
 - ❑ Lot #
 - ❑ Storage requirements (temperature etc.)
 - ❑ Expiration date
 - ❑ Date of use/disposal
 - ❑ Know your critical reagents (enzymes, probes, digestion and electrophoresis buffers) and perform QC checks as appropriate

Critical Molecular Assay Components

- ❑ Nucleic Acids: Prepare aliquots appropriate to workflow to limit freeze-thaw cycles
 - ❑ Primers and probes
 - ❑ dNTPs
 - ❑ Genomic DNA
 - ❑ 4-8°C
 - ❑ -15 to -25°C
- ❑ Enzymes
 - ❑ Benchtop coolers recommended
- ❑ Fluorescent reporters
 - ❑ Limit exposure to light
 - ❑ Amber storage tubes or wrap in shielding (foil)

Controls for Each Run

Appropriate positive, negative and no template controls should be included for each run of specimens being tested

Molecular Assay Controls

- ❑ Positive and negative controls:
 - ❑ Inhibitors
 - ❑ Component failure
 - ❑ Interpretation of results
 - ❑ Sources:
 - ❑ Residual positive DBS
 - ❑ PT samples
 - ❑ QC materials through purchase or exchange

- ❑ No template controls:
 - ❑ Nucleic acid contamination

Positive Controls

Ideally should represent each target allele used in each run

May not be feasible when:

- ❑ Highly multiplex genotypes possible
 - ❑ Systematic rotation of different alleles as positives
- ❑ Rare alleles
 - ❑ Heterozygous or compound heterozygous specimens

Positive Controls

- ❑ Assays based on presence or absence of product
 - ❑ Internal positive amplification controls to distinguish true negative from false due to failure of DNA extraction or PCR amplification
- ❑ PCR amplification product of varying length
 - ❑ Specimens representing short and long amplification products to control for differential amplification
- ❑ Quantitative PCR
 - ❑ Controls should represent more than one concentration
 - ❑ Control copy levels should be set to analytic cut-offs

In Newborn Screening

How can you control for presence of sufficient amount/quality of DNA for a PCR based test in a NBS lab?

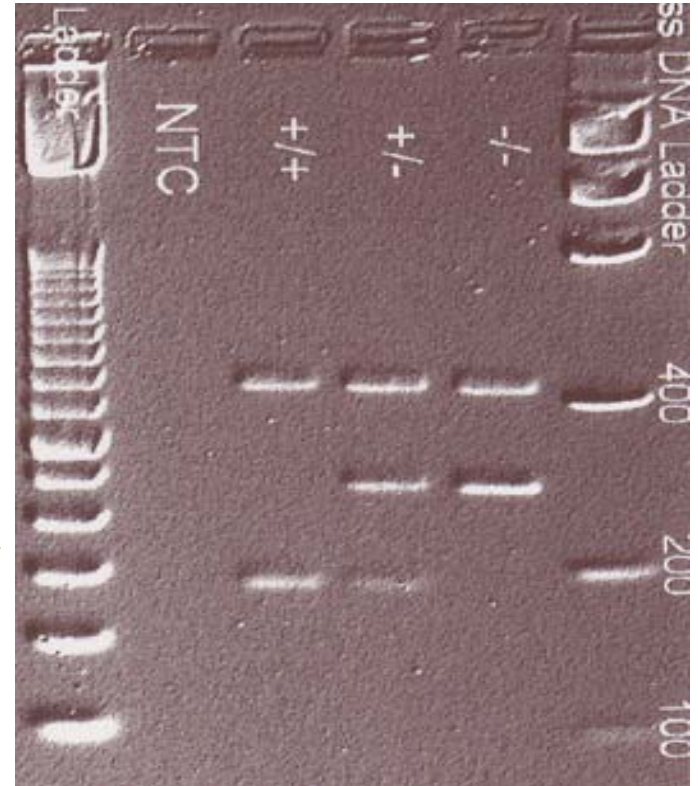
PCR with Internal Controls

Tetra-primer ARMS-PCR

Simultaneous amplification of:

- Positive amplification control
- Mutation allele
- Reference allele

Alternative to tetra-primer ARMS is to include an additional primer set to amplify a different control sequence



False Negative: ADO

Allele drop-out (ADO): the failure of a molecular test to amplify or detect one or more alleles

- ❑ Potential causes:
 - ❑ DNA template concentration
 - ❑ Incomplete cell lysis
 - ❑ DNA degradation
 - ❑ Non-optimized assay conditions
 - ❑ Unknown polymorphisms in target sites
 - ❑ Reagent component failure
- ❑ Major concern for screening laboratories
 - ❑ Confirmation of mutation inheritance in families may not an option

False Positives

- ❑ Potential causes:
 - ❑ Non-optimized assay conditions
 - ❑ Unknown polymorphisms in target sites
 - ❑ Gene duplications
 - ❑ Oligonucleotide mis-priming at related sequences
 - ❑ Pseudogenes or gene families
 - ❑ Oligonucleotide concentrations too high
 - ❑ Nucleic acid cross-contamination

Sample Acceptance and Tracking

- ❑ Special specimen acceptance criteria?
- ❑ Assign a unique code to each patient
- ❑ Use two patient-identifiers at every step of the procedure
- ❑ Develop worksheets and document every step
- ❑ Positive ID

Proficiency Testing

- ❑ Assessment of the Competence in Testing
- ❑ Required for all CLIA/CAP certified laboratories
- ❑ Performed twice a year
- ❑ If specimens are not commercially available alternative proficiency testing program has to be established (specimen exchange etc.)

Molecular Assay Proficiency Testing Material Sources

- ❑ CDC NSQAP
- ❑ UKNEQS
- ❑ EuroGentest
- ❑ CAP
- ❑ Maine Molecular
- ❑ SeraCare
- ❑ Corielle
- ❑ ECACC
- ❑ In-house samples
- ❑ Round-robin with other NBS laboratories

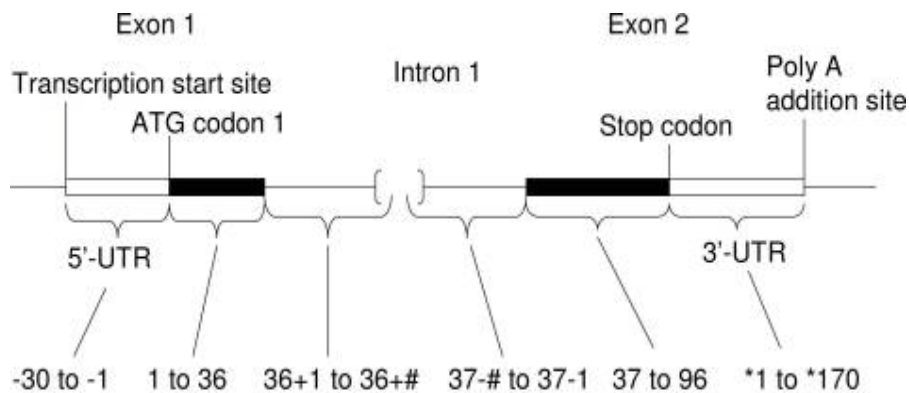
Mutation Nomenclature

❑ Uniform mutation nomenclature

- ❑ Den Dunnen & Antonarakis (2001) Hum Genet 109:121-124
- ❑ Den Dunnen & Paalman (2003) Hum Mutat 22:181-82
- ❑ Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>)

❑ Conventional notation should be retained for “established” clinical alleles

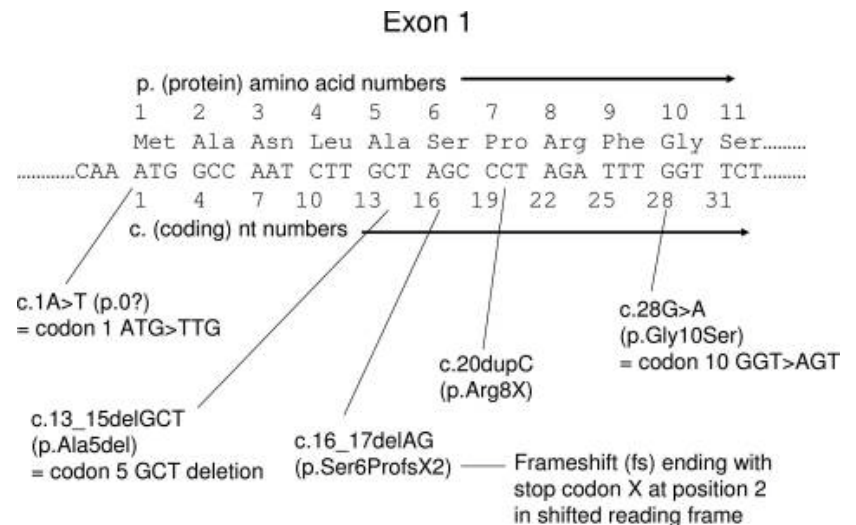
STANDARD NOMENCLATURE FOR GENES AND MUTATIONS



indicates any positive integer number

Standard mutation nomenclature based on a coding DNA sequence

Nucleotide numbering based on a coding DNA sequence



Source:
Ogino, et al (2007) J Mol Diagn 9:1-6

Examples of Mutation Nomenclature: CFTR

Commonly used colloquial nomenclature	DNA sequence change: NM_000492.3	Amino acid change (three-letter code)	Site of mutation (exon/intron)*	Type of mutation
5T/7T/9T polymorphism - 5T	c.1210-12[5]		Intron 8 (no. 9)	Splice site
1717-1G>A	c.1585-1G>A		Intron 10 (no. 11)	Splice site
Delta F508	c.1521_1523delCTT	p.Phe508del	Exon 10 (no. 11)	In-frame deletion
R553X	c.1657C>T	p.Arg553X	Exon 11 (no. 12)	Nonsense
3569delC	c.3437delC	p.Ala1146ValfsX2	Exon 18 (no. 21)	Frameshift
N1303K	c.3909C>G	p.Asn1303Lys	Exon 21 (no. 24)	Missense

*Conventional *CFTR* exon/intron numbering includes exons 6a and 6b, exons 14a and 14b, and exons 17a and 17b; for exon/intron numbers in parentheses, these exon pairs are numbered sequentially without modifiers such as '6a' and '6b.'

Additional Sources



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Medical Genetics: Translating Genes Into Health®

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Morbidity and Mortality Weekly Report
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Good Laboratory Practices for Molecular Genetic Testing for Heritable Diseases and Conditions

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