Molecular Laboratory Design and QA/QC Considerations

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Texas Department of State Health Services

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Laboratory Regulatory and Accreditation Guidelines

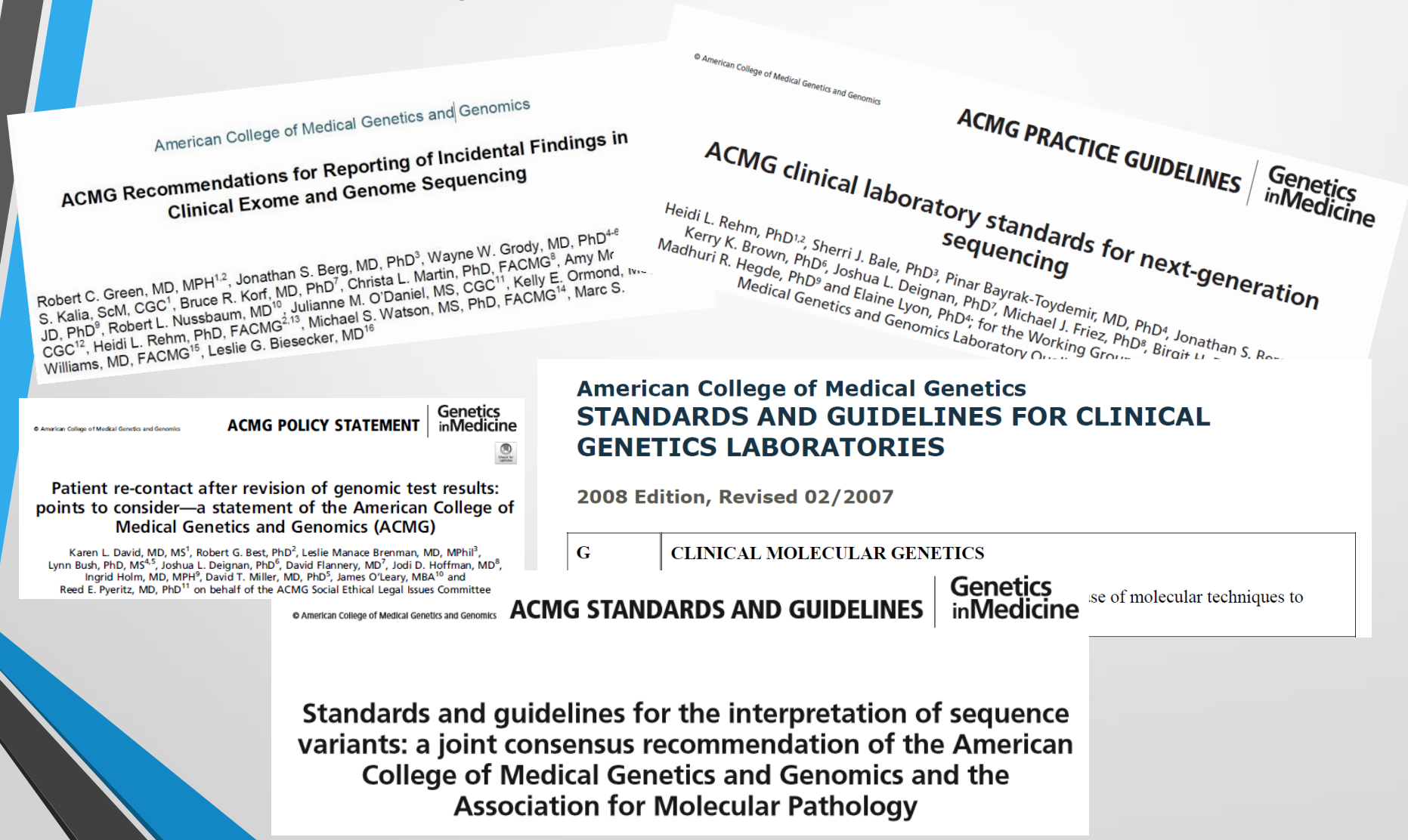
- US Food and Drug Administration (FDA):
  - Regulate manufacturers and devices under the Federal Food, Drug, and Cosmetic Act
  - Approve or clear kits for use in clinical testing (e.g. MiSeqDx Cystic Fibrosis 139-Variant Assay)
  - Analyte-specific reagent (ASR) regulation
  - FDA oversight for Laboratory Developed Test? – allow for further public discussion
- Clinical Laboratory Improvement Amendments (CLIA) - Centers for Medicare & Medicaid Services (CMS):
  - 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
  - Categorizes tests based on complexity – Nonwaived, High Complexity
  - No Molecular genetics specialty – under general requirements

Laboratory Regulatory and Accreditation Guidelines

- College of American Pathologists (CAP):
  - Laboratory General Checklist
  - All Common Checklist
  - Molecular Pathology checklist
- International Organization for Standardization standards
  - ISO 17025:2005 General requirements for the competence of testing and calibration laboratories
  - ISO 15189:2012 Medical laboratories -- Requirements for quality and competence
- State Specific Regulations
  - NY Clinical Laboratory Evaluation Program (CLEP)
  - WA Medical Test Site Licensure
  - CA Clinical Laboratory Licensure

Professional Guidelines

- American College of Medical Genetics and Genomics (ACMG)



Professional Guidelines

- Clinical and Laboratory Standards Institute (CLSI)

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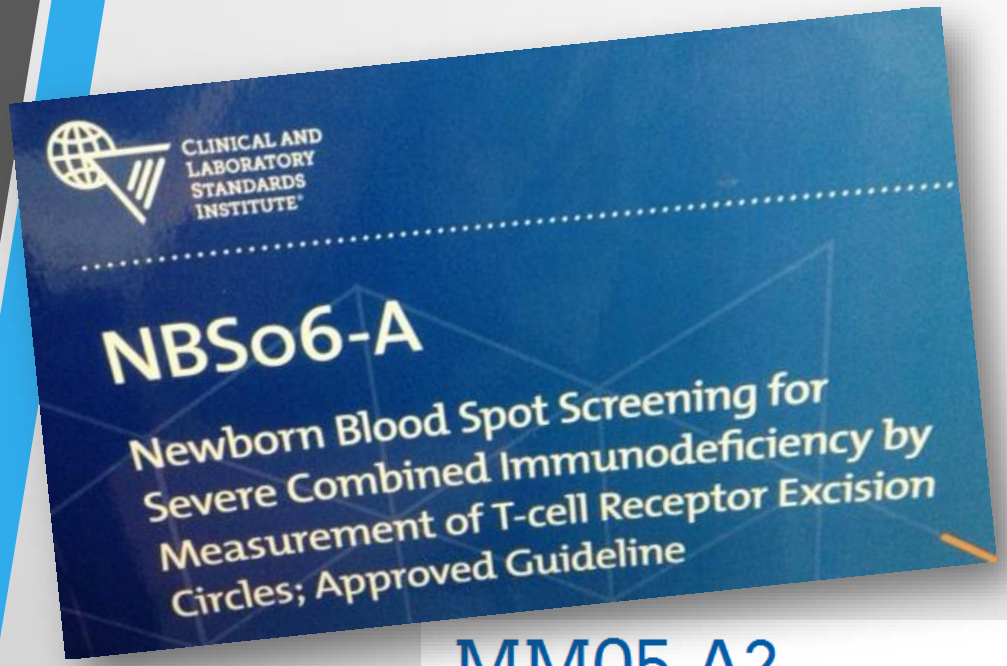


MM05-A2

Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline—Second Edition

Preview Sample Pages

This guideline addresses the performance and application of assays for gene rearrangement and translocations by both polymerase chain reaction (PCR) and reverse-transcriptase PCR techniques, and includes information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

Professional Guidelines

The Journal of Molecular Diagnostics

Volume 17, Issue 2, March 2015, Pages 107–117

Special article

Reporting Incidental Findings in Genomic Scale Clinical Sequencing—A Clinical Laboratory Perspective : A Report of the Association for Molecular Pathology

Madhuri Hegde[*,†,‡], Sherri Bale[*,§], Pinar Bayrak-Toydemir[*,¶,‖], Jane Gibson[*,**], Linda Jo Bone Jeng[*,††], Loren Joseph[*,‡‡], Jordan Laser[*,§§], Ira M. Lubin[*,¶¶], Christine E. Miller[*,‖], Lainie F. Ross[*,‖‖,***], Paul G. Rothberg[*,†††], Alice K. Tanner[*,†,‡], Patrik Vitazka[*,§], Rong Mao[*,¶,‖]

AMP Association for Molecular Pathology

Molecular Diagnostic Assay Validation

Update to the 2009 AMP Molecular Diagnostic Assay Validation White Paper

Members of the 2013 and 2014 Clinical Practice Committees of the Association for Molecular Pathology

CAP LABORATORY IMPROVEMENT PROGRAMS

College of American Pathologists' Laboratory Standards for Next-Generation Sequencing Clinical Tests

Nazneen Aziz, PhD; Qin Zhao, PhD; Lynn Bry, MD, PhD; Denise K. Driscoll, MS, MT(ASCP)SBB; Birgit Funke, PhD; Jane S. Gibson, PhD; Wayne W. Grody, MD; Madhuri R. Hegde, PhD; Gerald A. Hoeltge, MD; Debra G. B. Leonard, MD, PhD; Jason D. Merker, MD, PhD; Rakesh Nagarajan, MD, PhD; Linda A. Palicki, MT(ASCP); Ryan S. Robetorye, MD; Iris Schrijver, MD; Karen E. Weck, MD; Karl V. Voelkerding, MD

Molecular Laboratory Design

CLIA requirement

Part 493 – Laboratory Requirements
Subpart K – Quality Systems for Nonwaived Testing

Ensure that the physical plant and environmental conditions of the laboratory are appropriate for the testing performed and provide a safe environment in which employees are protected from physical, chemical, and biological hazards

CAP Molecular Pathology Checklist

MOL.35350 Carryover **Phase II**

Nucleic acid amplification procedures (*e.g.* PCR) are designed to minimize carryover (false positive results) using appropriate physical containment and procedural controls.

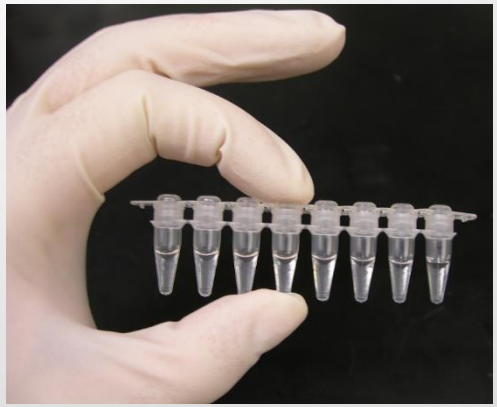
*NOTE: This item is primarily directed at ensuring **adequate physical separation** of pre- and post-amplification samples to avoid amplicon contamination. The extreme sensitivity of amplification systems requires that the laboratory take special precautions. For example, pre- and post-amplification samples should be manipulated in physically separate areas; **gloves** must be worn and frequently changed during processing; **dedicated pipettes** (positive displacement type or with aerosol barrier tips) must be used; and manipulations must minimize aerosolization. In a given run, specimens should be ordered in the following sequence: **patient samples, positive controls, negative controls** (including "no template" controls in which target DNA is omitted and therefore no product is expected). **Enzymatic destruction** of amplification products is often helpful, as is real-time measurement of products to avoid manual manipulation of amplification products.*

Evidence of Compliance:

- ✓ Written procedure that defines the use of physical containment and procedural controls as applicable to minimize carryover

Potential Sources of Contamination

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



What Happens If Lack Of Contamination Control

- Incorrect results
- Loss of creditability
- Require extensive cleanup
- Financial and performance impact

How to Control Contamination

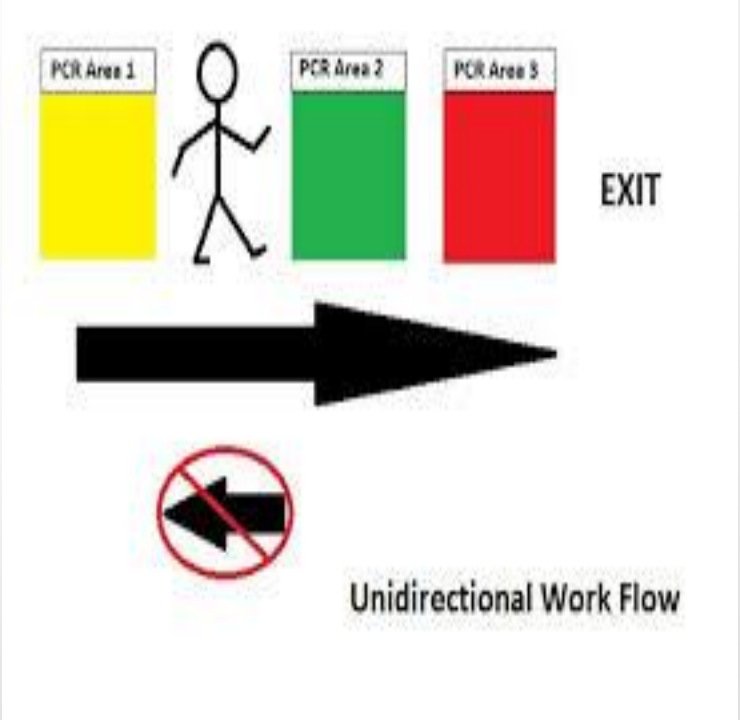
- Laboratory design
- Laboratory practices
- Chemical and enzymatic controls

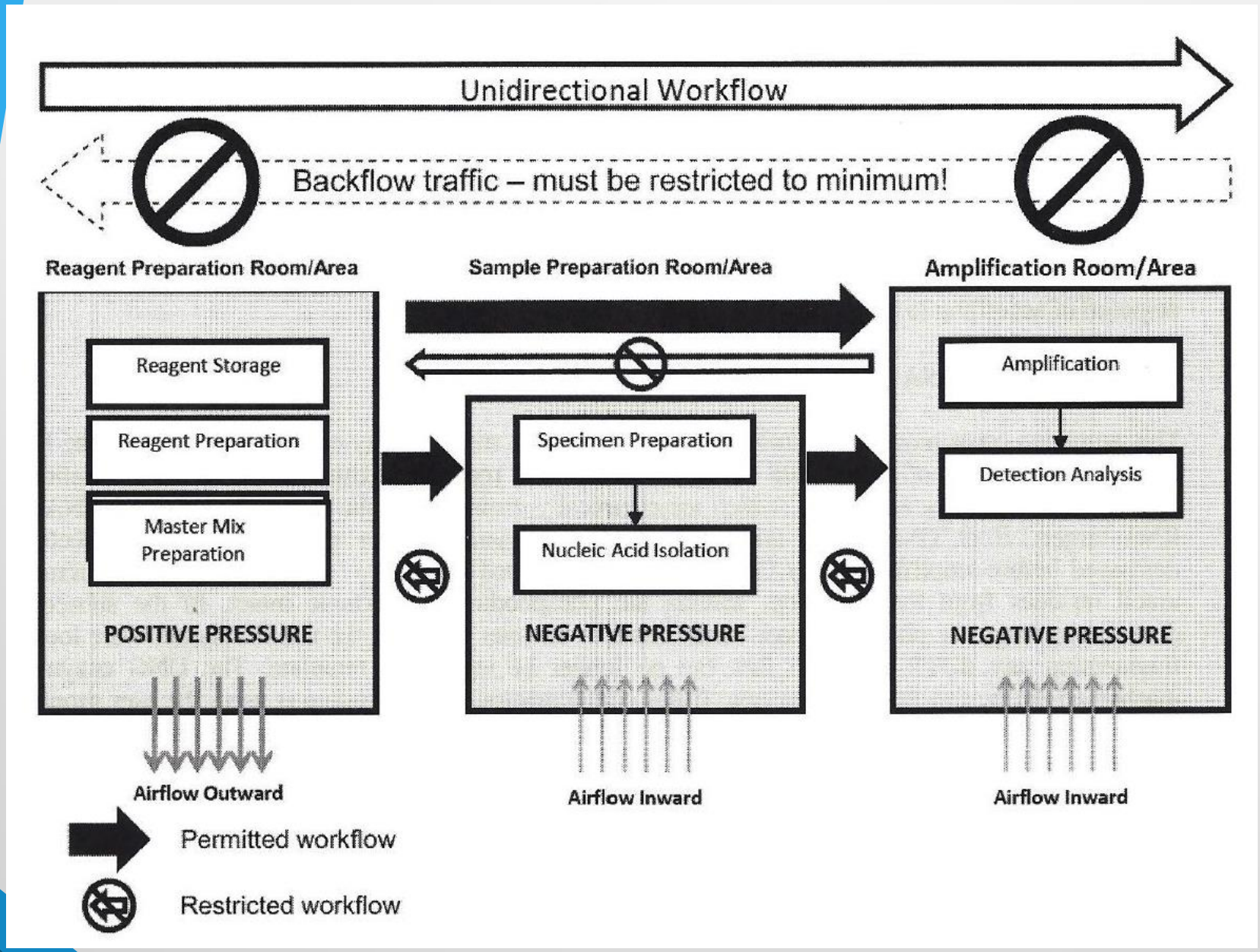
Setting Up a Molecular Laboratory

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

Unidirectional Flow

- Both personnel, including cleaning 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.





CLSI MM19-A Establishing Molecular Testing in Clinical Laboratory Environments

Features of the 3 Areas

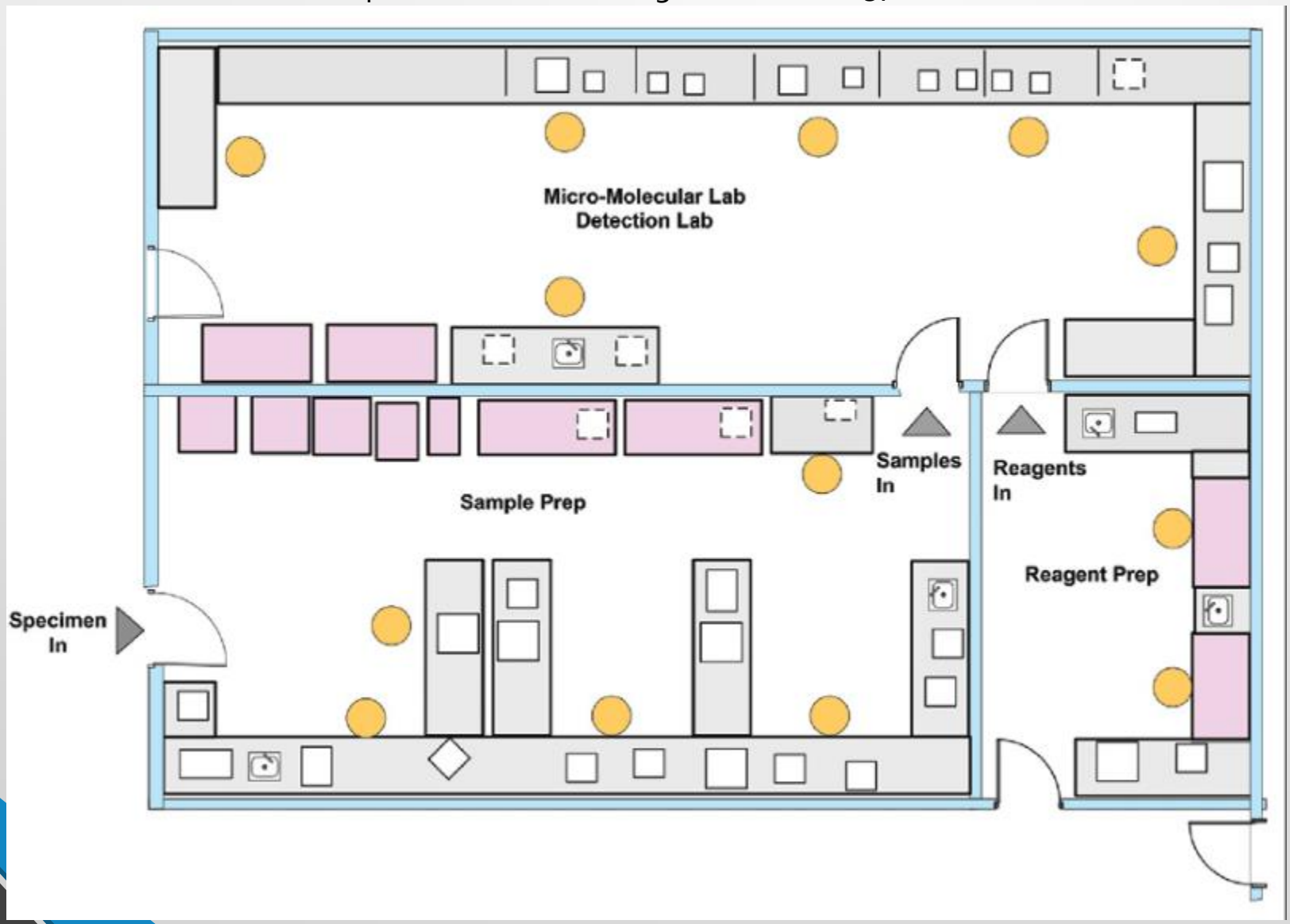
- Each area has separate sets of equipment and supplies
  - Refrigerator/freezer (manual defrost)
  - Pipettes, filtered tips, tubes, and racks
  - Centrifuge, timers, vortex
  - Lab coat (color-coded), disposable gloves, safety glasses, and other PPE
  - Cleaning supplies
  - Office supplies
  - Ventilation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

Features of the 3 Areas

- Air pressure
  - Reagent Prep – Positive
  - Sample Prep - Negative
  - Postamplification - Negative
- Reagent Prep – Single entrance, reagents used for amplification should not be exposed to other areas, no specimens
- Specimen Prep – Specimens should not be exposed to post-amplification work areas
- Size of each area should consider space for equipment and bench space needed for preparation

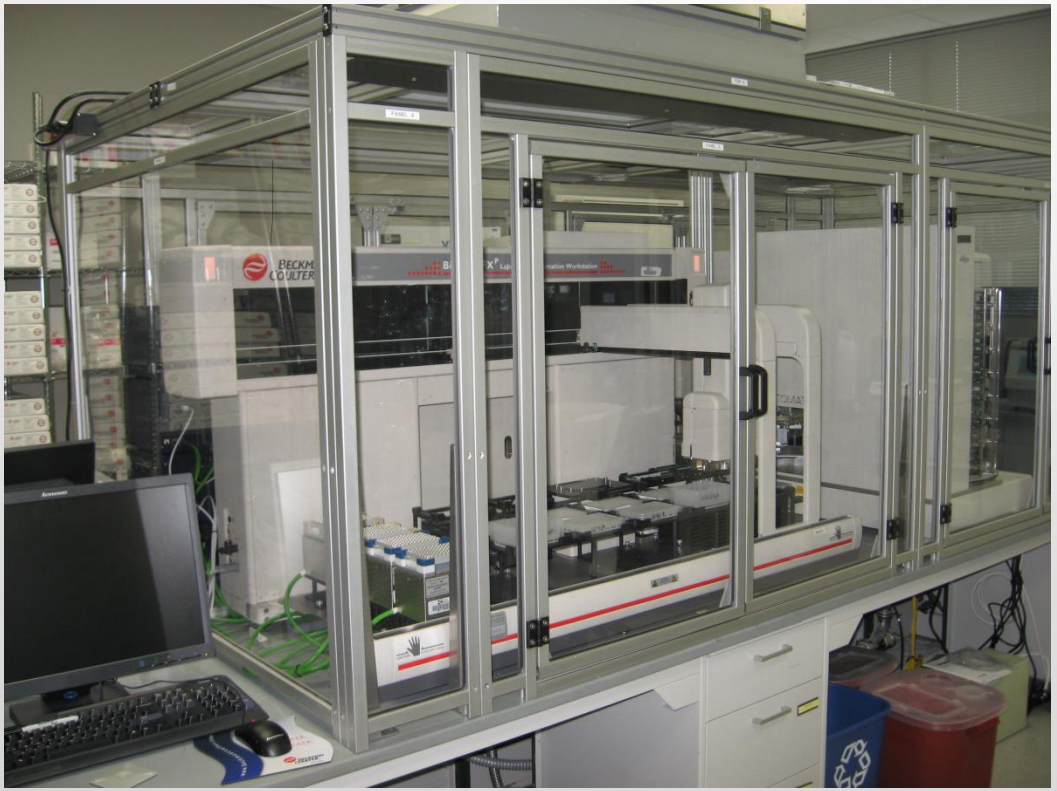
Laboratory Design Example

https://www.medlabmag.com/article/1570



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

Core Laboratory Concept

Core Lab

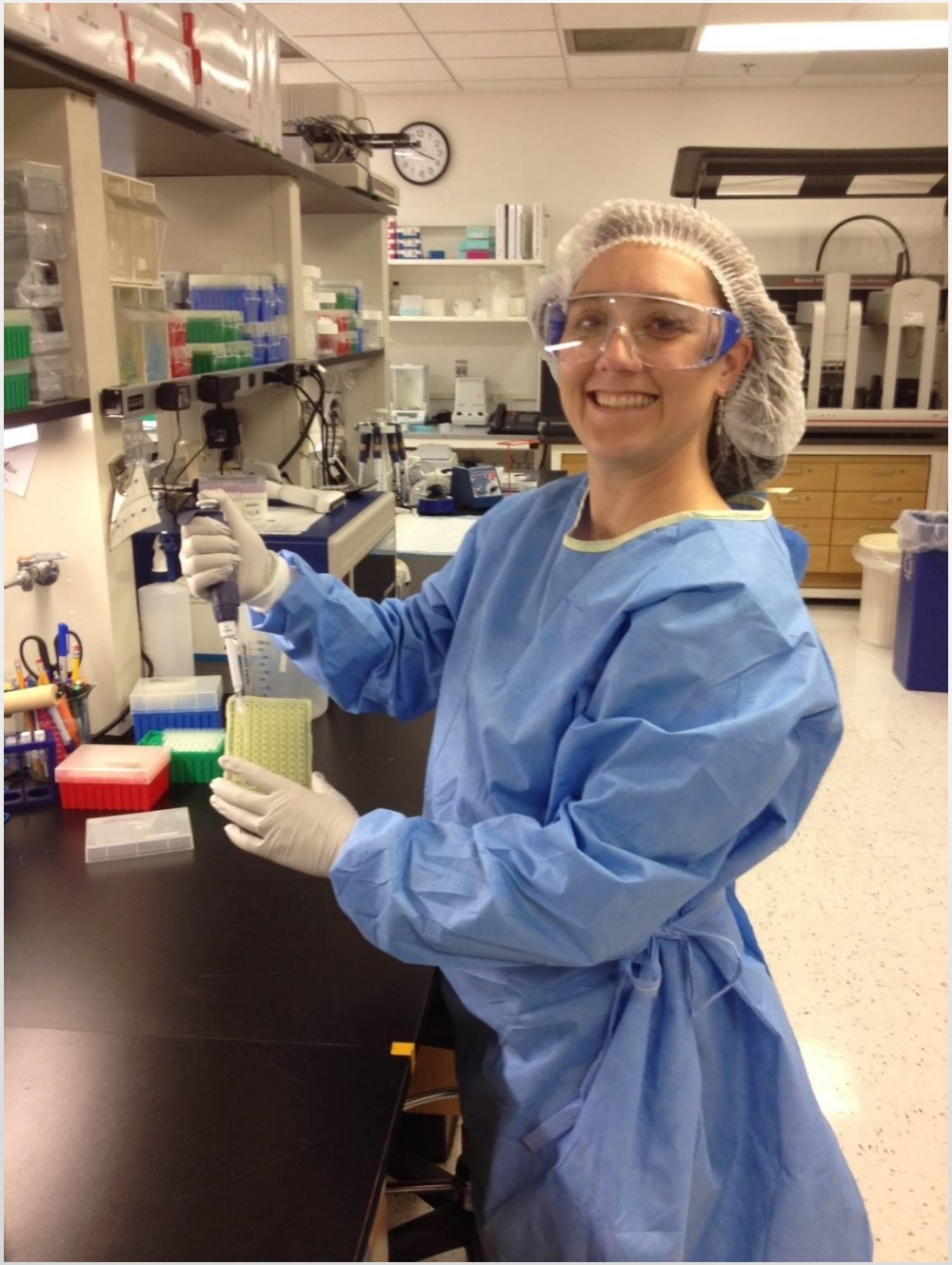
- On site – e.g. combine with microbiological testing
- Off site – e.g. academic institute

Other Laboratory Design Considerations

- Temperature and humidity requirements
- Water quality
- Electric outlet
- Back-up power system
- Eye wash
- Ergonomic assessment
- Need for storage area
- Need for waste disposal area

Laboratory Practices

- Open tubes carefully to avoid splashing
- Keep samples and reactions capped or covered as often as possible, and dispose them in a safe, contained place after use
- Use of positive displacement pipettes and disposable filtered pipette tips
- Avoid production of aerosols when pipetting
- Use of sterilized single-use plastic ware
- Use of cleanroom sticky floor mats
- Minimizes the risk of amplicon carry-over on clothing, hair and skin
  - Hairnet
  - Dedicated safety glasses
  - Disposable lab coat/gown, color-coded preferred
  - Gloves, need to change periodically
  - Shoe covers



More Laboratory Practices

- Clean punches between samples
- 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
- Use of electronic data system (flow of paper)
- Wipe test (swab test)
  - Monthly
  - Detect, localize, and remove contamination
  - Identify the source of the contamination

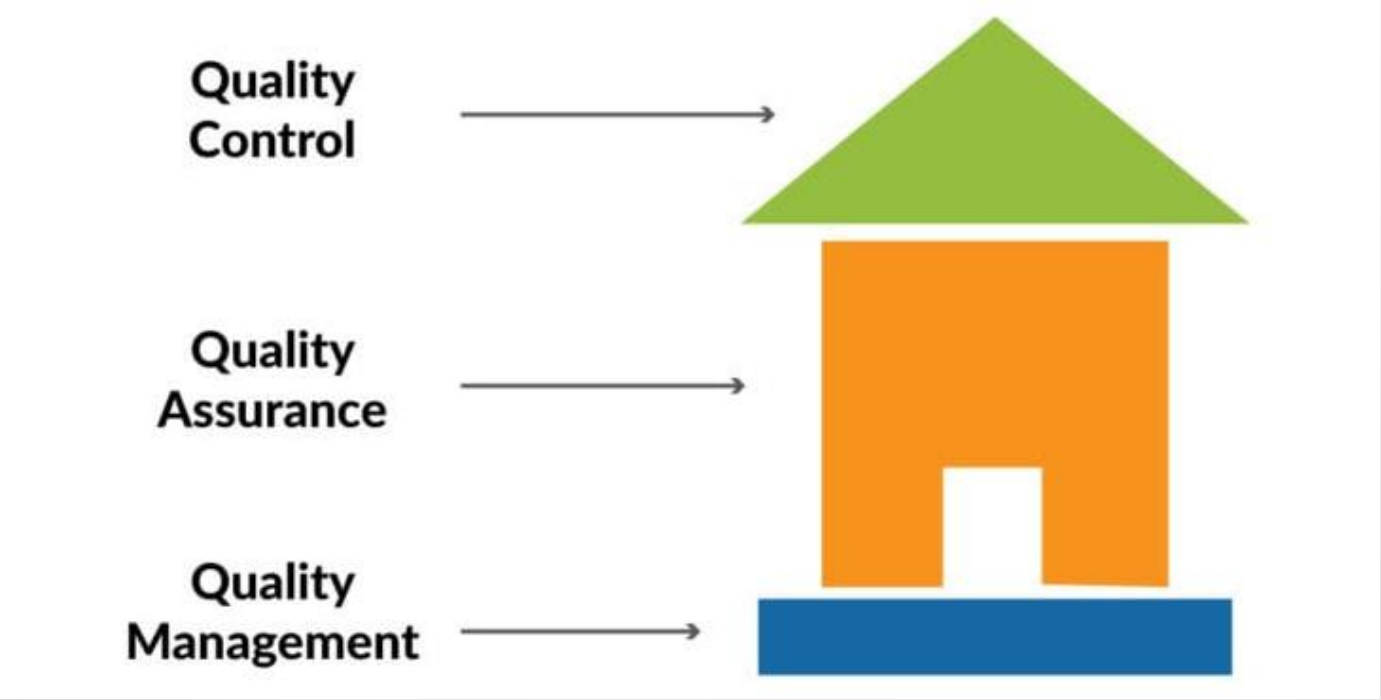
Decontamination Approaches

- Clean the work area & equipment 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, esp centrifuge and vortex
  - Clean the doorknobs, handle of freezers

Chemical and Enzymatic Controls

- Use of10% sodium hypochlorite solution (bleach), followed by removal of the bleach with ethanol and water.
- 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

Quality Assurance and Quality Control



CLIA Requirement

§493.1256 Standard: Control procedures.

- (a) For each test system, the laboratory is responsible for having control procedures that monitor the accuracy and precision of the complete analytic process.
- (b) The laboratory must establish the number, type, and frequency of testing control materials using, if applicable, the performance specifications verified or established by the laboratory as specified in §493.1253(b)(3).

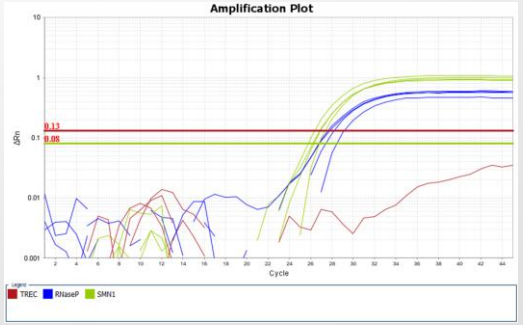
Quality Controls

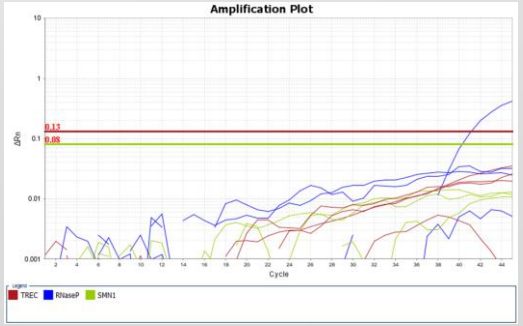
Monitor all steps of analytical procedure

- Types of Control
- Frequency and Number of Controls
- Evaluation of Controls and Calibrators

Types of Controls

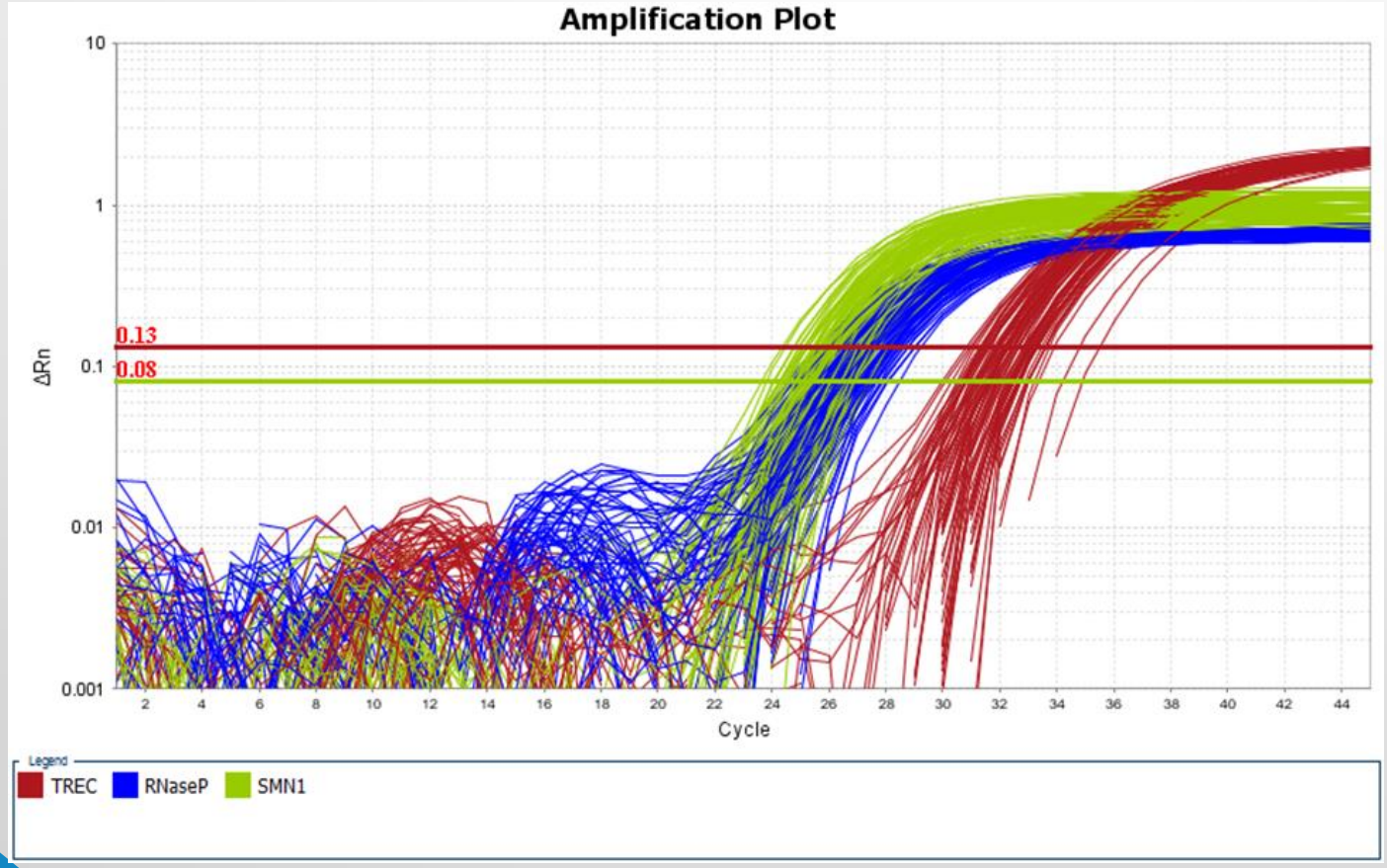
- Internal Control
  - Internal positive amplification controls to detect failure of DNA extraction or PCR amplification
    - Reagent or equipment issues
    - Integrity of DNA sample
    - Presence of inhibitory substance
- External Control
  - Positive control (e.g. SCID-like)
  - Normal control (wild type)
  - No template control (extraction blank)
  - Blank





Internal Controls

Reference gene (e.g. RNaseP)



External Controls

- Positive and normal controls:
  - Inhibitors
  - Component failure
  - Interpretation of results
  - Sources:
    - Residual DBS
    - PT samples
    - QC materials
- No template controls and Blanks:
  - Nucleic acid contamination during extraction
  - Nucleic acid contamination during PCR

Frequency and Number of External Controls

- Based on risk
- CLIA Regulation:
  (3) At least once each day patient specimens are assayed or examined perform the following for—
  - (i) Each quantitative procedure, include two control materials of different concentrations;
  - (ii) Each qualitative procedure, include a negative and positive control material;

Frequency and Number of External Controls

- Ideally should represent each target allele and include in each run, but may not be feasible when:
  - Highly multiplex genotypes
    - Systematic rotation of different alleles as positives
    - Specimens representing short and long amplification products to control for differential amplification
  - Sanger or Next-Generation sequencing
- Quantitative PCR
  - External controls should represent more than one concentration, e.g. low and high positives and negative, covering the analytical measurement range
  - Daily run or with each runs
- After equipment maintenance, new operator, new reagent lot/shipment

Evaluation of Controls

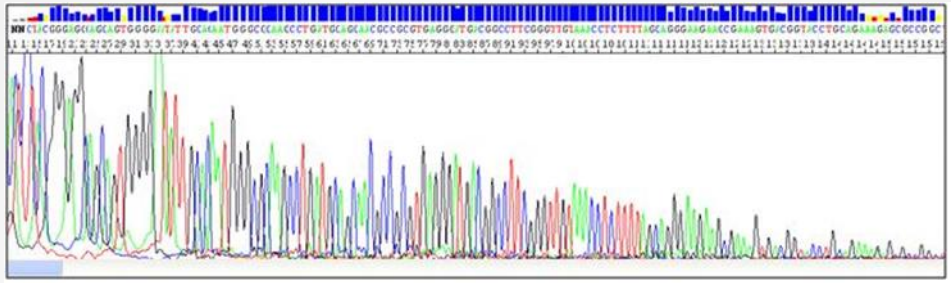
- Pass/Fail Criteria – established during validation study
  - Parameters
    - Specific PCR product bands
    - Specific DNA fragments
    - Quantity or Ct of reference gene
    - Quantity or Ct of targeted marker
  - Threshold
    - Presence or absence of DNA bands
    - Above or below LoB
    - Above or below cut-offs
    - Within Mean± 2SD, Mean ± 3SD, or Mean± 10%
  - % of controls acceptable
  - Impact the entire run or only affected samples

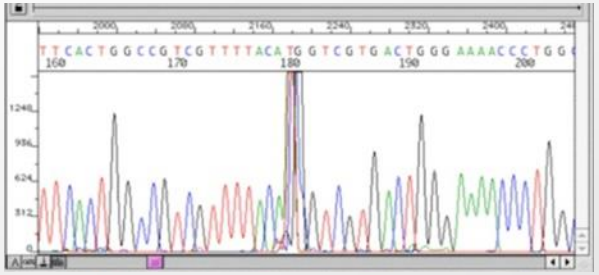
Calibrator

- Number, type, and concentration of calibration materials
- Criteria for acceptance
  - Within Mean± 2SD, Mean ± 3SD, or Mean± 10%
  - Slope, R2, and Y-intercept of Calibrator curve
- Calibration verification
  - Change of reagent lots
  - After major maintenance or service
  - At least every six months

Additional QC for Sequencing

- Sanger
  - Peak intensity
  - Baseline fluctuation
  - Signal to noise ratio
  - Peak shape….





- Next Generation
  - Sequencing depth
  - Base quality score
  - % of base above a specified quality threshold
  - % on-target aligned reads……

Critical Molecular Assay Components

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

Instrument Maintenance and Calibration

MOL.49520 Thermocycler Temperature Checks **Phase II**

Individual wells (or a representative sample thereof) of thermocyclers are checked for temperature accuracy before being placed in service and at least annually thereafter.

NOTE: A downstream measure of well-temperature accuracy (such as productivity of amplification) may be substituted to functionally meet this requirement. For closed systems this function should be performed as a component of the manufacturer-provided preventive maintenance.

Evidence of Compliance:

- ✓ Written procedure for verification of thermocycler accuracy **AND**
- ✓ Records of thermocycler verification

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 and Reference Material Sources

- CDC NSQAP
- CAP
- In-house samples
- Round-robin with other NBS laboratories
- Corielle
- European Collection of Authenticated Cell Cultures (ECACC)
- United Kingdom National External Quality Assessment Service (UK NEQAS)
- EuroGentest
- Acrometrix
- Advanced Biotechnologies
- Asuragen
- Diagnostic Hybrids
- Horizon Discovery
- Invivoscribe
- LGC Standards
- Maine Molecular Quality Controls
- Molecular Controls
- QIAGEN Marseille (formerly Ipsogen)
- Qnostics
- Seracare Life Sciences
- Zeptometrix

CAP Requirement on TAT

CBG.20140 Out-of-Range/Invalid Results **Phase II**

There is a policy for reporting positive (out of range) or invalid results to the submitting location and other appropriate entities to allow for patient follow-up within a timeframe appropriate to ensure maximum health benefit.

NOTE: Positive results include those results that are outside of the expected range of testing results established for a particular condition. Invalid results include situations where the laboratory is unable to complete the screening process due to an unsuitable specimen, test, or incomplete information. The findings must be communicated in a manner consistent with the urgency of the intervention needed. For situations requiring repeat screening or confirmatory testing, the laboratory must clearly communicate the timing of the actions to be taken.
Results must be reported to the submitting location (at minimum) within 7 days of specimen receipt and within 3 days for specimens received for tests requiring additional action (e.g. invalid or positive).The records should indicate when results were reported and who received the results. In cases where the testing laboratory is responsible for documenting that a return specimen has been received and analyzed, appropriate records should attest to specimen receipt, testing and result reporting.

CAP Requirement on Cutoff Verification

MOL.34516 Qualitative Cut-Off Verification **Phase II**

For qualitative tests that use a cut-off value to distinguish positive from negative, the cut-off value is verified with every change in lot or at least every six months.

NOTE: The threshold value that distinguishes a positive from a negative result must be verified with every change in lot (eg, new master mix), instrument maintenance, or at least every six months. Note that a low-positive control that is close to the threshold value can satisfy this checklist requirement, but must be external to the kit (eg, weak-positive patient sample or reference material prepared in appropriate matrix).

Evidence of Compliance:

- ✓ Written procedure for verification of the cut-off value **AND**
- ✓ Records of verification of cut-off value at defined frequency

Periodic Review of QA/QC

- Turnaround time - % of specimens meet TAT
- Failed run log
  - % of runs failed
  - Causes – look for pattern or trend in QC materials, exons, reagent, equipment, staff
- Total number of tests
- Update variant database
- Diagnosed cases
  - Compare our results with clinical results
  - Frequency of variant alleles in diagnosed cases
  - Any missed variants
  - Age of diagnosis
  - Incidence rate
- PT performance

TEXAS Department of State Health Services

Periodic Review of QA/QC

Monthly Median Values	
Median RNaseP Ct	26.596
Median TREC Ct	33.794
Median TREC Quantity	1080.364

Monthly Median Controls Values			
	RNaseP Ct	TREC Ct	TREC Quantity
HCB	26.809	33.629	1198.596
MCB	26.940	34.271	766.739
LCB	27.099	35.117	429.634
SCID-Like	26.949		

Controls % Failed		
	Total Failed	% Failed
HCB	0	0.00%
MCB	0	0.00%
LCB	3	1.44%
SCID-Like	0	0.00%
NTC	0	0.00%
EBC	0	0.00%

Total qPCR Assay Run Failures	
Retest	4
Initial	14
Total	18

qPCR Assay Run % Failed by Type					
	Controls	Standard Curve	Other	Total Failed	%Failed
Retest	1	1	2	4	16.00%
Initial	1	11	2	14	7.61%
Total	2	12	4	18	8.61%

Extraction Plate % Failed				
	EBC > Cutoff	Accidental	Technician Error	Total Failed
Retest	0	0	0	0
Initial	0	1	0	1
Total	0	1	0	1

Negative Controls with TREC amplification		
	Total w/ TREC	%
SCID-Like	5	1.20%
NTC	0	0.00%
EBC	3	0.36%

Mean Standard Curve QC Values					
	Mean	SD	%CV	Min (-3SD)	Max (+3SD)
Slope	3.352	0.073	2.18%	3.133	3.571
Y-Intercept	43.956	0.299	0.68%	43.060	44.851
R2	0.974	0.008	0.84%	0.949	0.998

Mean TREC Standard Curve Ct's							
	16129	4032	2016	1008	504	252	150
Mean TREC Ct	29.796	31.902	32.937	33.923	34.857	35.894	36.619
Std Dev	0.237	0.293	0.344	0.399	0.427	0.464	0.569
%CV	0.80%	0.92%	1.04%	1.18%	1.22%	1.29%	1.55%
+3SD	30.507	32.780	33.968	35.120	36.137	37.286	38.326
-3SD	29.085	31.024	31.906	32.727	33.577	34.502	34.913

Cutoff Analyzer					
	Total	Newborn	Follow-up	Low BW	Normal BW
Number of Specimens	61382	31553	29829	2206	55393
Median TREC Quantity	1084.51	1005.54	1175.97	773.18	1087.82
Median TREC Ct	33.77	33.91	33.63	34.25	33.77
Median RNaseP Ct	26.60	26.17	26.97	26.87	26.56

Percentiles

TREC Percentiles	TREC Quantity					RNaseP Ct					RNaseP Percentiles
Percentiles (%)	Total	Newborn	Follow-up	Low BW	Normal BW	Total	Newborn	Follow-up	Low BW	Normal BW	Percentiles (%)
99.00	215.88	186.83	260.00	55.46	238.24	27.80	27.36	27.97	28.19	27.77	95.00
99.50	158.97	130.80	198.71	38.65	182.33	28.31	28.00	28.42	28.94	28.25	99.00
99.90	58.08	50.14	70.13	10.87	68.23	28.53	28.34	28.61	29.53	28.46	99.50
99.95	46.01	40.21	56.77	6.56	55.45	29.19	29.41	29.06	31.18	29.00	99.90

TREC Percentiles	TREC Ct				
Percentiles (%)	Total	Newborn	Follow-up	Low BW	Normal BW
99.00	36.13	36.31	35.81	38.02	35.98
99.50	36.61	36.87	36.27	38.73	36.36
99.90	38.01	38.26	37.82	40.64	37.80
99.95	38.41	38.72	38.04	41.53	38.16

12/11/2017: RBC Buffer Lot change from 10/03/17 to 10/31/17-- No significant change observed between lots
12/02/2017: Buffer B lot change from 09/13/17 to 10/11/17-- No significant change observed between lots
12/21/2017: Buffer A lot change from 10/11/17 to 11/08/17-- No significant change observed between lots
12/30/2017: Buffer B lot change from 10/11/17 to 11/08/17-- No significant change observed between lots

Take Home Messages

- Separate laboratory spaces for Reagent Prep, Sample Prep, and Amplification and Detection
- Precautions and special laboratory practices must be made to minimize the risk of contamination
- A Quality Management Plan to monitor the quality of testing process, detect daily errors, and identify trends should be in placed for each new test before it's implemented.
- Continuous quality improvement is essential