Molecular Diagnostics

An Introduction and Overview of fairly ^Good Practices

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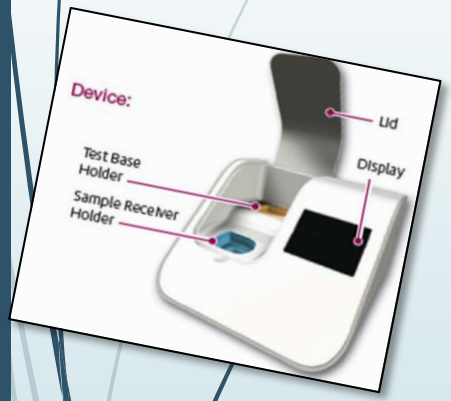
Molecular Awesomeness!!!!

Easy!

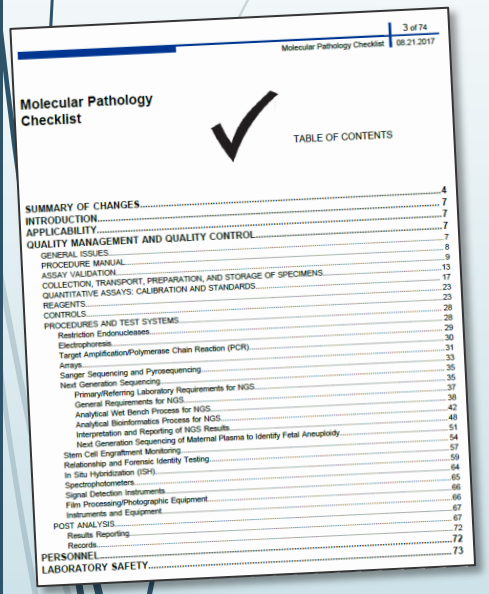


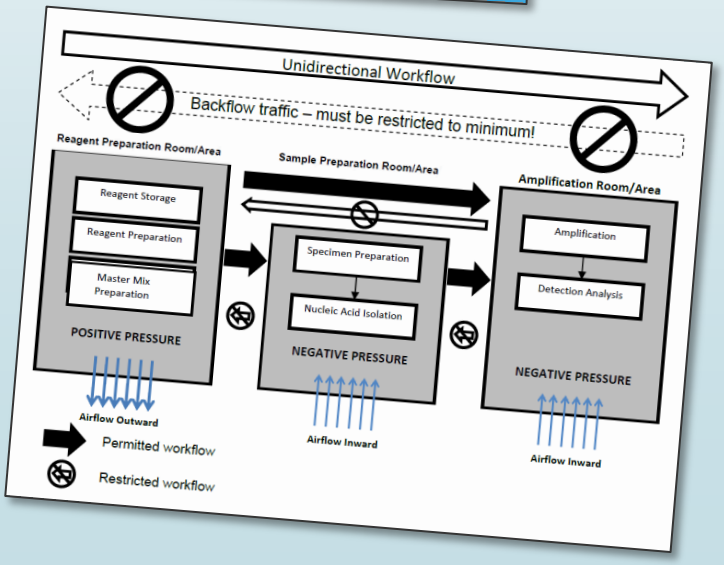
Sample to Answer!

Great Patient Care!!!

AAAAAGGGHHH!! Reality!!!

Contamination!





Multiplexing!

Inhibition!

My assumptions

- You're experienced laboratory professionals.
  - Mostly microbiologists. I know, it's not all of you, but I couldn't resist a few microbiology-jokes.
- You're bringing in molecular testing, or planning to soon.
- You're going to mostly be doing FDA-approved tests.
  - I'll mention LDT, but not focus on them.
- You're not molecular gurus; if you are, YOU oughta be teaching this session.

Learning Objectives

- Participants should be able to:
  - Describe the basic work-flow of molecular diagnostic testing, including established and emerging methods.
  - Describe some major amplification and detection methods.
  - Recognize the properties of analytes that make them candidates for molecular testing.
  - Describe types of molecular testing platforms.
  - Articulate best practices in molecular testing.
  - Create quality improvement goals for molecular diagnostics.

What is Molecular Diagnostics?

- Analysis of DNA or RNA for diagnostic purposes. Molecular diagnostics have found widespread application with the advent of ***amplification methods*** (PCR and related approaches) and now with ***Next-Generation Sequencing*** (NGS) methods just coming into use.
- Huge scope
  - From single-target molecular detection of pathogens…
  - To pharmacogenomic analysis of metabolism genes for drug dosing…
  - To whole genome sequencing for cancer therapeutics and disease susceptibility and God knows whatall.

Conventional (Amplification) Molecular Diagnostic Testing

- Specimen
- DNA / RNA Extraction
- Amplification of Target
- Detection of amplified target
- Interpretation and Clinical Use

Why Amplify?

- Sensitivity for infections
  - Can detect small numbers of organisms or other targets
  - Can even detect dead or damaged or unculturable organisms
- Speed
  - Can be very fast (15 min and up)

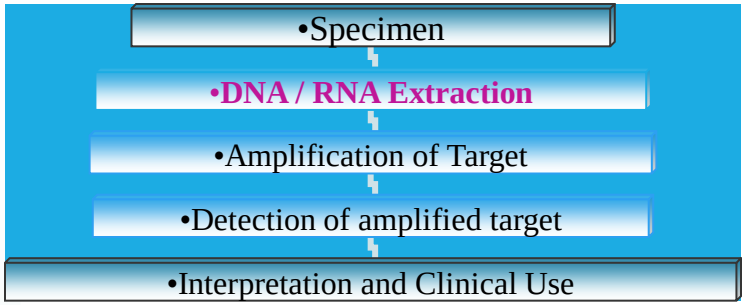
Why Amplify, continued

- Targets
  - Test for things there's no other way to test
  - Uncultivable bugs
  - Genetics
    - Pharmacogenomics
    - Prenatal testing
    - Hypercoagulability, etc.
  - Oncology
    - Diagnostic / Tumor type markers
    - Minimal residual disease
    - Therapeutic targets

Why Not Amplify?

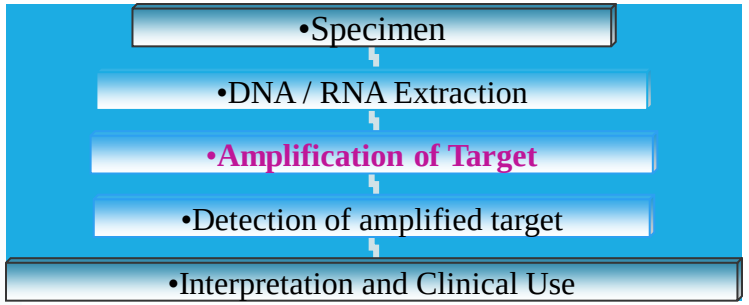
- Clinical significance?
- Technical problems
  - Contamination
  - Inhibition
- Cost
- COST
- CO$T
- Not as bad as all that anymore…mostly.

Extraction



- DNA/RNA Extraction
  - Depends on:
  - Specimen source (blood, CSF, stool)
  - Target organism (human tumor, CMV, M. tuberculosis)
  - Target nucleic acid (DNA, RNA)
- Increasing automation
  - Magnetic or other separation methods.

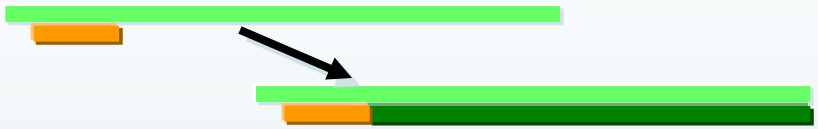
Amplification



- Nucleic Acid Amplification means taking a small number of targets and copying a specific region many, many times.
- NAAT, NAT, etc; commonly-used abbreviations

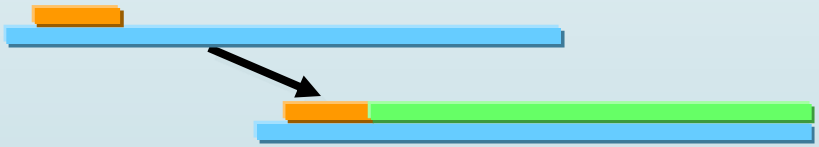
Amplification Enzymology

- DNA polymerase
  - makes DNA from ssDNA, requires priming

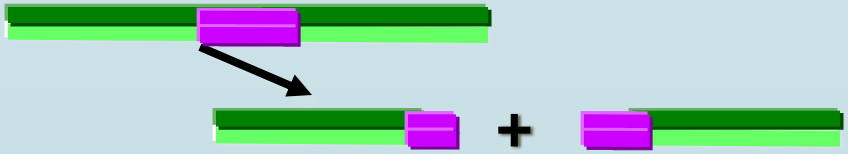


- RNA polymerase
  - makes RNA from dsDNA, requires specific start site

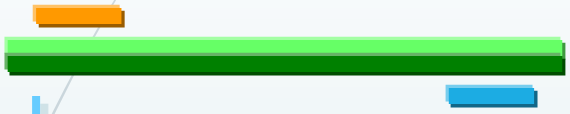
- Reverse transcriptase
  - makes DNA from RNA, requires priming



- Restriction endonucleases
  - cut DNA in a sequence specific manner



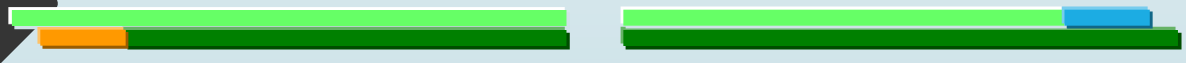
Polymerase Chain Reaction (PCR)



Target DNA
+
Primer oligonucleotides (present in excess)

Split DNA strands (95°C 5 min), then allow primers to bind (40-70°C)

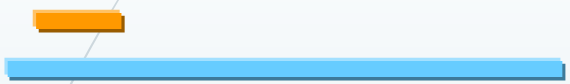
DNA polymerase extends the primers (40-80°C) to produce two new double-stranded molecules



Repeat the split-bind-extend cycle

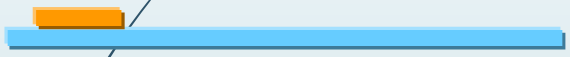
This 'short product' amplifies exponentially in subsequent split-bind-extend cycles, driven by the temperature changes in a 'thermal cycler'.

Reverse Transcriptase PCR (RT-PCR)



Target ***RNA*** +
Primer oligonucleotide

Primer binding (RT - 37°C)



Reverse Transcriptase (RT) makes a DNA copy of the RNA target

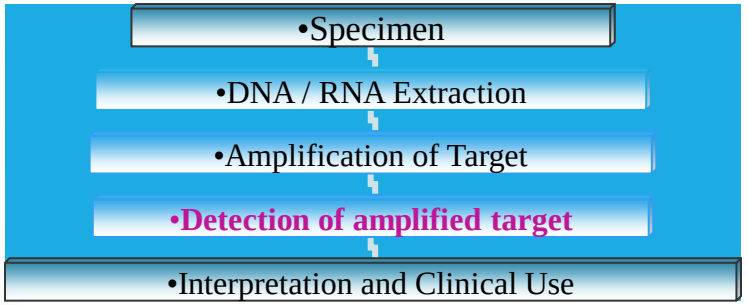
The DNA copy is used in a PCR reaction

PCR

Other Amplification Methods

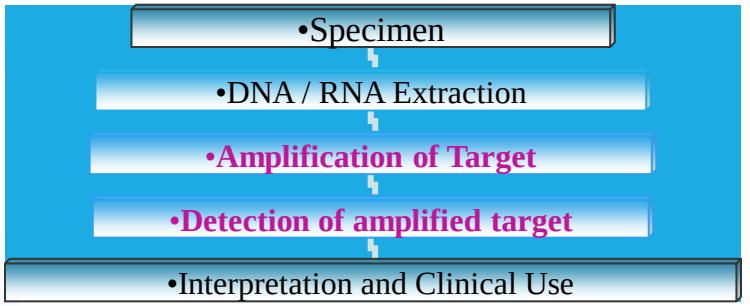
- PCR isn't all there is!
  - Transcription-mediated amplification (TMA)
  - Loop-mediated isothermal AMPlification (LAMP)
  - Nicking-Enzyme Amplification Reaction (NEAR)
  - Many Others
  - Isothermal technologies decrease the complexity of the instrument required.

Detecting PCR Products in the Old Days



- Gel electrophoresis (± Southern blotting)
- Enzyme-linked assays
- Hybridization Protection/chemiluminescent assay
- Increasingly, a multitude of specific formats available, to serve market and technical needs

Real-Time PCR



- Combination
  - Detection
  - Amplification
- RT-PCR Instruments monitor product formation by detecting change in fluorescence in a tube or well during thermal cycling.
- Frequently use PCR for amplification
  - Robust
  - Off-patent

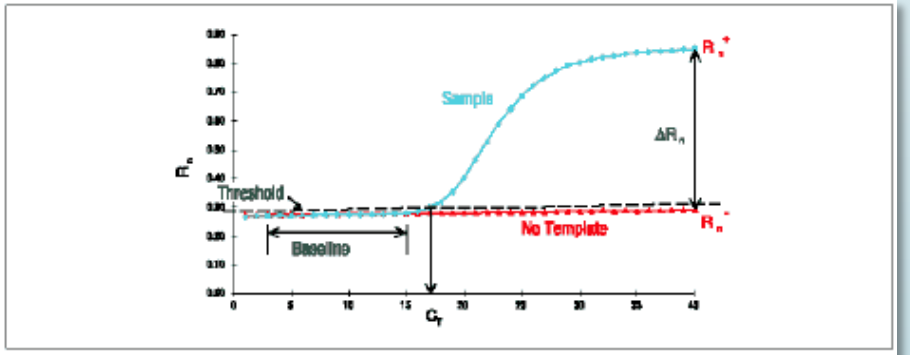
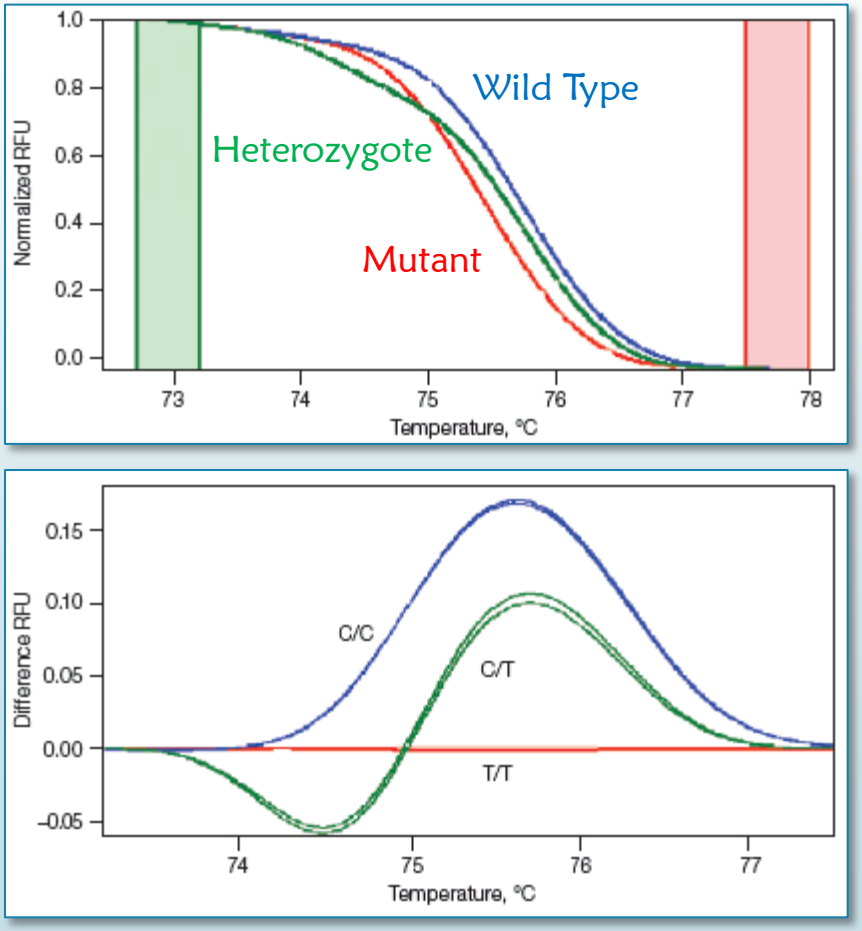


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNA/RNA Real-Time Quantitative PCR bulletin).

Real-Time PCR Instruments

- Contain three functional components
  - A thermal cycler
    - Mostly a single cycler that cycles all the tubes / wells at the same time
    - The SmartCycler and GeneExpert have individually controllable cycler elements.
  - Fluorescent detection system
    - The number of fluorescent detection channels determines how many different probes you can use.
    - Many different detection chemistries to serve different purposes.
    - Melt-curve analysis can distinguish alleles.
    - An internal amplification control is a must.
  - A computer to run the components, interpret the data, etc.



Melting curve analysis; from Bio-Rad.com

Types of amplification-Based Molecular Tests

- Single-target (small number of targets)
  - Except for the 'molecular' bit, not unlike other lab tests.
- Multiplex (more targets)
  - As the number of targets increases, QC and validation / verification increase in complexity.
- Qualitative vs Quantitative
- Mutation / sequence variant detection
  - How many? How detected?
  - Threshold for detecting variants?

Categories of Molecular Instruments

- Discrete Pieces
  - Extraction instrument + thermal cycler + detection system
  - Extraction instrument + real-time PCR system.
  - Example: Roche Taqman systems, Abbott m2000, QiaSymphony + Focus cycler, many combinations possible.

Sample to Answer Instruments

- Sample-to-Answer
  - High-throughput batch-mode
    - Combine extraction and analysis with substantial automation of sampling, interpretation, and reporting.
    - Examples: Roche 4800, 6800, others
  - Low-throughput random-access
    - Typically test for acute-care markers (respiratory viruses, etc.)
    - Some CLIA-waived
    - Examples: Alere Liat, Biomerieux BioFire, Cepheid GeneXpert, Roche Liat, others
  - High-throughput random-access
    - Glom several to many sample-to-answer modules together, maybe add some improved sample-handling.
    - Example: Cepheid Infiniti
- The lines do blur, and will continue to.
- Yes, all you manufacturer's people out there, this is an oversimplification and vilely disserves your product line. You're welcome to consider it a conspiracy.

Managing molecular diagnostics

- All the sorts of things you do for conventional testing…plus items specific to molecular testing.
- Specimen Management
- Validation – Verification
- Process / Quality control
- Proficiency Testing
- Contamination Control
- Quantitative Tests
- Reporting
- Personnel and Competencies
- NOTE: If you're only doing molecular testing for microbial targets, you use the Molecular section of the CAP Microbiology checklist. If you test other targets, you use the Molecular checklist.

Specimen collection / handling / storage and stability

- Collection
  - Pay particular attention to the possibilities of specimen cross-contamination. Patients with HCV may have viremia in excess of 30 million/ml. Respiratory viruses may contaminate surfaces for prolonged periods of time – especially in flu season!
  - Carryover studies on automatic aliquoting systems.
- Specimen Handling
  - Is the volume sufficient to the test?
  - Does the test have urgent TAT requirements (e.g. HSV on CSF)?
- Specimen Storage
  - As with other analytes, nucleic acid targets may have limited stability.
    - E.g. HIV in the plasma
    - RNA targets tend to be relatively unstable.

Verification – Validation

- Accuracy – Precision – Reportable Range – Reference Interval – (For LDT Cutoffs –LLOD – Interfering Substances – Clinical Performance)
- Specimen types
  - If using specimens other than FDA-approved; e.g. bronch specimens for a test approved for sputum.
  - Need to show that inhibition isn't a problem; that sensitivity is preserved; and that new interferences (different normal microbiota; contamination by host DNA in CF; etc.) don't impact results.
  - Note potential interferences in different sample types; formalin, alcohols, heparin, solvents, drugs.

More Verification and Validation

- Verification of extraction
  - Does the method use an internal control to prove that the lysis/extraction steps worked?
  - If not, establish the reliability of the extraction method; if you change extractions, reverify.
  - Or find controls that verify lysis/extraction.
- Reverification of primers/ probes in LDT for currently circulating pathogen strains.
  - Flu changes yearly; other pathogens may also change periodically.
- Accuracy; at least 20 samples, at least 18 within the reportable range of the test.
- Interpretive guidelines
  - Tell providers what the limits of detection are, what interferences are, and (sometimes) what positives / negatives mean.

From CLSI MM-19A Establishing Molecular Testing in Clinical Laboratories.

Table 10. Validation Parameters. Adapted from Jennings L, Van Deerlin VM, Gulley ML. Recommended principles and practices for validating clinical molecular pathology tests. *Arch Pathol Lab Med*. 2009;133(5):743-755. Used with permission from *Archives of Pathology & Laboratory Medicine*. Copyright 2009. College of American Pathologists.[82]

Parameter	Applies to:		Approved CLSI Guidelines
	Qualitative	Quantitative	
Accuracy	X	X	EP12[83] EP15[84] MM03[85]
Trueness		X	EP09[86]
Precision		X	EP05[87] EP15[84]
Reproducibility	X		EP12[83]
Robustness	X	X	
Linearity		X	EP06[88]
Reportable range		X	
Reference range		X	C28[81]
Interfering substances	X	X	
Sensitivity	X	X	
Specificity	X	X	
LoD	X	X	EP17[89] MM06[78]
Limit of quantification		X	EP17[89]

Strategies for verifying multiplex tests

- What if your test detects 20 targets??!!
- Let's see…20 targets x 20 samples each x 2 analyzers = Maybe not.

Multiplex Test Verification Example

Day 1

- Pool #1: 600µl of Adenovirus + 600µl of Influenza A subtype H1
- Pool #2: 600µl of Influenza B + 600µl of PIV4 + 600µl Coronavirus OC43
- Pool #3: 600µl of HRV/HEV + 600µl of Influenza A subtype H3 + 600µl of Coronavirus 229E

Day 3

- Pool #4: 600µl of PIV1 + 600µl of PIV2 + 600µl of *M. pneumoniae*
- Pool #5: 600µl of Influenza A subtype H1-2009 + 600µl of PIV3
- Pool #6: 600µl of RSV + 600µl of Coronavirus NL63 + 600µl of hMPV

Day 5

- Pool #7: 600µl of *B. per*

Commercial control materials seeded into sample matrix

	ANALYZER		
	A	B	C
Day 1	Pool #1: ALL DETECTED Pool #3: ALL DETECTED	Pool #1: ALL DETECTED Pool #2: ALL DETECTED	Pool #2: ALL DETECTED Pool #3: ALL DETECTED
Day 2	Pool #1: ALL DETECTED Pool #2: ALL DETECTED	Pool #2: ALL DETECTED Pool #3: ALL DETECTED	Pool #1: ALL DETECTED Pool #3: ALL DETECTED
Day 3	Pool #4: ALL DETECTED Pool #6: ALL DETECTED	Pool #4: ALL DETECTED Pool #5: ALL DETECTED	Pool #5: ALL DETECTED Pool #6: ALL DETECTED
Day 4	Pool #4: ALL DETECTED Pool #5: ALL DETECTED	Pool #5: ALL DETECTED Pool #6: ALL DETECTED	Pool #4: ALL DETECTED Pool #6: ALL DETECTED
Day 5	Pool #7: ALL DETECTED	Pool #7: ALL DETECTED	
Day 6		Pool #7: ALL DETECTED	Pool #7: ALL DETECTED

Conclusion: **Method is sufficiently accurate for clinical use.** All targets expected to be detectable were detectable.

More Multiplex Verification

- Do verify precision with a range of targets.

II. Precision

The VRL assessed precision by performing 20 day QC using [redacted] quality control material. Using the [redacted] Control Panel M210 v1.1, the VRL assessed all targets on all instruments for 20 days.

Results are as follows:

20 DAY QC	M211 Panel	M212 Panel
Day 1	Detected	Detected
Day 2	Detected	Detected
Day 3	Detected	Detected
Day 4	Detected	Detected
Day 5	Detected	Detected
Day 6	Detected	Detected
Day 7	Detected	Detected
Day 8	Detected	Detected
Day 9	Detected	Detected
Day 10	Detected	Detected
Day 11	Detected	Detected
Day 12	Detected	Detected
Day 13	Detected	Detected
Day 14	Detected	Detected
Day 15	Detected	Detected
Day 16	Detected	Detected
Day 17	Detected	Detected
Day 18	Detected	Detected
Day 19	Detected	Detected
Day 20	Detected	Detected

M211 contains: Adenovirus, Human Metapneumovirus, Human Rhinovirus/Enterovirus, Influenza A H1-2009, Influenza A H3, Parainfluenza Virus 1, Parainfluenza Virus 4

M212 contains: Coronavirus 229E, HKU1, NL63, OC43, Influenza A H1, Influenza B, Parainfluenza Virus 2, Parainfluenza Virus 3, Respiratory Syncytial Virus, *Bordetella pertussis, Chlamydophila pneumoniae,* and *Mycoplasma pneumoniae*.

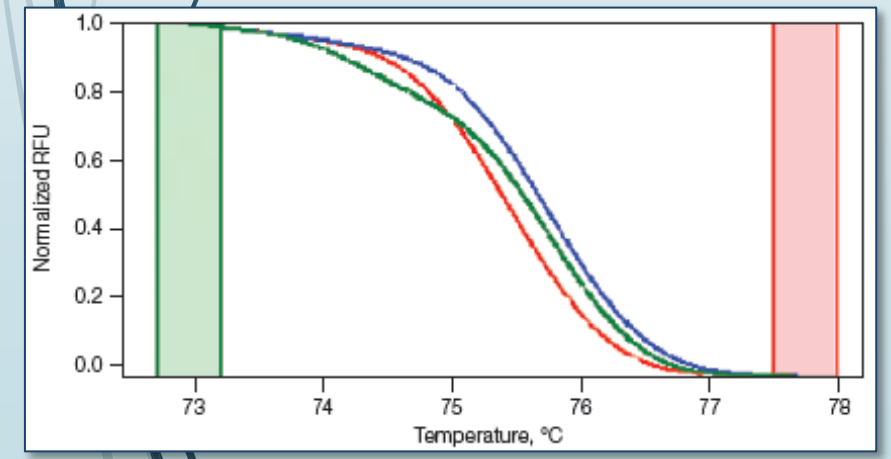
Conclusion: **Method is sufficiently precise for clinical use.** All targets in the [redacted] Control Panels were detectable each day for twenty consecutive days on all three instruments.

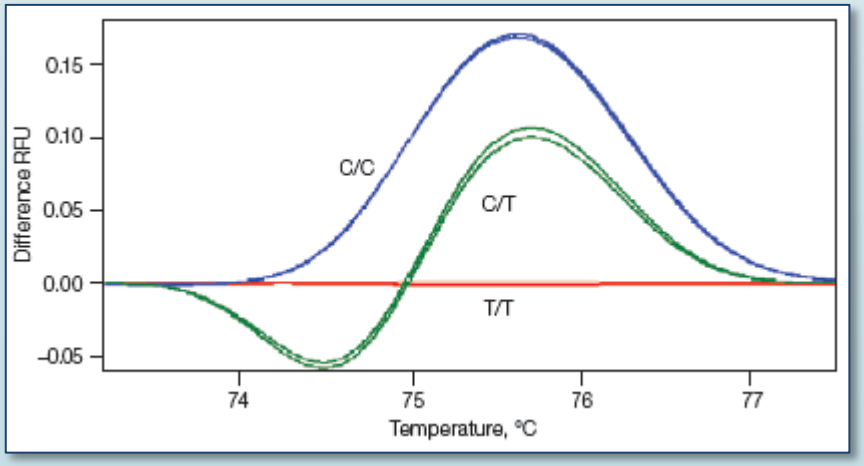
Process / Quality control

- If you save extracts – 'derivative material' – then they must be labeled and identified.
- Daily QC or IQCP
  - Note that some molecular systems with internal controls are eligible for IQCP.
- Multiplex QC
  - Important to control all targets; if not possible each time QC is run, rotate targets systematically.
- Reagent verification for multiplex tests
  - Test all targets
- Extraction assessment
  - RNA based testing and sample degradation.
  - Use an extraction control unless it's internal.
- Cutoff verification periodically

More Process Control

- Specific issues
  - Group B strep: When screening during pregnancy, must perform enrichment culture.
  - Culture for TB regardless of amplified result
  - Unexpected results in genetic testing – mother testing negative for fragile X with sons known to be positive.
  - Technique-specific – for electrophoresis, restriction digestion, melt-curve analysis, arrays, sequencing.



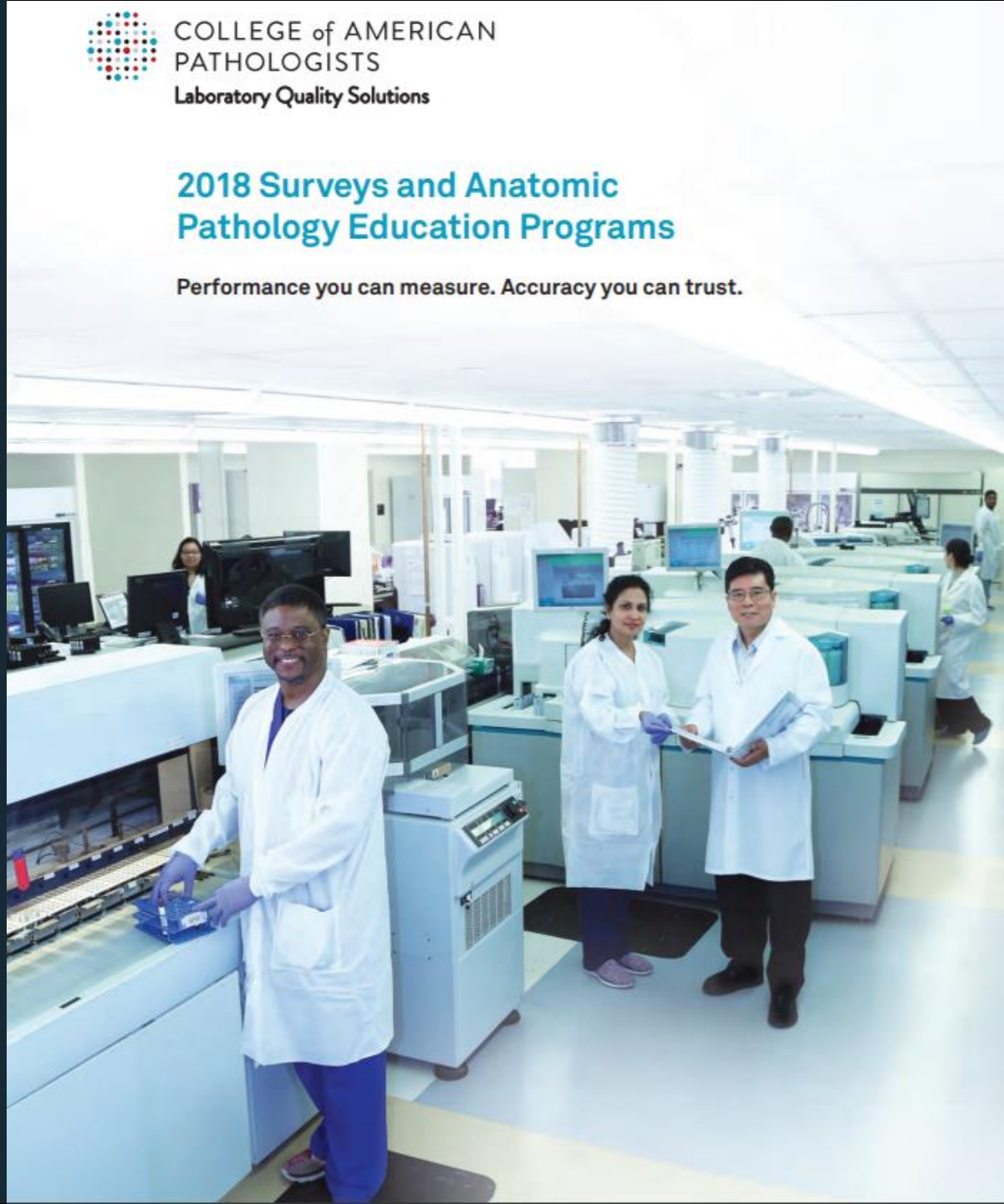


Process – Quality Control Cont.

- Instrument-specific issues
  - Signal detection equipment
    - Background detection / correction
    - Multiplex signal bleed / color correction
  - Thermal cyclers
    - Periodic calibration and cycling verification.

Proficiency Testing

- Yes.
  - It's expensive.
  - It's important.
  - You have to do it.



Amplification's Achilles Heel -- Contamination!

- What happens when you make 10^6 copies of a single short sequence in a 100μl reaction?
  - You end up with 10^4 copies/μl
  - What happens when you pop the top off a microcentrifuge tube?
    - ...or pipet anything
    - ...or vortex anything
    - ...or...
- You create aerosols…
  - Droplet nuclei with diameters from 1-10 μm persist for hours/days
  - Each droplet nucleus contains amplified DNA
  - Each amplified molecule can initiate a new amplification reaction
- …and droplets
  - That can contaminate hands, pipettes…

Sources of Contamination…

- Specimens
  - Cross contamination
- Amplification product contamination
  - Laboratory surfaces
  - Ventilation
  - Reagents/supplies/pipettes
  - Hair, skin, and clothes of lab personnel

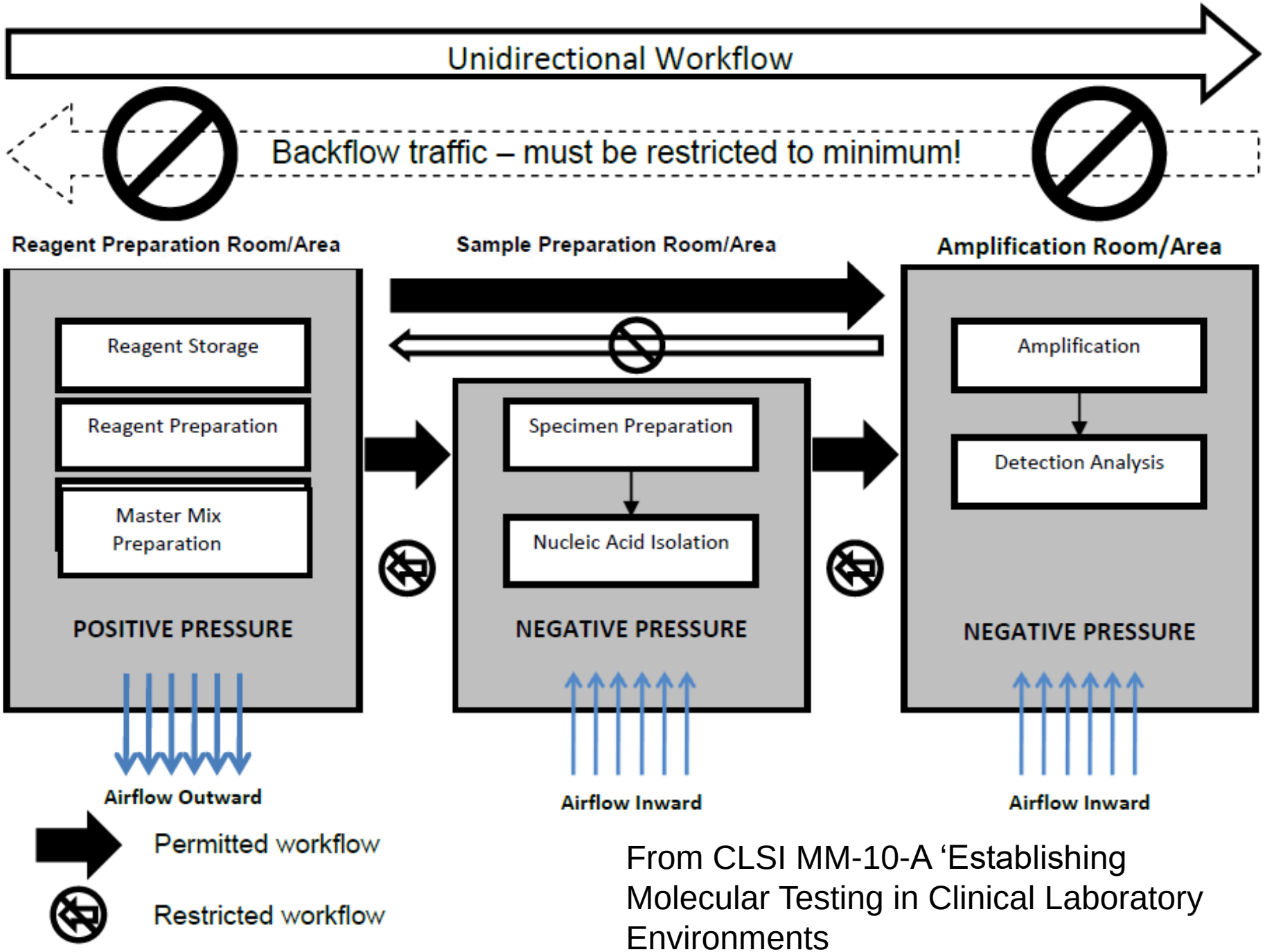
Contamination Control

- Include monitoring positivity rates, wipe tests.
  - Regular cleaning protocols and monitoring.
- Specimen handling
  - Aliquoting, residual samples
  - Pipette cleaning and calibration
- Carryover and containment
- Do you need to do all this with a simple, self-contained molecular platform?
  - Maybe not; but be careful, be aware of the problem, be meticulous.

Ways to Prevent Contamination

- Meticulous technique
  - Hoods, aerosol-resistant pipettes, physical separation of work areas with unidirectional workflow

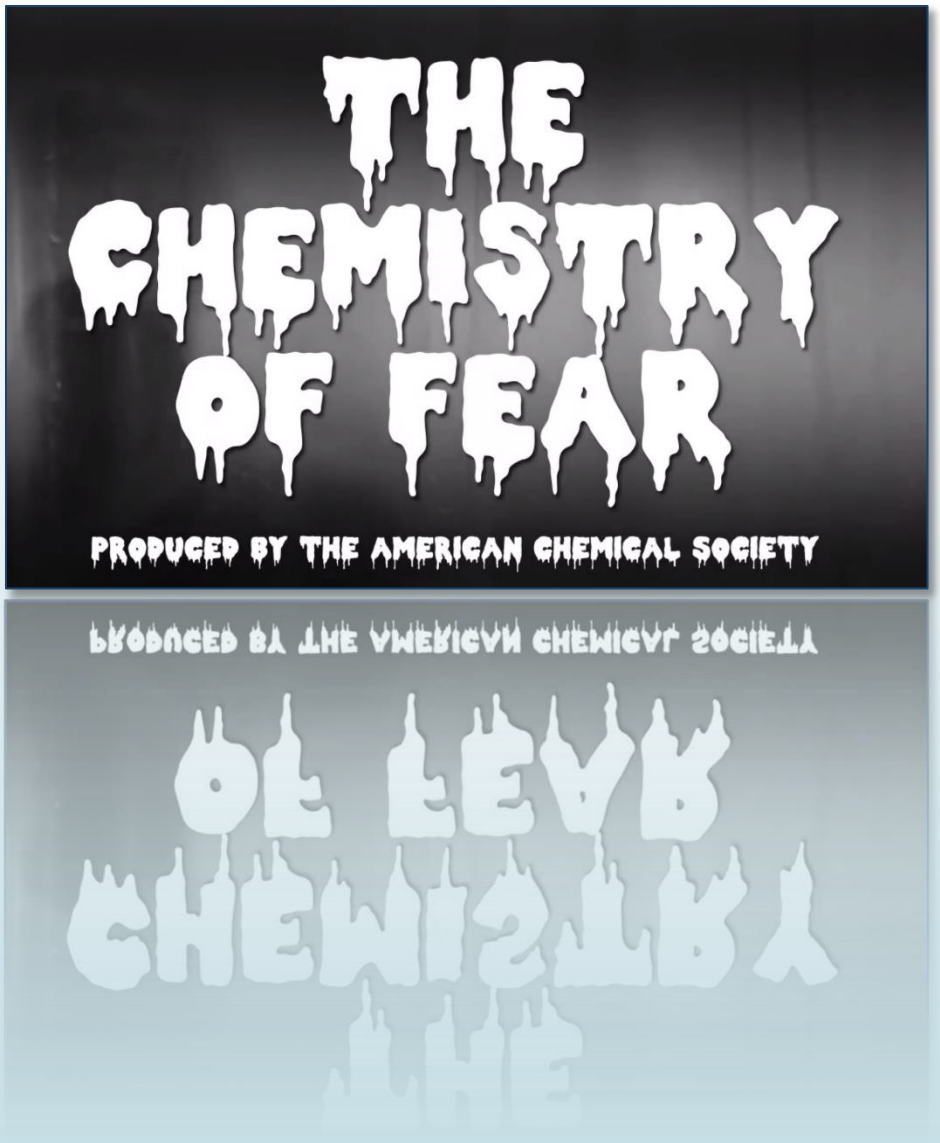
- Assay design
  - avoid opening tubes for reagent addition, etc.
  - reactions that produce RNA products
  - negative controls
  - real-time assays with closed-tube detection



From CLSI MM-10-A 'Establishing Molecular Testing in Clinical Laboratory Environments

Quantitative Tests

- Quantitative molecular tests are basically fancy Chemistry assays.
  - Yes. Be afraid.
  - Talk to your chemist. They love this stuff.
- Require periodic calibration verification / verification of reportable range.
- Calibration and calibration materials.



Reporting

- If using Analyte Specific Reagents or Laboratory Developed Test, require a disclaimer.
  - See CAP checklist for recommended wording.
- Director sign-off if an interpretive component is involved.
- Special Issues
  - Each area has some specific issues; one-over-lightly is all there's time for.
  - Genetics
  - Oncology
  - Pharmocogenomics

Genetic testing

Table 16. Mutation Composition of the ACMG CF Screening Panel

ΔI507	N1303K	R1162X	2184delA
ΔF508	R117H	W1282X	2789(+5)G>A
A455E	R334W	621(+1)G>T	3120(+1)G>A
G85E	R347P	711(+1)G>T	3659delC
G542X	R553X	1717(−1)G>A	3849(+10kb)C>T
G551D	R560T	1898(+1)G>A	Reflexes

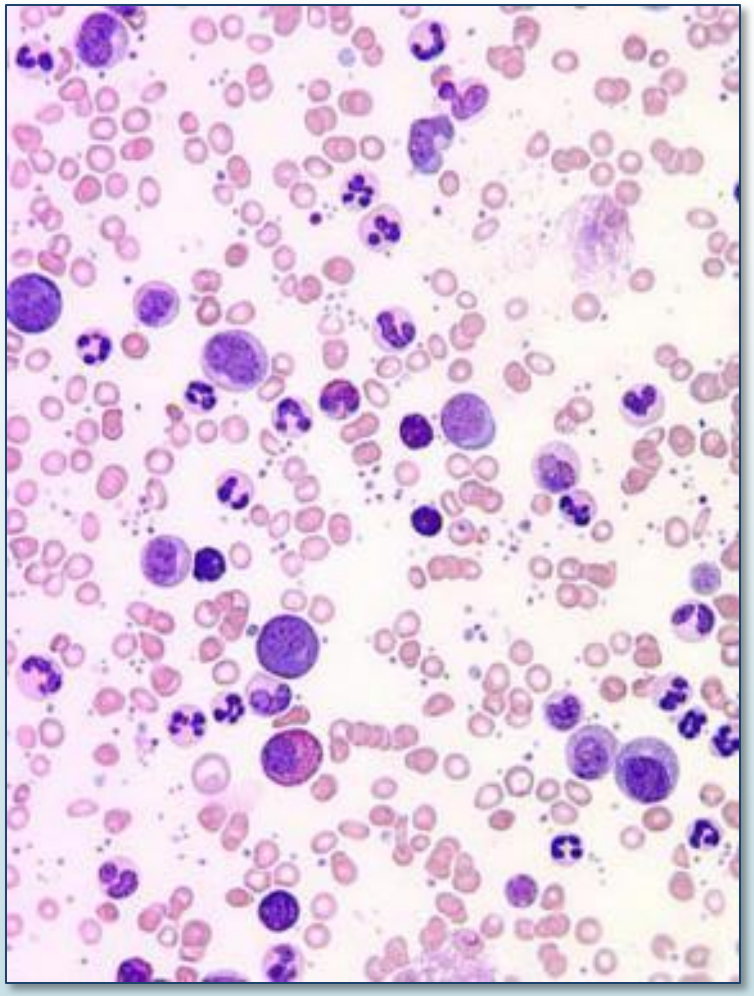
- Is specific consent required? how many variants did we test; can our method miss important variants? Penetrance, severity, inheritance for complex disorders. Counseling recommendation. Privacy issues and family testing.
- Standardized names of genes
- Correlation with histology / cytology when those specimens are tested.
- CF: Must test the 23 recommended mutations, and have arrangements to send others when clinically indicated. Complex algorithms.
- Thrombophilia; Factor V Leiden and Factor II, FDA-approved methods available.
  - Risk varies with homo-versus heterozygosity at each locus; and with mutations in one versus both loci.
  - There are rarer polymorphisms which may be risk-associated.
  - Beware testing blood in liver or stem-cell transplant patients. Factors made in liver.

Example: Hemachromatosis

- 2 common mutations are C282Y and H63D.
- Variable penetrance and severity; C282Y more likely to be significant, homozygotes more likely to be affected than heterozygotes.

Oncology

- Tumor / tissue heterogeneity
  - What proportion of the allele is significant?
- Jak2 V617F
  - Found in polycythemia vera and in myelofibrosis; occasionally in other myeloid diseases.
  - Qualitative to assess for possibility of disease; quantitative testing may reflect prognosis.
- BCR-ABL Fusion
  - Used for diagnosis, and quantitatively to follow therapy for CML.
- Tissue-based testing
  - MUST have procedures to verify that tumor is present in sample.
  - MUST define tumor percentage required for valid sampling / analysis.
  - A complex area.



CML: from https://emedicine.medscape.com/article/199425-workup#c8

Pharmacogenomics

Table 20. Pharmacogenomic Assays

Drug or Indication	Pharmacogenomic Assays
Tamoxifen and antipsychotic drugs	Cytochrome P450, *CYP2D6*, and *CYP2C19*
Warfarin	*CYP2C9* and vitamin K receptor complex 1 *(VKORC1)*
Irinotecan	*UGT1A1*
Clopidogrel, proton pump inhibitors	Cytochrome P450, *CYP2C19*
Statin drugs	Kinesin-like protein-6 *(KIF6)*
Abacavir	HLA-B*5701
Carbamazepine, phenytoin	HLA-B*1502
Allopurinol	HLA-B*5801

- Testing for drug metabolism and efficacy.
- Interpretation is complex; reports which give guidance to providers are essential.
- For many drugs, clinical value is an evolving area.

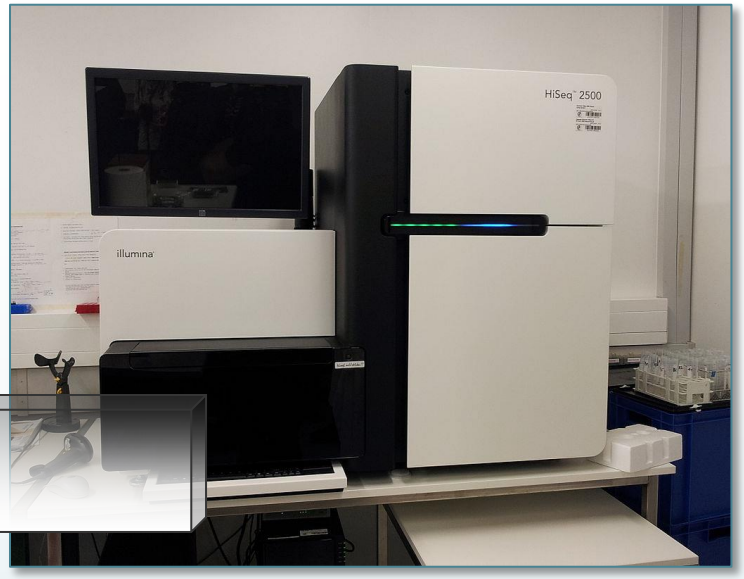
Infectious Diseases

- Testing for diagnosis
  - Optimized for sensitivity usually
    - When is sensitive too sensitive – *C. difficile*
  - Sometimes quantity important; CMV in transplant.
  - Sometimes used after culture; e.g. blood culture assessment tests, BS testing.
- Testing for management
  - Viral load
  - Genotyping for therapeutics
- Testing for risk assessment
  - HPV; high-risk genotypes associated with cervical cancer
- Testing for infection control
  - MRSA screening, others.

Personnel & Competencies

- For use of the MOL checklist:
  - 'The section director/technical supervisor of the molecular pathology laboratory is a pathologist, board-certified physician in a specialty other than pathology, or doctoral scientist in a chemical, physical, or biologic science, with specialized training and/or appropriate experience in molecular pathology.'
- 'The molecular pathology general supervisor is qualified as one of the following.'
  - Person who qualifies as a section director/technical supervisor; or
  - Bachelor's degree in a chemical, physical, biological, or clinical laboratory science or medical technology with at least four years of experience (at least one of which is in molecular pathology methods) under a qualified section director
- Competencies
  - Develop molecular competencies around contamination control and other specifics of molecular testing.

NGS Testing



- Specimen
- DNA / RNA Extraction
- Amplification / Selection of Targets, Parallel Sequencing
- Analysis of Sequence Data
- Interpretation and Clinical Use

Image from https://commons.wikimedia.org/wiki/File:Illumina_HiSeq_2500.jpg

Target Amplification / Selection

- Few NGS methods are yet entirely target-agnostic; most use some form of amplification or selection of target nucleic acid.
- Validation/verification studies need to prove that clinically-relevant targets aren't lost.

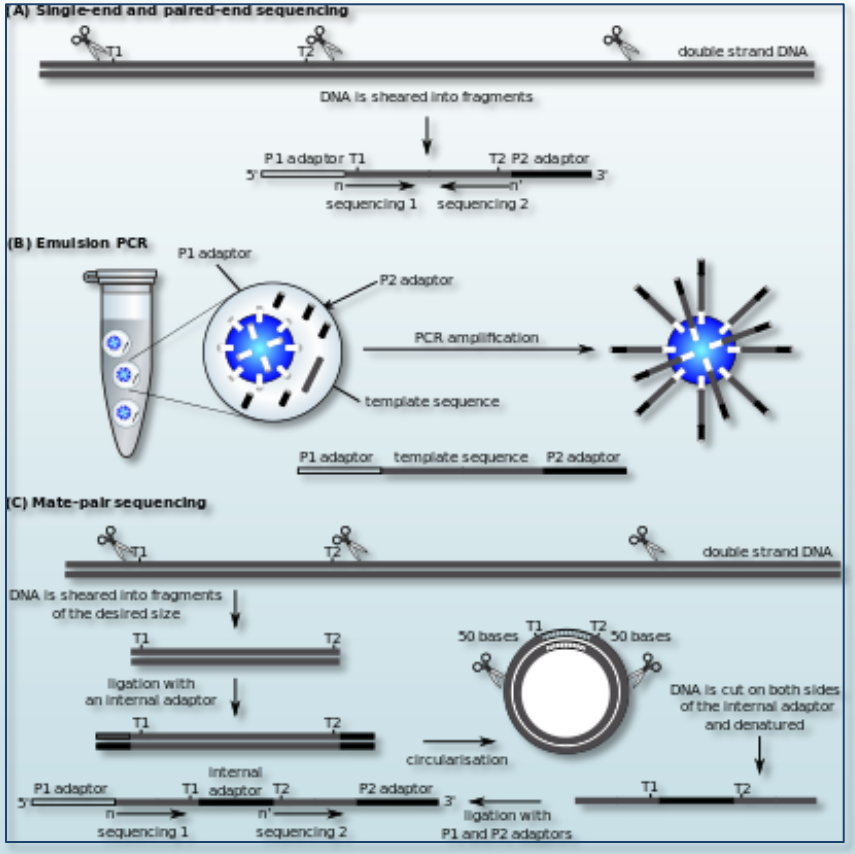


Image from https://commons.wikimedia.org/wiki/File:Library_preparation_for_the_SOLiD_platform.svg

Parallel Sequencing

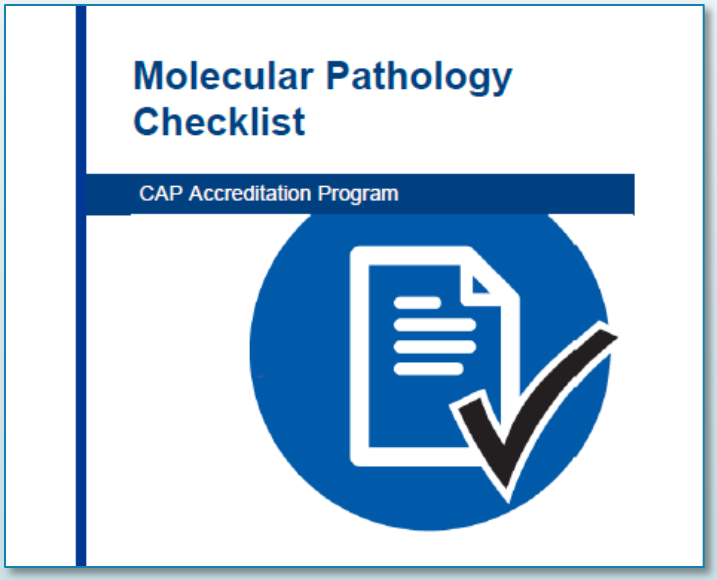
- Variables include:
  - Read length: some applications requite longer read-lengths; some can be done with quite short.
  - Sequencing depth: how many-fold sequencing of the targets do you need for accuracy / sensitivity to minor variants?
  - Sensitivity to homopolymers, indels, ability to accurately handle sequence repeats.

NGS Quality, briefly

- Very few NGS assays are yet FDA-approved.
  - LDT rules apply.
- Complex testing must be validated and quality-controlled from beginning to end.
- Many labs split pieces of the workflow between referral services.
  - Need policy for selection and monitoring.
- Extraction QC must include relevant parameters, e.g. size and amount of DNA/RNA expected.
- Test must be validated for the range of samples and targets expected.
- The bioinformatics element must be documented and quality-controlled. Updates / upgrades to the tools must be tracked and verified.
- Interpretive criteria must be defined. How do you 'call' significant findings? What do you do with findings that are novel or of uncertain significance? What do you do with findings unrelated to the disease you're primarily testing for?
- Some results need confirmation.
- Special topics
  - Plasma testing for fetal abnormalities
  - Stem-cell engraftment monitoring
  - Identity testing
  - Minimal residual disease testing
  - Etc.

Resources

- CLSI MM-19-A 'Establishing Molecular Testing in Clinical Laboratory Environments'
- CAP Microbiology and Molecular Pathology checklists



Acknowledgements

- David Peaper M.D., Ph.D. and Danielle Plank MB(ASCP)^{CM} for multiplex verification scheme. Also for being really, really good at this stuff and teaching me about it.