



A PRACTICAL GUIDE TO MOLECULAR MICROBIOLOGY ASSAYS IN THE CLINICAL LABORATORY

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DISCLOSURES

- Yale University: Employment
- VA CT Healthcare: Employment
- BD: Honoraria / Advisory Board
- All of the relevant financial relationships listed for this individual have been mitigated.
- I will use brand names and refer to companies throughout, but do not imply support or favor.
- Collectively, labs I direct use most vendors on the market in some way.

OVERVIEW & GOALS

Overview

- Part 1 – The Basics
 - Overview of fundamentals of molecular methods
 - Overview of regulatory basics
- Part 2 – More complicated stuff
 - Multiplexing
 - Sequencing
- Part 3 – More practical stuff
 - Validation / Verification / Workflow
 - Cases

Goals

- Provide an overview of modern molecular methods in medical microbiology
- Provide a framework for evaluating molecular methods in the clinical microbiology laboratory
- Review scenarios and cases for questions relating to molecular methods including assay selection and verification / validation

SPEAKER SUMMARY – WHO AM I?

- Direct two labs performing internal reference testing for hospitals and clinics throughout Connecticut (YNHH) and New England (Old VISNI)
- Past member of CAP Microbiology Committee
- Current Vice Chair of CAP Accreditation Committee
- NYS CLEP Director for Yale New Haven Hospital Labs
- Leader (among others) of YNHHS COVID-19 response including lab testing, sample collection network, and stewardship
- Leader (among others) of development of in-house bacterial WGS via NGS

Test Category	FY2025 Volume
Routine and Specialized Cultures	322,983
Susceptibility Testing Panels	76,902
Molecular	188,408
High Throughput NAAT	93,078
Quant. Viral Loads	4,207
STAT Random Access NAAT#	81,201
LDT NAAT	9,387
Bacterial WGS	535*
Serology / Antigen / Other	79,706

AUDIENCE SUMMARY – WHO ARE YOU?

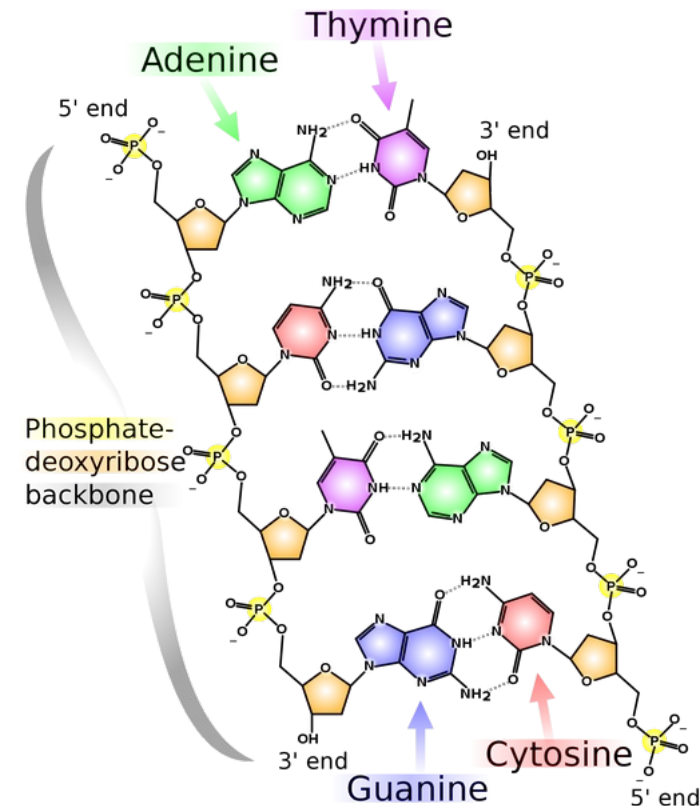
- Who is here? – Lab directors? Pathologists? Managers / Supervisors? Molecular Specialists? Laboratory Scientists? Industry medical affairs? Industry scientific affairs?
- What kind of labs? – Commercial? AMCs? Health Systems? Community hospitals?
- What kind of testing?
 - Modified FDA
 - LDTS with ASR? Open Channel? Home brew?
 - FDA approved?
 - Sequencing? NGS?
- Experience with molecular? – Manual extraction? Pour your own gels? Ethidium Bromide?
- Test menu? – Respiratory? GI? C.diff? GAS? M.tb? Blood Culture? ME?

Assumptions:

- *Most are not looking to stand up a highly complex, LDT-focused lab from scratch*
- *Most have limited choices in terms of platforms at your disposal*

FUNDAMENTAL PRINCIPLE: COMPLEMENTARY BASE PAIRING

- Complementary base pairing is the basis for all nucleic acid testing including amplified testing
- Complementary sequences are used for:
 - Primer and probe design
 - Amplicon capture to matrices
 - “Bridging” of amplicon to detection method
- Energy of complementary bonds determine assay conditions (e.g. salts and temperatures including T_m)
- Inappropriate matches / mismatches affect assay sensitivity and specificity



FUNDAMENTAL PRINCIPLE: OLIGONUCLEOTIDE LABELING AND CONJUGATION

- Radioactive isotopes were incorporated into DNA, but fluorescent molecules, enzymes, biotin, ferrocene, etc. are added to DNA primers or oligonucleotide probes to facilitate detection
- DNA oligonucleotides can be bound to substrates to create arrays or other systems to physically demarcate sequences
- These principles are the basis for most real-time PCR methods, Luminex, ePlex, Verigene / Diasorin, etc.

< Amplification >



Genome DNA
Master mix
Taq DNA polymerase

Reaction condition
93°C, 30sec.
↓
65°C, 30sec.
↓
72°C, 30sec., 40cyc.

< Hybridization >



Amplified DNA
Bead mixture
Hybridization Buffer

Reaction condition
95°C, 2min.
↓
52°C, 60min.

< SA-PE (Streptavidin-phycoerythrin) Reaction >



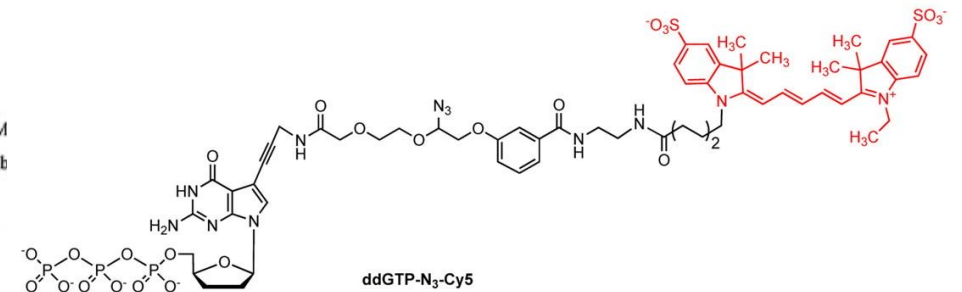
SA-PE
Phosphate buffer

Reaction condition
52°C, 60min.

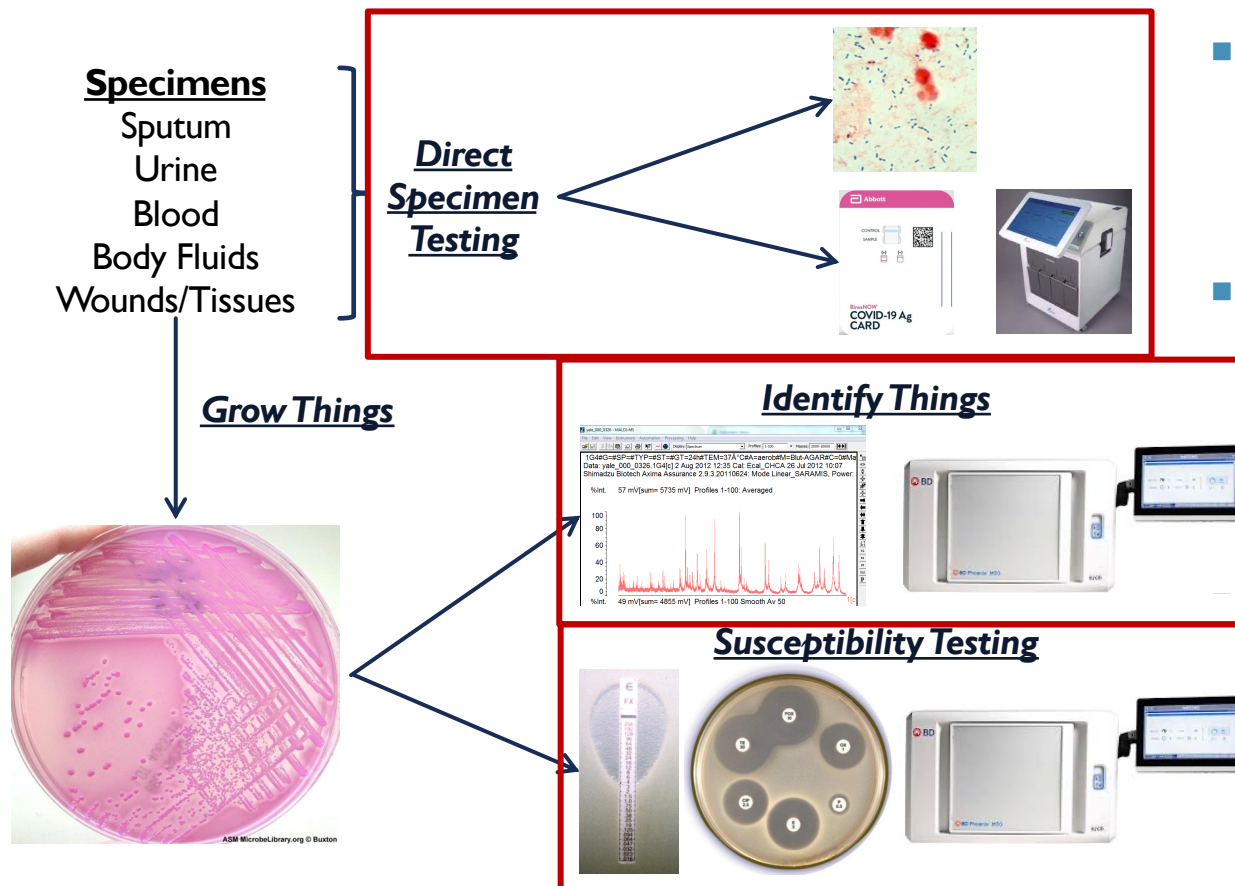
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Ma
lab



CLINICAL MICROBIOLOGY WORKFLOW & TASKS



- Direct assessment of specimen
 - Gram or other stains
 - Direct specimen NAAT or antigens
 - Growth and isolation on culture media
 - Generally requires at least 18 to 36 hours
 - Laboratory protocols designed to isolate clinically relevant organisms ASAP
- Recovered Isolates → Identification & Susceptibility
- ID and AST require pure colonies (not mixed)
 - May require additional growth (another 18 hours) esp. for AST

ROUTINE MEDICAL MICROBIOLOGY

Questions

- For this disease, is there one or only a few causative organisms?
- Will these organisms grow in a reasonable time frame using commonly available media?
- Are organisms present in sufficient numbers to grow directly from a specimen or is enrichment needed?
- For this body site, are there normal flora that must be considered?

Challenges

- Slow growing organisms
- Antibiotic treated patients
- Turn-around-time for critical results
- Non-cultivable organisms
- Normal flora

Goal: Identify disease-causing organisms quickly while retaining clinically relevant information

WHY MOLECULAR METHODS?

- Clinically significant infections and/or TAT Critical
- Current methods inadequate
 - Specialized culture techniques
 - Fastidious / uncultivable organisms
 - Slow growing organisms
 - Antibiotic treated patients
- Definite pathogens OR definite epidemiologic significance
- Is a “yes” or “no” answer sufficient?

IMPROVED PERFORMANCE OF PCR: 2009 H1N1

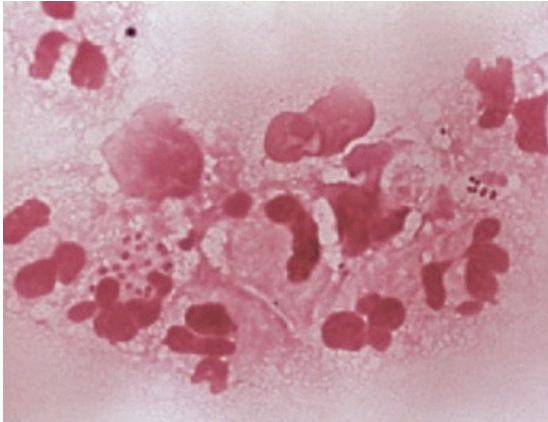
	% Sensitivity	% Specificity
DFA	80.4	99.2
R-Mix rapid culture	96.9	100
Rapid Ag	46.4	100
<i>J Clin Viro</i> v45 p146; 500 respiratory specimens before 2009 vH1N1		

	% Sensitivity	% Specificity
DFA	46.7	94.5
R-Mix rapid culture	88.9	100
Rapid Ag	17.8	93.6
RVP (PCR)	97.8	100
<i>J Clin Viro</i> v45 p191; 288 respiratory specimens from 2009 vH1N1		

Note: Viral genetic and antigenic changes reduce test sensitivity and targeted tests need updating. Yale School of Medicine

A QUICK CASE

- A 5 year old with T=101.2°F, altered mental status, and purpura on legs



	Tube 1	Tube 4	Normal Range
Protein	100	98	<50 mg/dL
Glucose	30	33	40 – 70 mg/dL
WBC	1300 (95% PMN)	1260 (97% PMN)	<6 cells / μ L
RBC	3	2	0 cells / μ L

CASE FOLLOW UP

- Cultures were “No Growth to Date” @ 18 hours
- LP had been delayed, and vancomycin and ceftriaxone had been given 2 hours before LP
- What additional testing can be done?
 - Sent out for BioFire ME Panel → *Neisseria meningitidis* detected

THE BIOFIRE MENINGITIS/ENCEPHALITIS PANEL MENU

Overall 94.2% Sensitivity and 99.8% Specificity¹

Sample Type: Cerebrospinal Fluid (CSF) collected by lumbar puncture

BACTERIA:

- *Escherichia coli* K1
- *Haemophilus influenzae*
- *Listeria monocytogenes*
- *Neisseria meningitidis*
- *Streptococcus agalactiae*
- *Streptococcus pneumoniae*

YEAST:

- *Cryptococcus* (*C. neoformans*/*C. gattii*)

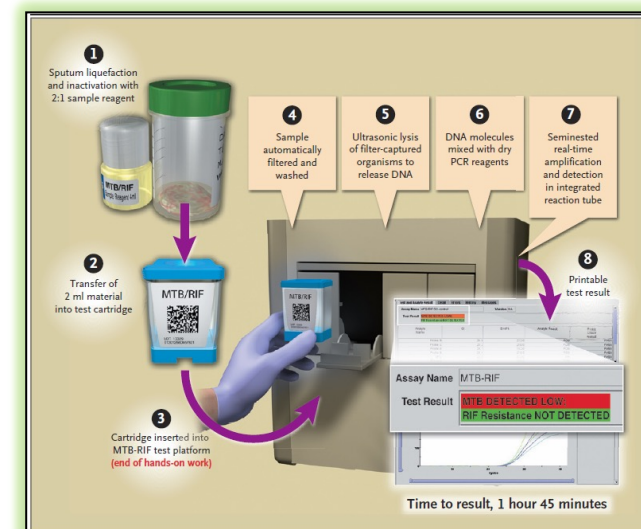
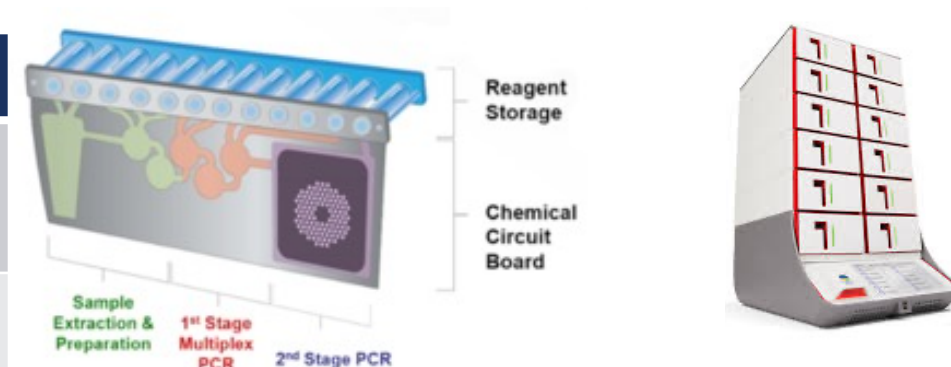
VIRUSES:

- Cytomegalovirus (CMV)
- Enterovirus (EV)
- Herpes simplex virus 1 (HSV-1)
- Herpes simplex virus 2 (HSV-2)
- Human herpesvirus 6 (HHV-6)
- Human parechovirus (HPeV)
- Varicella zoster virus (VZV)

DIRECT MOLECULAR TESTING OF SPECIMENS

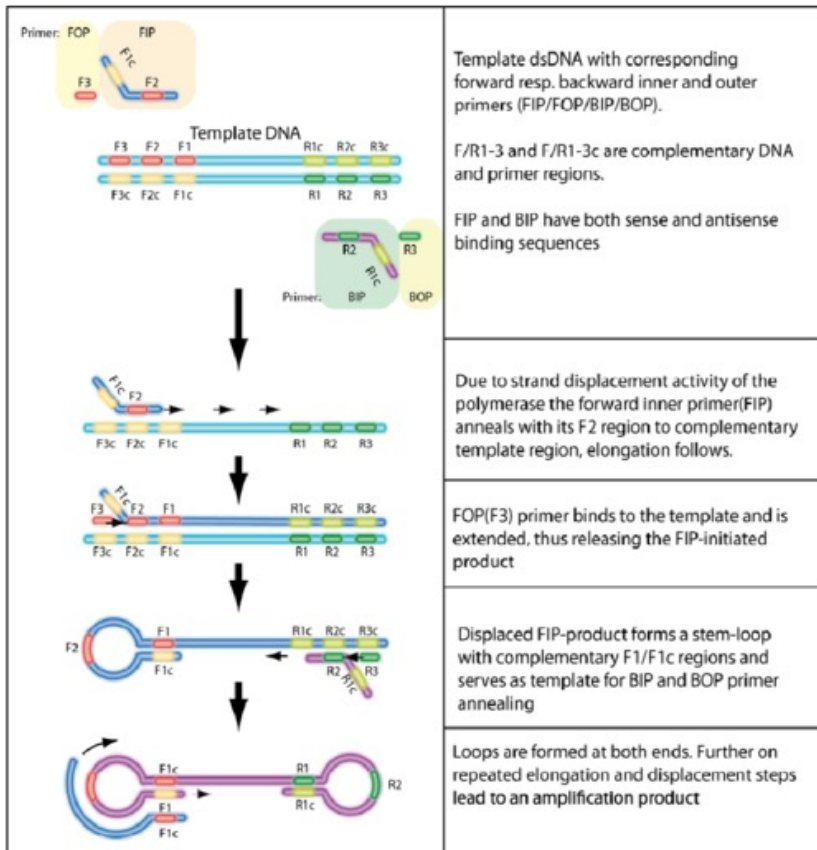
Advantages	Disadvantages
Sensitive and Specific (if designed well)	More expensive
Can be rapid (~20 min)	Limited “Multiplex” capacity (Changing)
Can be done at point-of-care	Must have knowledge of targets
Can be quantitative (viral loads)	Requires specialized equipment
Does not require viable organisms	Risk of (insidious) contamination
Does not require growth <i>in vitro</i>	Not necessarily more sensitive

Looks for pathogen nucleic acid → Potentially replacing culture or antigen techniques in select circumstances

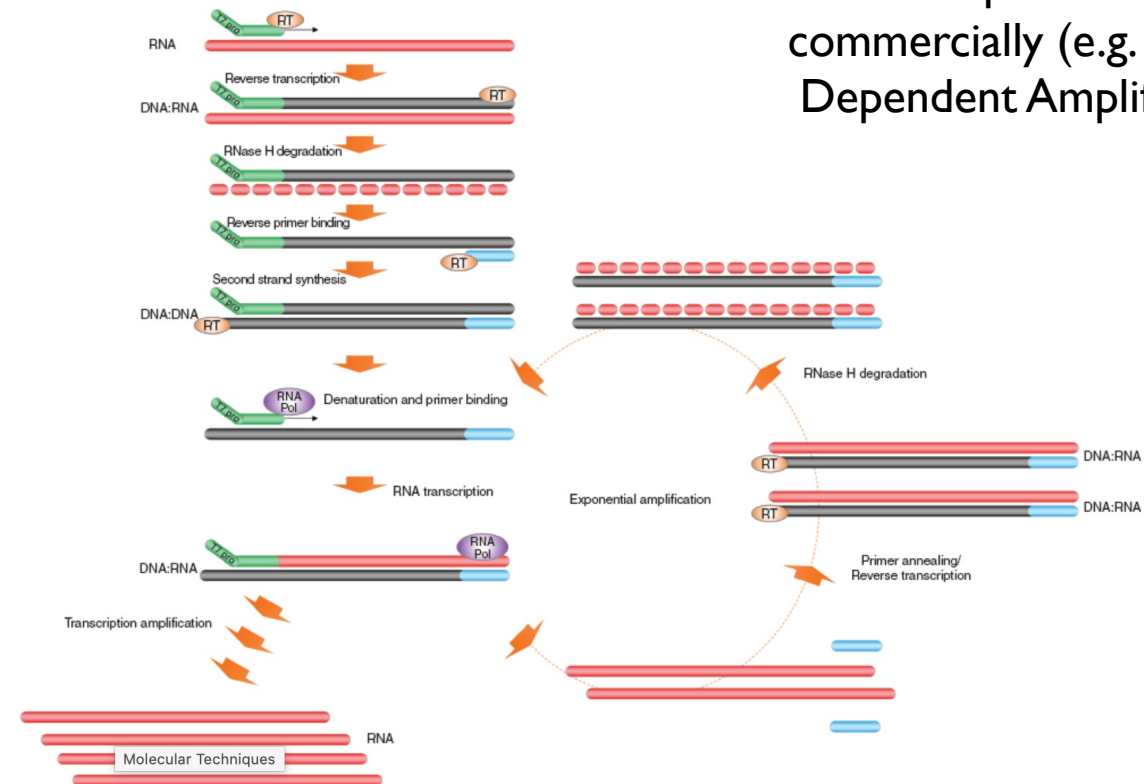


ISOTHERMAL AMPLIFICATION METHODS

LAMP

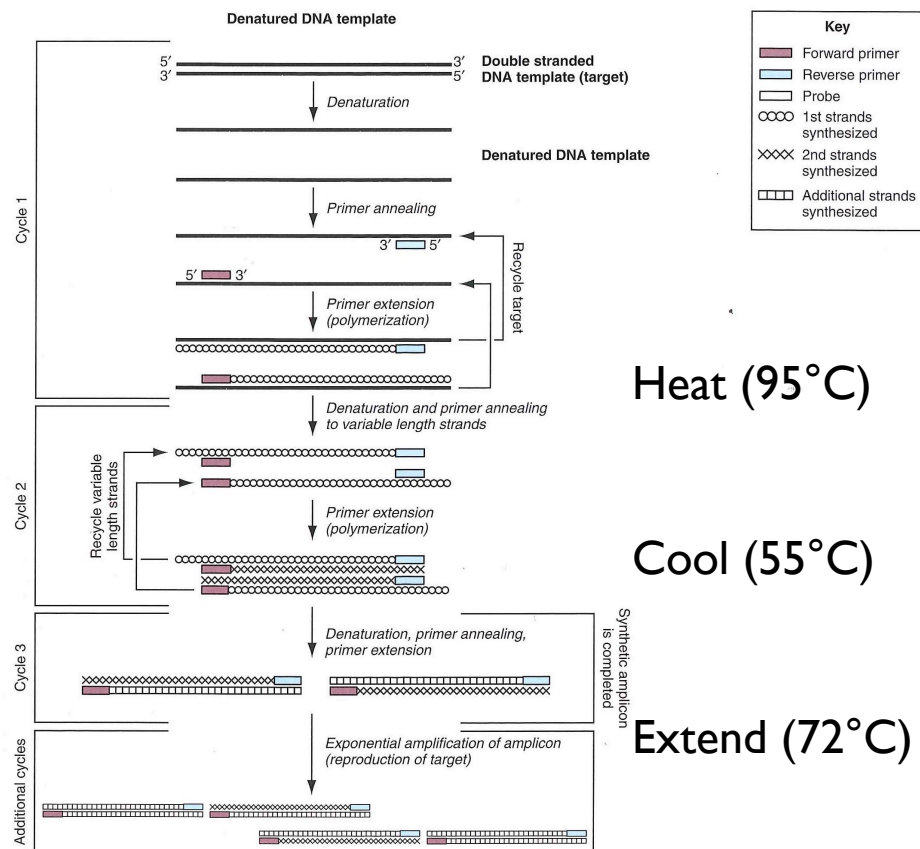


TMA



Other isothermal methods developed and used commercially (e.g. Helicase Dependent Amplification)

POLYMERASE CHAIN REACTION (PCR)



# of Cycles	# of Copies
1	2
2	4
3	8
4	16
5	32
6	64
7	128
//	//
35	34×10^9

PCR V. ISOTHERMAL METHODS

PCR

- Very well understood and established method → Easier to design primers and probes
- Nucleic acid extraction allows variety of specimens to be tested
- Requires more sophisticated instruments incl. extraction
- *Hologic PCR COVID Assay = 3/16/2020*

Isothermal

- Much simpler equipment (in theory) a single temperature heat block.
- Can be more rapid than PCR in some cases.
- More work to create probe sets and establish chemistry of assay.
- Less robust reagent set for optimization
- *Hologic TMA COVID Assay = 5/14/2020*

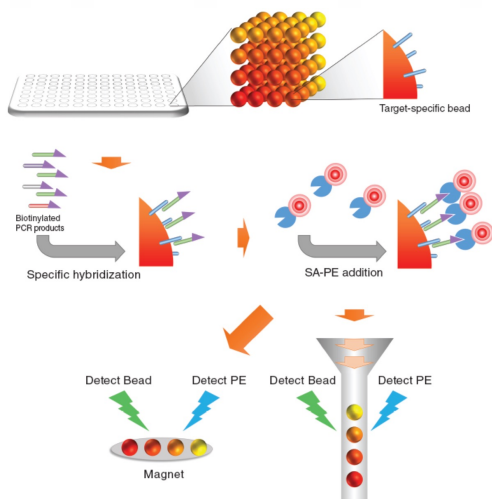
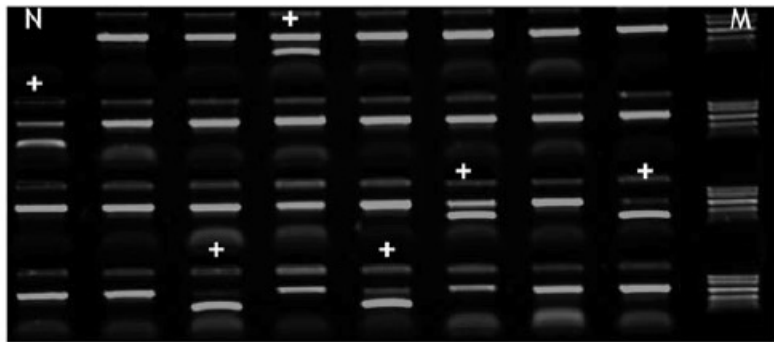
ONCE AMPLIFIED, HOW TO DETECT NUCLEIC ACID?

End Point	Real Time
Gels	DNA Binding Dyes (e.g. SYBR)
Arrays	Hydrolysis Probes (e.g. TaqMan)
Beads	FRET Probes, Scorpion Probes, Molecular Beacons, etc.
Mass Spectrometry	Hybridization protection
Hybridization protection	

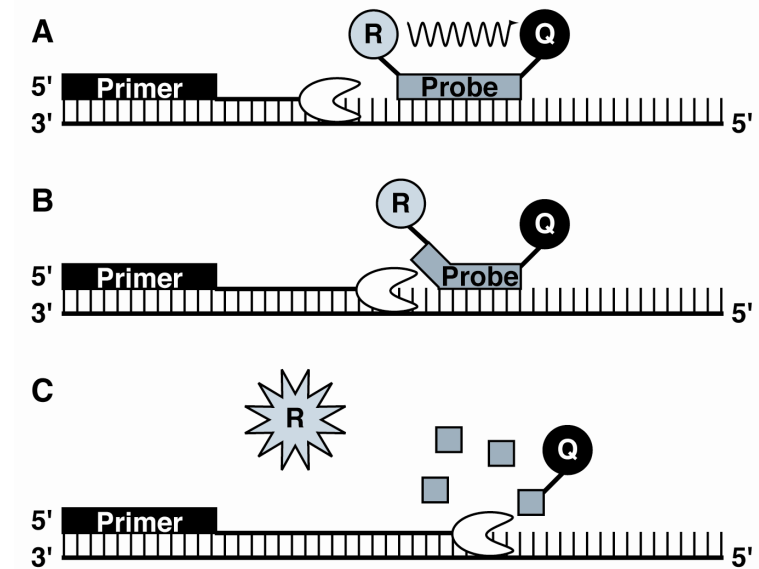
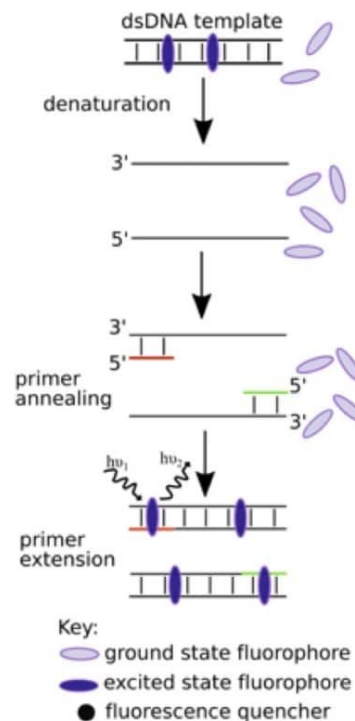
- Different detection methods allow different degrees of multiplexing
- End Point PCR has higher risk of contamination
- Real Time detection requires more expensive equipment and reagents

PCR DETECTION METHODS

End-Point PCR → Gels and Beads Real Time PCR → Binding Dyes and Probes



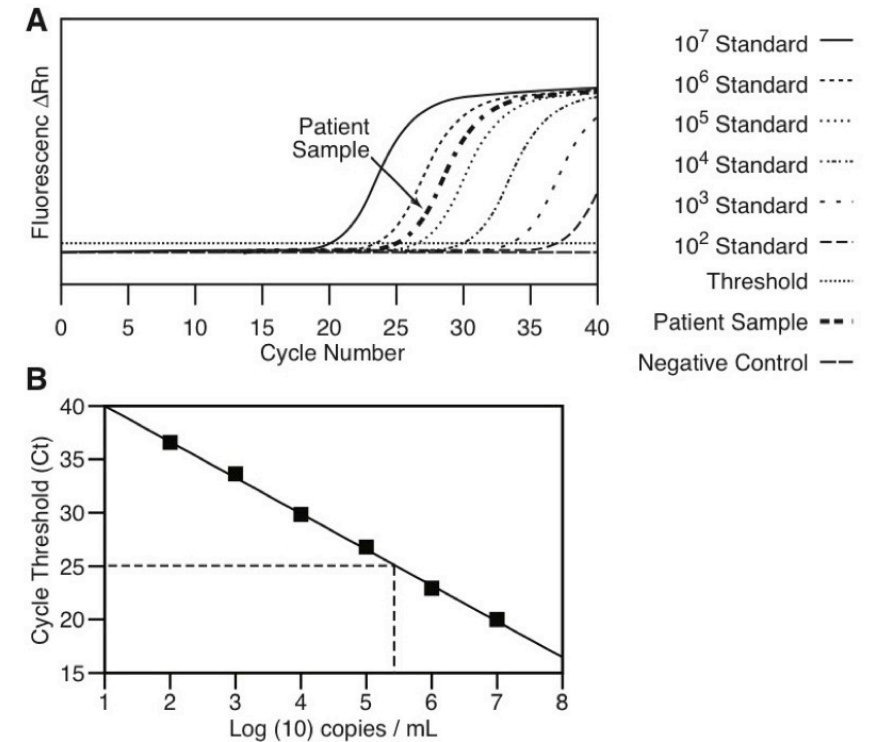
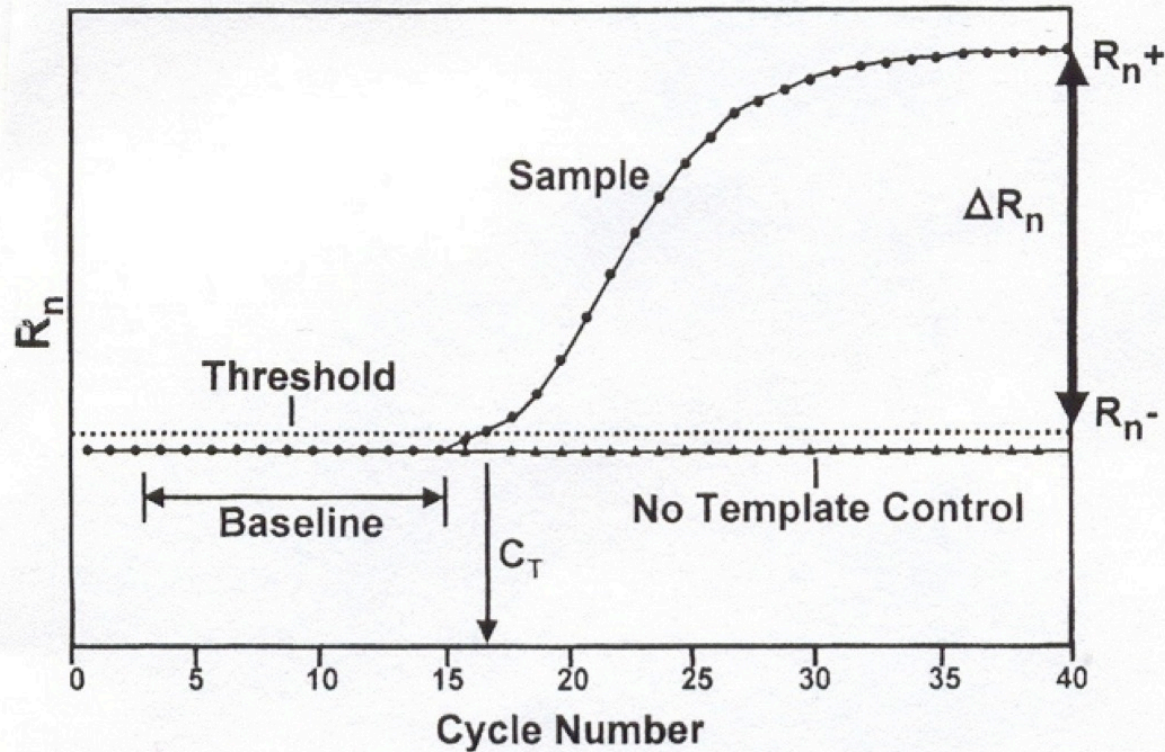
Fluorescent dye-based real-time PCR



Probes Include:

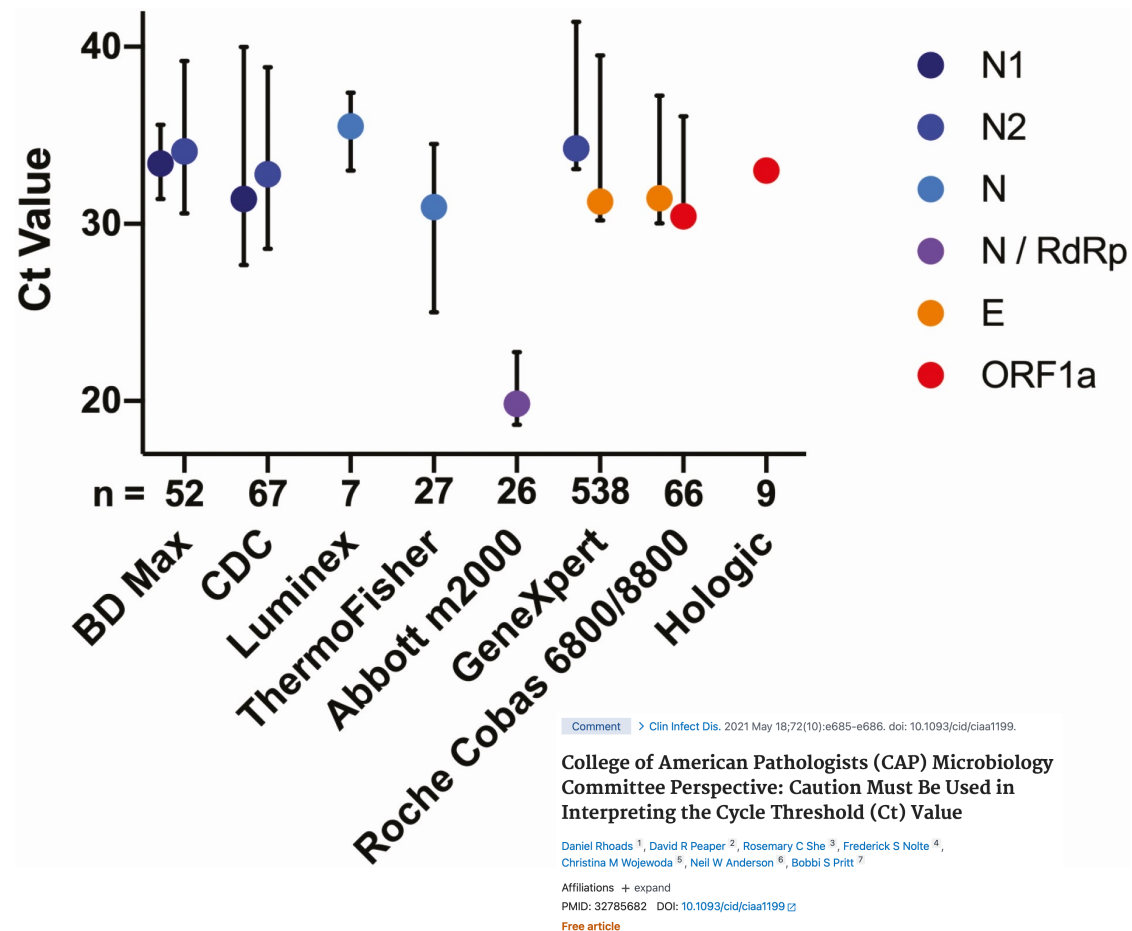
- Hydrolysis Probes (Shown)
- Molecular Beacons
- Scorpion Probes

“REAL TIME” NAAT: QUALITATIVE AND QUANTITATIVE



Signal (R = reporter) is emitted in “real-time” as target is amplified. C_T is cycle number at which the threshold is crossed to give a **positive** result.

CAVEATS ABOUT CT VALUES



- *Should* be consistent for a given sample on a given test system
- Can vary between assays for same analyte and between specimens from same patient
 - Micro specimens are heterogenous
- Assays behave oddly at / near / below LOD

ASSAY PERFORMANCE AT LOD – QUANTUM EFFECTS

Table 12 LoD determination using USA-WA1/2020 strain

Strain	Concentration [TCID ₅₀ /mL]	Total valid results	Hit rate [%]^		Mean Ct*	
			Target 1	Target 2	Target 1	Target 2
USA-WA1/2020 (stock concentration 2.8E+05 TCID ₅₀ /mL)	0.084	21	100	100	31.0	33.0
	0.028	21	100	100	31.8	34.1
	0.009	21	100	100	32.7	35.2
	0.003	21	38.1	100	33.5	36.4
	0.001	21	0	52.4	n/a	37.9
	0.0003	21	0	14.3	n/a	37.2
	0.0001	21	0	9.5	n/a	38.5
	0 (blank)	10	0	0	n/a	n/a

^ All replicates where Target 1 was positive were also positive for Target 2.

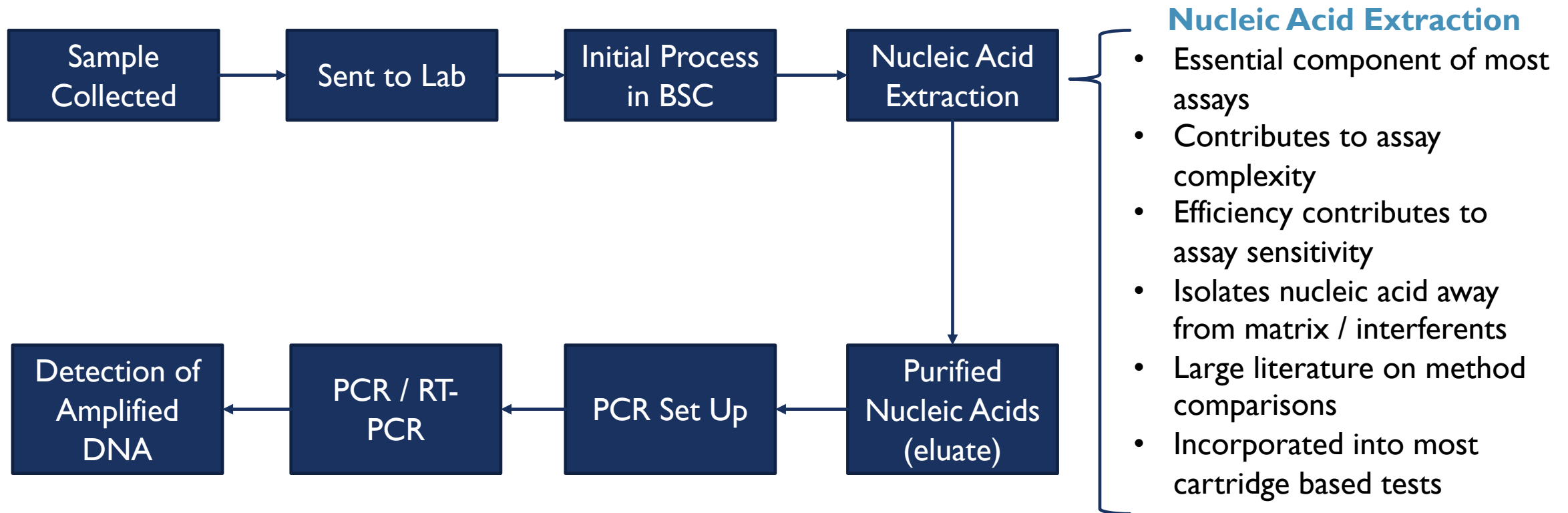
* Calculations only include positive results.

Table 13 Probit predicted 95% hit rates using USA-WA1/2020 strain

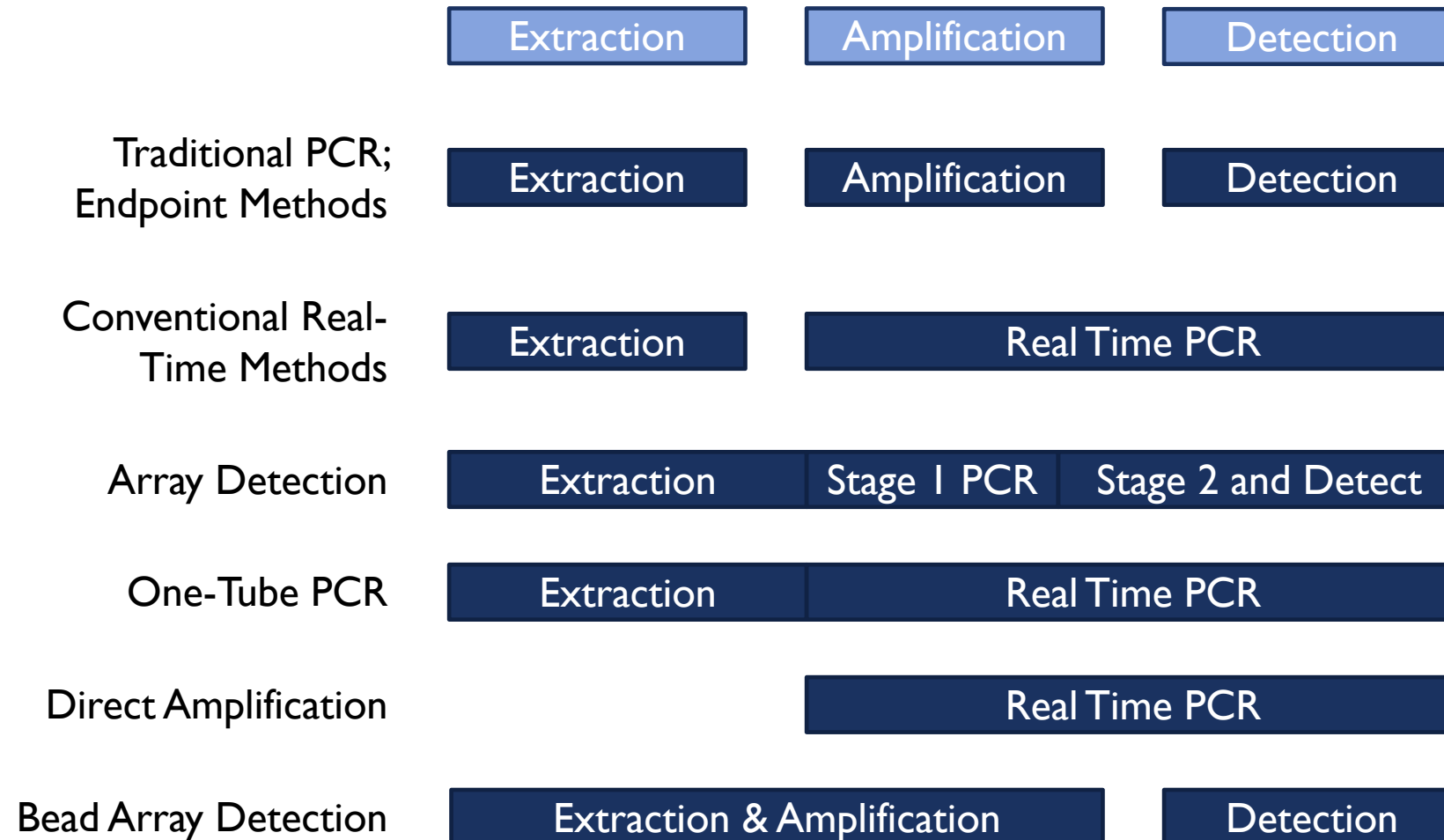
Strain	Probit Predicted 95% Hit Rate [TCID ₅₀ /mL]	
	Target 1	Target 2
USA-WA1/2020 (stock concentration 2.8E+05 TCID ₅₀ /mL)	0.007 (95% CI: 0.005 – 0.036)	0.004 (95% CI: 0.002 – 0.009)

- Implications for low positive / false positive assessment
- Low positives may not repeat even if true positives
- My bias is to get a new, better sample if it matters and is possible

TYPICAL MOLECULAR MICRO (PCR) WORKFLOW



MOLECULAR MICRO PCR ASSAY FORMATS



PCR CONSIDERATIONS - COMMERCIAL KITS OFTEN INCLUDE

- **IC fails, target negative** → invalid; suspect inhibition or extraction failure → repeat.
- **NTC positive** → contamination in amplification reagents/workspace → halt, investigate.
- **Extraction blank positive** → invalidate associated positives.
- **External positive fails** → run invalid; do not report.
- **UNG/dUTP** carryover prevention to enzymatically destroy prior amplicons.

CURRENT NAAT METHODS

Assay Format	Highly (>4) Multiplexed	Random Access (STAT)	Point of Care	Turn-Around-Time
Traditional PCR w/End Point	Yes	No	No	>8 hrs
Conventional Real-Time	No	No	No	>8 hrs
Array Detection	Yes	Yes	Yes	~1 hr
One-Tube PCR	No → Yes	Yes	Yes	20 min – 1 hr
Direct Amplification	No	Yes	No	~1 hr
Bead Array	Yes	Yes	No	~3 hr
Isothermal Methods	No	Yes	Yes	20 min – ~3 hr

POTENTIAL PITFALLS OF NAAT

Event	Assay Implications	Outcomes
Primer binding site mutations/variations	Inefficient or no amplifications	False Negatives
Probe binding site mutations/variations	Inefficient or no detection	False Negatives
Contamination of workspace with amplicon	Nucleic acid present in negative samples	False Positives
Inhibitors present in sample	Inefficient or no amplification	False Negatives
Highly similar primer binding sites among related organisms	Amplification of “wrong” pathogen	False Positives
Probe Degradation	Late, unusual signal	False Positives



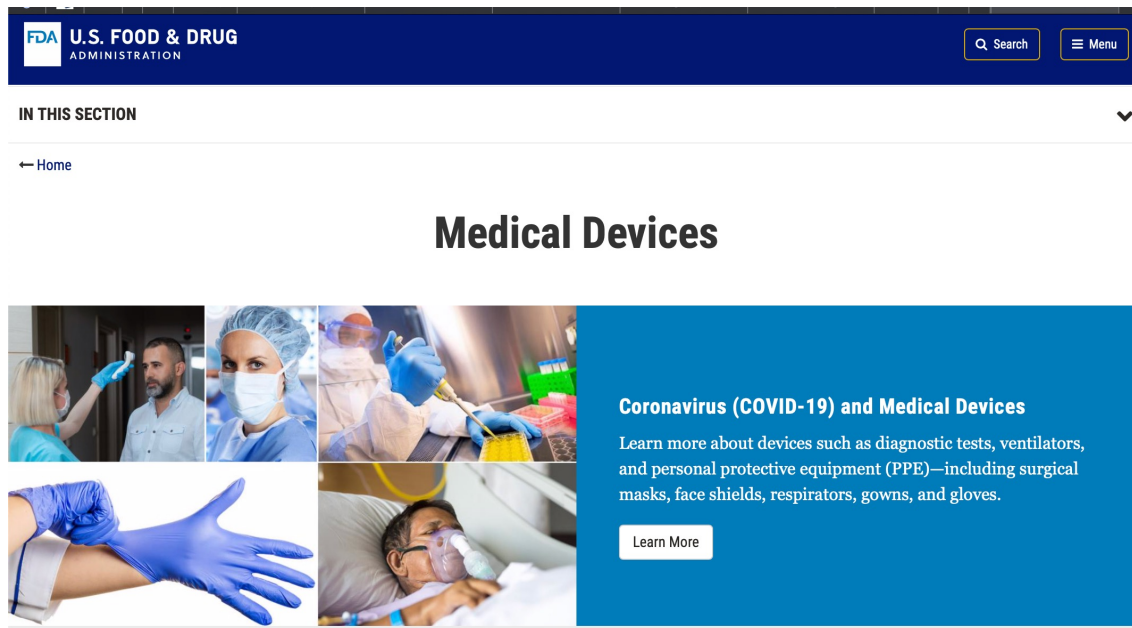
REGULATORY BASICS



HOW ARE LABS REGULATED?

- National accreditation organizations (AOs) offer accreditation programs that are recognized by CMS for purposes of certifying the compliance.
- CMS “deems” these accredited health care facilities as having satisfied the health and safety standards component of the Medicare certification process by virtue of their accreditation through a CMS-recognized “deemed status” program.
- CMS can do spot inspections or post-inspection validations.
- Accreditation organizations (AO)
 - AABB
 - American Osteopathic Association (AOA)
 - American Society for Histocompatibility and Immunogenetics (ASHI)
 - COLA (Commission on Office Laboratory Accreditation)
 - College of American Pathologists (CAP)
 - The Joint Commission
- States with CMS approved State Laboratory Programs
 - New York
 - Washington

HOW ARE LAB TESTS REGULATED?



- Per Section 201(h) of the Food, Drug, and Cosmetic Act, a device is:
- An instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including a component part, or accessory which is:
 - recognized in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them,
 - **intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or**
 - intended to affect the structure or any function of the body of man or other animals, and which does not achieve its primary intended purposes through chemical action within or on the body of man or other animals and
 - which does not achieve its primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of its primary intended purposes. The term "device" does not include software functions excluded pursuant to section 520(o).

FDA DEVICE CLASSES

- Class I General Controls
 - With Exemptions
 - Without Exemptions
- Class II General Controls and Special Controls
 - With Exemptions
 - Without Exemptions
- Class III General Controls and Premarket Approval
- FDA-approved, FDA-cleared, FDA-authorized, don't mean the same thing but I will probably inadvertently use these inappropriately at some point.

CLASS I – GENERAL CONTROLS

- Subject to the least regulatory control.
- They present minimal potential for harm to the user and are often simpler in design than Class II or Class III devices. Class I devices are subject to "General Controls" as are Class II and Class III devices.
- General controls include:
 - Establishment Registration of companies which are required to register under 21 CFR Part 807.20, such as manufacturers, distributors, repackages and relabelers.
 - Medical Device Listing with FDA of devices to be marketed.
 - Manufacturing devices in accordance with Good Manufacturing Practices (GMP) in 21 CFR Part 820.
 - Labeling devices in accordance with labeling regulations in 21 CFR Part 801 or 809.
 - Submission of a premarket notification [510(k)] before marketing a device.
- Examples of Class I devices include elastic bandages, examination gloves, and hand-held surgical instruments. Also: microbiology culture media, rapid biochemical tests

CLASS II – SPECIAL CONTROLS

- General controls alone are insufficient to assure safety and effectiveness, and
- Existing methods are available to provide such assurances.
- Subject to General and Special Controls, e.g.:
 - Special labeling requirements
 - Mandatory performance standards
 - Postmarket surveillance.
- Include Micro AST platforms
- E.g.: Class II Special Controls Guidance Document: Antimicrobial Susceptibility Test (AST) Systems; Guidance for Industry and FDA.
- Must demonstrate Substantial Equivalence to existing device unless *de novo* pathway
- Submitted and ***cleared*** through 510(K) process

CLASS III – PREMARKET APPROVAL

- The most stringent regulatory category for devices.
- Devices for which insufficient information exists to assure safety and effectiveness solely through general or special controls.
- Usually support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential, unreasonable risk of illness or injury.
- Premarket approval is the required process of scientific review to ensure the safety and effectiveness of Class III devices.
- Can be novel tests but must demonstrate clinical utility
- Submitted and **approved** through premarket authorization (PMA) pathway

NEW PATHWAY – DE NOVO

What Is a De Novo Classification Request?

The De Novo request provides a marketing pathway to classify novel medical devices for which [general controls](#) alone, or general and [special controls](#), provide reasonable assurance of safety and effectiveness for the intended use, but for which there is no legally marketed [predicate device](#). De Novo classification is a risk-based classification process.

Devices that are classified into class I or class II through a De Novo classification request (De Novo request) may be marketed and used as predicates for future premarket notification [[510\(k\)](#)] submissions, when applicable.

WHY DOES THIS MATTER?

- Manufacturers and FDA meet to discuss regulatory pathways for devices
- Class III Devices requiring PMA require much larger, more complicated, more expensive clinical trials to demonstrate clinical utility and safety
- Class II Devices require more traditional trials focused more on analytic validity
- Emergency Use Authorization is an exception with specific criteria

- Most molecular tests for infectious diseases are Class II devices with 510(K).
- De novo pathway allows for 510(K) like trials for new analytes
- Fewer Class III / PMA Devices

HOW IS COMPLEXITY DETERMINED?

- Tests are classified as:
 - Highly complex
 - Moderately complex
 - Waived
 - Provider performed microscopy
- The FDA classifies all tests that are cleared / approved
- All non-FDA-approved tests* are considered highly complex
- All modified FDA-approved tests are considered highly complex
- High and Moderate complexity are defined by points system
- Seven Criteria for assessment (all defined in CLIA):
 - Knowledge
 - Training and Experience
 - Reagents and Materials Preparation
 - Characteristics of operational steps
 - Calibration, QC, and PT materials
 - Test system troubleshooting and equipment maintenance
 - Interpretation and judgment
- 1, 2, or 3 points awarded based on defined criteria
- 1 = less complex; 3 = more complex
- Score $\leq 12 \rightarrow$ Moderately Complex
- Score $> 12 \rightarrow$ Highly complex

FDA APPROVED / CLEARED ASSAY / EUA AUTHORIZED

- Labeled for in vitro diagnostic use (IVD)
- Two key things from FDA approval
 - Indications for use: what samples, what clinical conditions, how to interpret test
 - Instructions for use: how to actually perform test
 - How to process samples
 - How to extract nucleic acids (restricted to those methods)
 - What controls to run and how often
 - How to interpret results

Alinity m

HSV 1 & 2 / VZV AMP Kit

Created May 2024

REF 09N61-095
53-608319/R1

CUSTOMER SERVICE: 1-800-553-7042
CUSTOMER SERVICE INTERNATIONAL:
CALL YOUR ABBOTT REPRESENTATIVE

Instructions must be carefully followed. Reliability of assay results cannot be guaranteed if there are any deviations from these instructions.

NAME

Alinity m HSV 1 & 2 / VZV Assay

- Alinity m HSV 1 & 2 / VZV AMP Kit (09N61-095)
- Alinity m HSV 1 & 2 / VZV CTRL Kit (09N61-085)

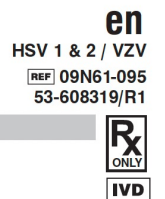
INTENDED USE

The Alinity m HSV 1 & 2 / VZV assay is an in vitro real-time polymerase chain reaction (PCR) assay for the qualitative detection and differentiation of Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2) and Varicella Zoster Virus (VZV) DNA from clinician-collected cutaneous or mucocutaneous lesion swab specimens from symptomatic patients suspected of active herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus infection. The Alinity m HSV 1 & 2 / VZV assay is intended to aid in the diagnosis of herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus active cutaneous or mucocutaneous infections. Negative results do not preclude herpes simplex virus type 1, herpes simplex virus type 2 or varicella-zoster virus infections and should not be used as the sole basis for diagnosis, treatment or other management decisions.

The Alinity m HSV 1 & 2 / VZV assay is not intended for use with cerebrospinal fluid (CSF) or to aid in the diagnosis of HSV or VZV infections of the central nervous system (CNS). The Alinity m HSV 1 & 2 / VZV assay is not intended for use in prenatal screening.

SUMMARY AND EXPLANATION OF THE TEST

HSV-1 and HSV-2: HSV-1 and HSV-2 are large double-stranded DNA viruses of the family *Herpesviridae*. HSV-1 and HSV-2 are genetically and antigenically distinct forms of HSV. Typically, HSV-1 is transmitted through oral-to-oral contact. HSV-2 is sexually transmitted and is the main cause of genital herpes.¹⁻³ HSV infections in humans can cause lesions at a variety of cutaneous and mucocutaneous sites. These lesions can be a result of the primary infection by the virus or they can result from a reactivation of the latent virus, causing recurrent episodes of the disease. The HSV DNA can be



The Alinity m System is designed to be a random access analyzer that can perform the Alinity m HSV 1 & 2 / VZV assay in parallel with other Alinity m assays on the same instrument.

HSV-1, HSV-2, and VZV DNA from specimens are extracted automatically onboard the Alinity m System using the Alinity m Sample Prep Kit 2, Alinity m Lysis Solution, and Alinity m Diluent Solution. The Alinity m System employs magnetic microparticle technology to facilitate nucleic acid capture, wash and elution. The resulting purified nucleic acids are then combined with the liquid unit-dose activation reagent, liquid unit-dose amplification reagents, and Alinity m Vapor Barrier Solution, and transferred by the instrument to an amplification/detection module for PCR amplification, and real-time fluorescence detection.

Assay controls are tested at or above an established minimum frequency to help ensure that instrument and reagent performance remain satisfactory. During each control event, a negative control and a positive control are processed through sample preparation and PCR procedures that are identical to those used for specimens. Assay controls are used to demonstrate proper sample processing and assay validity.

The Alinity m HSV 1 & 2 / VZV amplification reagents include primers and probes that amplify and detect an exogenous internal control (containing plasmid DNA). Amplification and detection of the internal control demonstrates proper sample processing. The internal control is used to demonstrate assay validity.

The possibility of nucleic acid contamination on the Alinity m System is minimized because:

- Aerosol barrier pipette tips are used for all pipetting. The pipette tips are discarded after use.
- PCR amplification and detection is carried out automatically in a sealed reaction vessel.
- Disposal of the reaction vessel is performed automatically by the Alinity m System.

For additional information on system and assay technology, refer to the Alinity m System Operations Manual, Section 3.

REAGENTS

Kit Contents

Alinity m HSV 1 & 2 / VZV AMP Kit

List No. 09N61-095

The Alinity m HSV 1 & 2 / VZV AMP Kit is composed of 2 boxes of

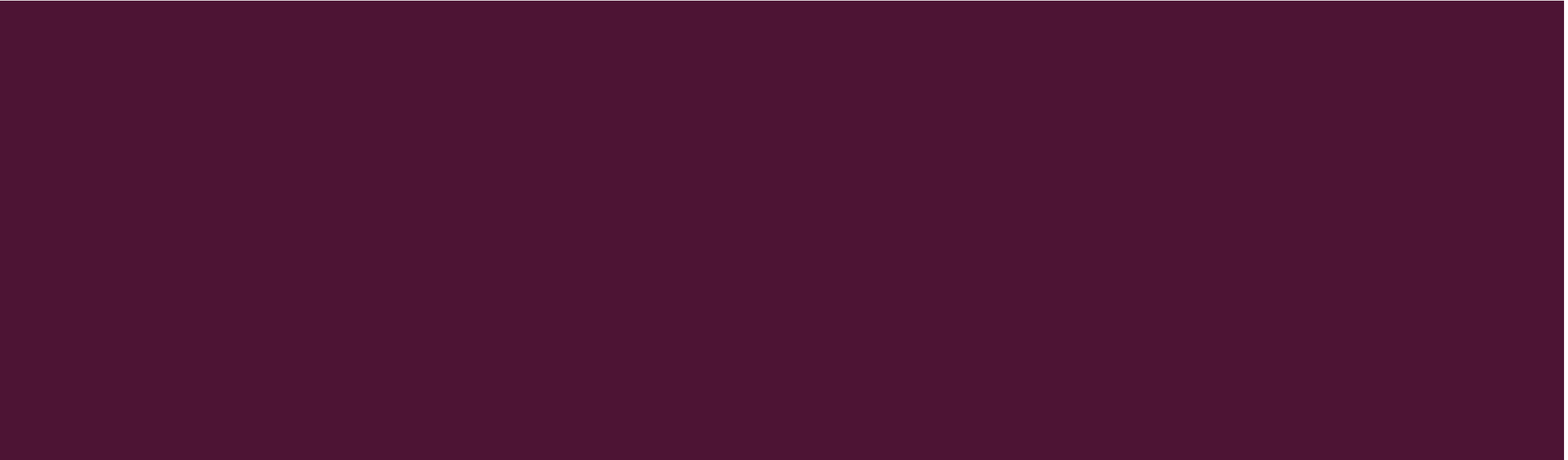
HOW IS ALL OF THIS RELATED?

- Device classifications are defined by the FDA
- The Device classification (I, II, III) will determine the FDA submission process
 - Critical tests may require PMA submission
 - Less critical tests may get away with 510(K)
- Once approved / cleared, it will be determined if the test is Waived, Moderately Complex, or Highly Complex
 - Class III Devices can be Waived (e.g. POC HIV test)
 - Tests can have different classifications based on specimen (e.g. POC HIV test for whole blood (waived) vs. plasma (moderate complexity))
- Non-approved / cleared tests are highly complex by definition
- Test complexity determines lab staffing and test performance

We mostly work with moderately complex, Class II devices cleared by the 510(K) process.



MULTIPLEXING AND SEQUENCING

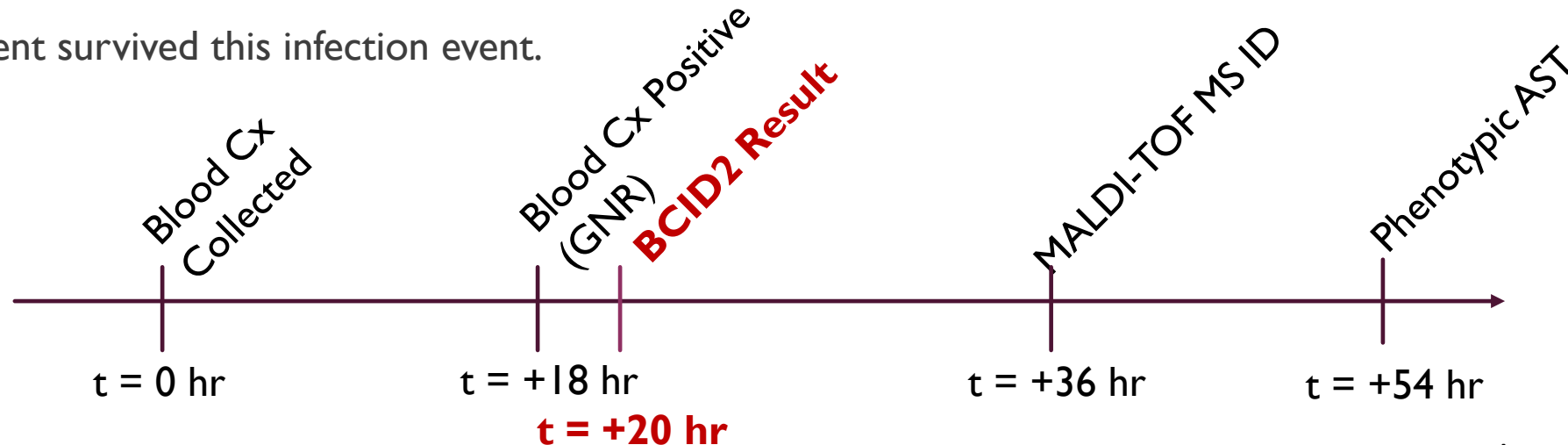


CULTURE IS THE ULTIMATE MULTIPLEX ASSAY

- Culture can recover thousands of different potentially disease causing (or contaminating) organisms
- However, a relatively small subset of organisms is responsible for most of our positive cultures
 - We specifically assess for some pathogens in cultures while not reporting others (e.g. Mixed or Normal Microbiota)
 - Most disease-causing organisms are reasonably well known / well characterized
- Bridging the gap between dedicated / low-plex NAAT and full complexity of culture requires higher order multiplexing
- This gives rise to our “Syndromic Panels” which are both amazing and intermittently frustrating

CASE – THE ANECDATA IS STRONG

- A premature infant (born at 23 weeks) in the NNICU developed signs and symptoms of sepsis and meningitis.
- Blood cultures grew GNR.
- BioFire BCID2 panel was run and identified *E. coli* with CTX-M gene POSITIVE.
- Patient was switched to meropenem from ceftriaxone.
- Phenotypic ceftriaxone was resistant 2 days later.
- Patient survived this infection event.



SINGLEPLEXING V. MULTIPLEXING

- There are many competing considerations for assay optimization → Some compromise may be required
 - Extraction Method
 - Ideal method may vary by DNA v. RNA, Bacteria v. Virus 1 v. Virus 2 v. Fungi
 - Amplification Conditions
 - Annealing temperature, enzyme + dNTP + salt concentrations
- These factors can, in theory, be individually optimized for single-plex assays
- Adding more targets, across more diverse organisms may require compromise
- Test systems may have some underlying decisions baked in

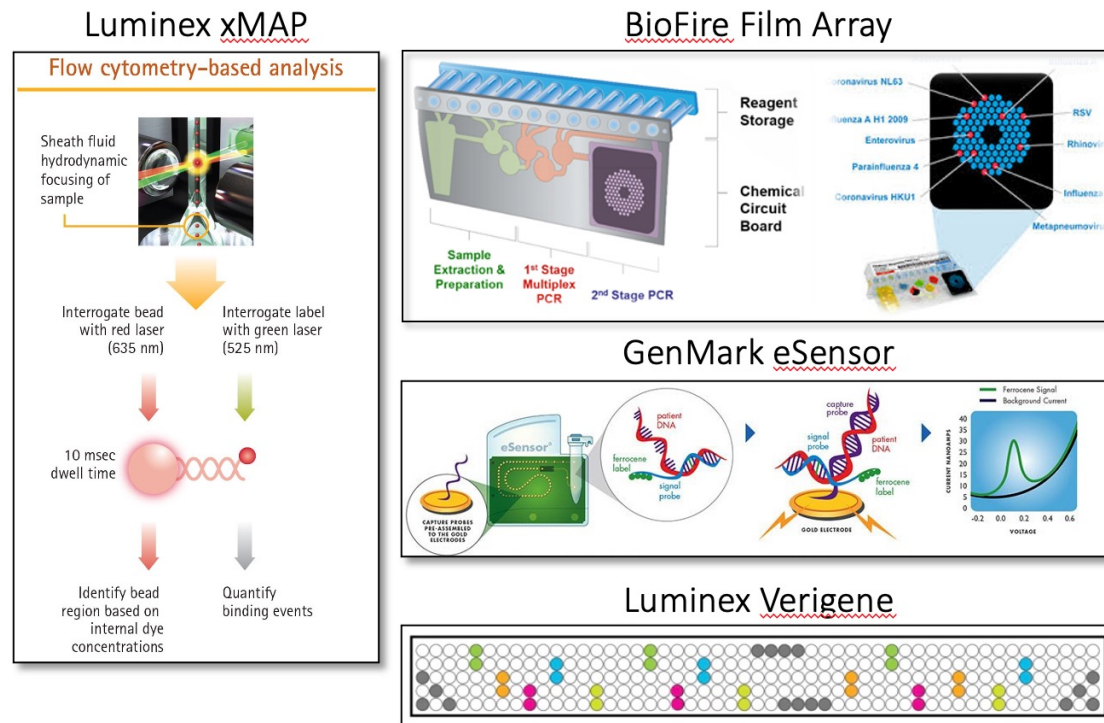
In theory, a well designed singleplex assay should be “better” than a less optimized / more compromise laden multiplex assay

FLUOROPHORE OVERLAP LIMITS MULTIPLEXING

- Most real-time PCR and other NAAT are based on detecting fluorescent signals
- Most current instrumentation is limited to 2 to 5 signals in a given assay / sample
 - This is normally OK:
 - Gonorrhea / Chlamydia; Flu A / Flu B / RSV / COVID-19; HSV-1 / HSV-2 / VZV; M.tuberculosis
- Detection of more than 4 targets requires either: 1) multiple assays or 2) specially designed instruments and assays

Color group	Dye	Wavelength (nm)	
		Excitation	Emission
Blue	FAM	495	520
Green	TET	521	536
	JOE	529	555
	VIC	538	554
	HEX	535	556
Yellow	Cy3	549	566
	NED	546	575
	TAMRA	557	583
	ABY	550	580
Orange	ROX	586	610
	CAL Fluor Red 610	590	610
	JUN	606	617
Red	Cy5	646	669

HIGH ORDER MULTIPLEXING

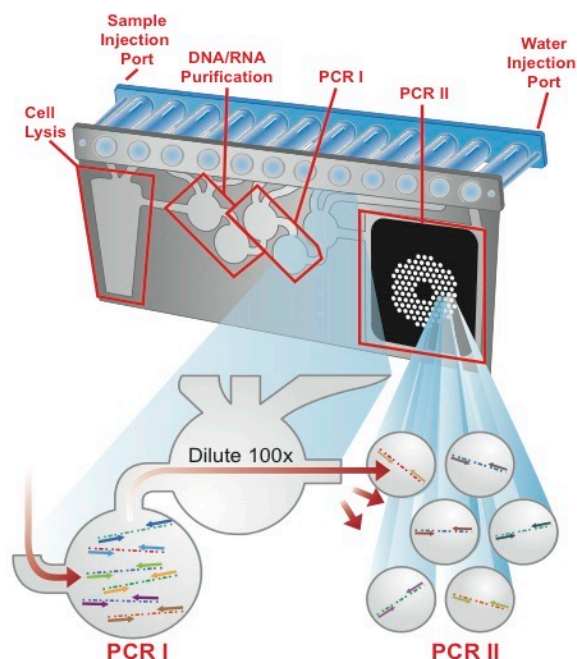


- Stacked 4+plex assays used by some manufacturers (e.g. BD, Hologic)
- Physical location of amplification or detection is used for higher order multiplexing
- Physical location is known from immobilized sequence specific oligos
- Each uses a different approach to detect amplified material

MNAAT PANELS IN CLINICAL PRACTICE

- Upper respiratory virus, blood culture and diarrhea panels widespread
- Pneumonia, CSF, and joint fluid panels available
- In nearly all cases, cost is additive (does not replace conventional testing) and may require instrument acquisition.
- Limited studies showing comprehensive PCR increases rate of bacterial detection, but:
 - The added detections were mostly covered by empiric therapy.
 - The other added detections would need phenotypic AST to guide therapy.
 - Lack of quantitation makes interpretation difficult (is it useful to know they were culture negative?)
- Implementation is essential to realize any advantages to panel esp. integration of antimicrobial stewardship

BIOFIRE PNEUMONIA PANEL – HOW TO ADDRESS QUANT ?



The FilmArray® Pneumonia Panel

Investigational Use Only

Sample Type: Sputum, Endotracheal Aspirate, and Bronchoalveolar Lavage

34 Targets in One Test

The FilmArray Pneumonia Panel will test for a comprehensive set of 27 pathogens and 7 antibiotic resistance markers. The FilmArray Pneumonia Panel will identify the most common bacterial, viral, and fungal pathogens associated with various types of community/hospital-acquired pneumonia. Quickly identifying the probable causative agent helps determine how a healthcare provider chooses to treat a lower respiratory tract infection.

Panel Menu

Bacteria

Semi-Quantitative Bacteria
Acinetobacter calcoaceticus-baumannii complex
Serratia marcescens
Proteus spp.
Klebsiella pneumoniae group
Enterobacter aerogenes
Enterobacter cloacae
Escherichia coli
Haemophilus influenzae
Moraxella catarrhalis
Pseudomonas aeruginosa
Staphylococcus aureus
Streptococcus pneumoniae
Klebsiella oxytoca
Streptococcus pyogenes
Streptococcus agalactiae

Atypical Bacteria

Qualitative Bacteria
Legionella pneumophila
Mycoplasma pneumoniae
Chlamydia pneumoniae

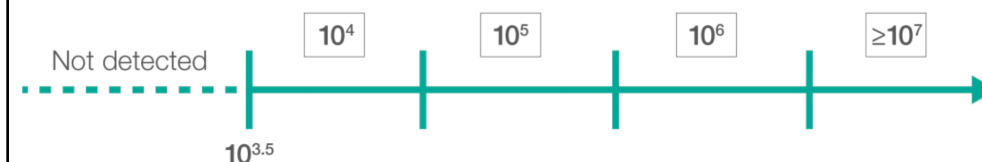
Viruses

Influenza A
 Influenza B
 Respiratory Syncytial Virus
 Human Rhinovirus/Enterovirus
 Human Metapneumovirus
 Parainfluenza virus
 Adenovirus
 Coronavirus
 Middle East Respiratory Syndrome Coronavirus

Antimicrobial Resistance Genes

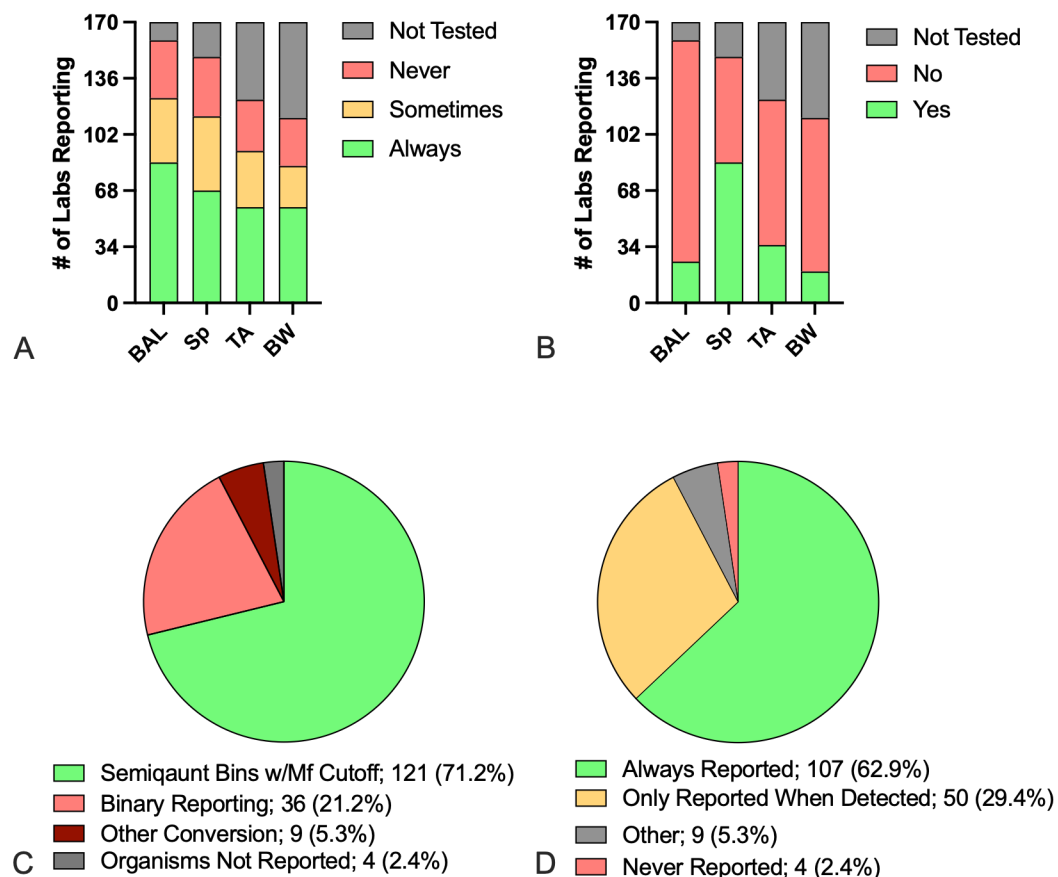
macA/C and MREJ
 KPC
 NDM
 Oxa48-like
 CTX-M
 VIM
 IMP

Values reported by the BioFire Pneumonia Panel



- Semi-quantitative bins for some bacterial respiratory pathogens
- Does not correlate well with I+ → 4+
- Other best-practices and regulatory considerations to implementation

CHALLENGES WITH COMPLEX ASSAYS



Survey from CAP IDPN Survey 2023-C

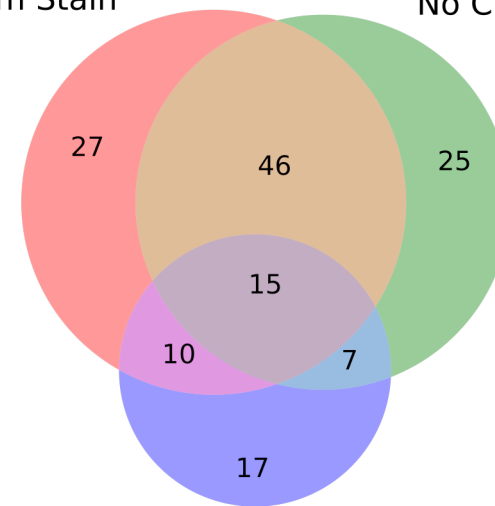
Laboratory responses for A) Culture performance, B) Gram stain performance, C) Test reporting, D) Molecular AST reporting,

Considered Gram Stain + Culture + Mfr IFU as best practice

Deviations from Package Insert and/or Best Practice

No Gram Stain

No Culture



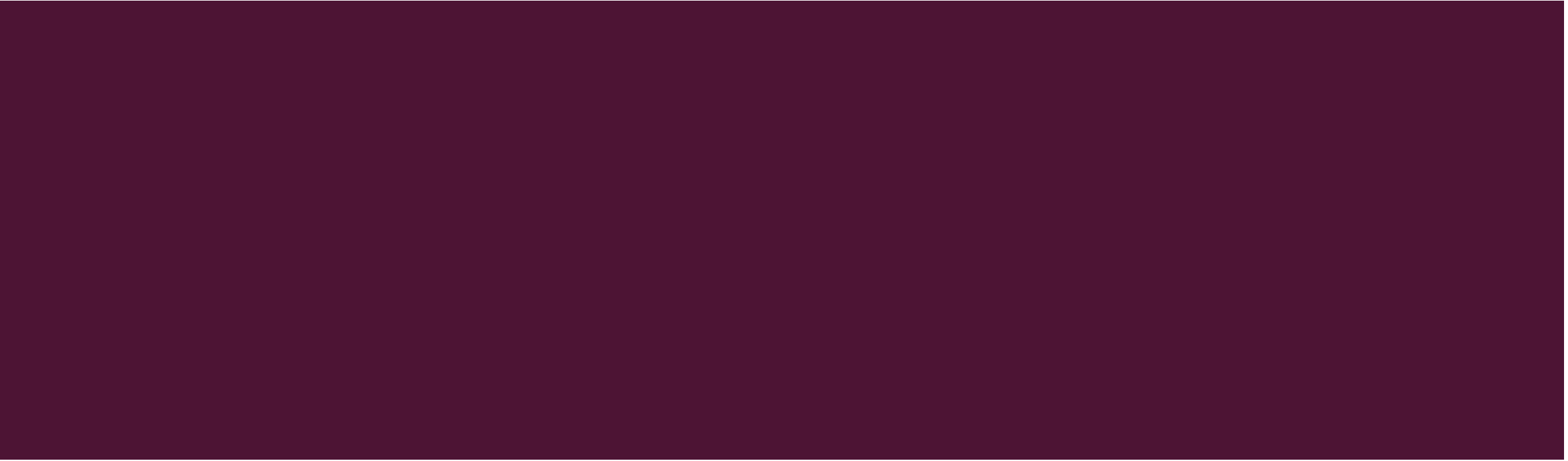
15 / 170 labs deviated from all
23 / 170 labs followed all

Altered Reporting

Yale School of Medicine



SEQUENCING BASED METHODS



CONSIDERATIONS FOR MICROBIAL SEQUENCING

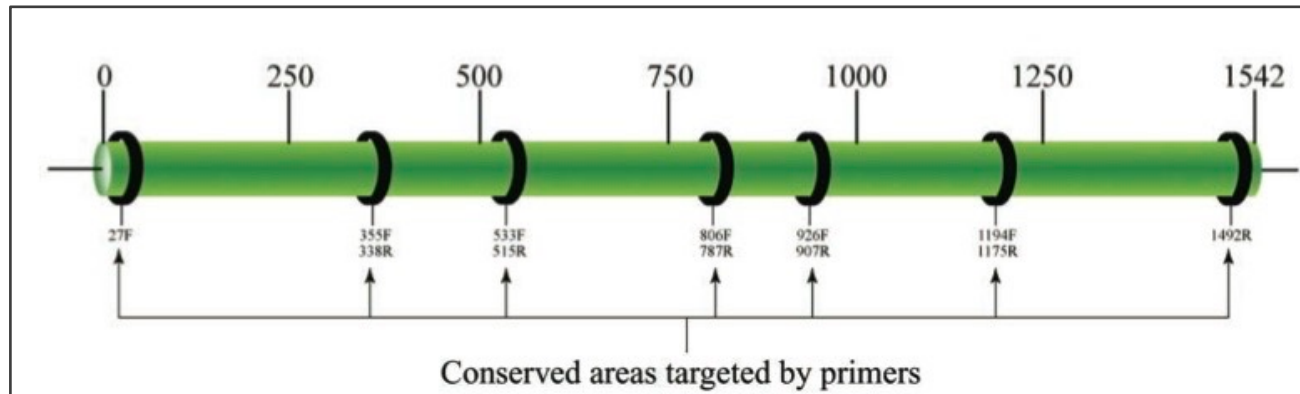
- Why are we sequencing?
 - Clinical suspicion, no cultures, organisms seen on stain
- What are we sequencing?
 - Recovered isolates that have failed ID?
 - Fresh tissue or body fluids?
 - Formalin Fixed Paraffin Embedded (FFPE) tissue?
- What organisms are we worried about?
 - Bacteria (16S), Fungi (ITS or others), AFB (16S, rpoB, hsp65, or others)
- Is there a normal microbiota to consider at the body site?
 - Sanger sequencing does not work well for mixtures of organisms → may require NGS

A CASE

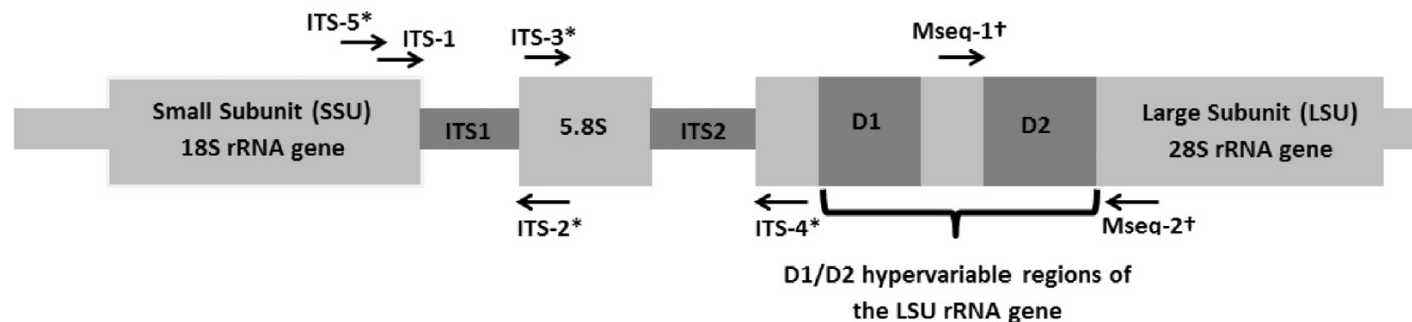
- 45 year old woman with recurrent breast abscesses
- Purulent fluid was sent to the laboratory for Gram stain and culture
- Gram stain revealed 3+ WBC and mixed skin flora
- 18 hr plate read was mixed skin flora
- 36 hr plate read demonstrated 3+ small, creamy colony in excess of normal flora
- MALDI-TOF ID failed on initial culture and sub-culture
- How to ID?

16S rDNA Sequencing identified *Corynebacterium kroppenstedtii*

TARGETS FOR MICROBIAL SEQUENCING: 16S AND FUNGAL RNA COMPLEX



- Other loci can be used esp. for fungi
- 16S Does not typically provide adequate species resolution of Mycobacteria
- May need to supplement with other targets (e.g. RPO-beta, HSP65)

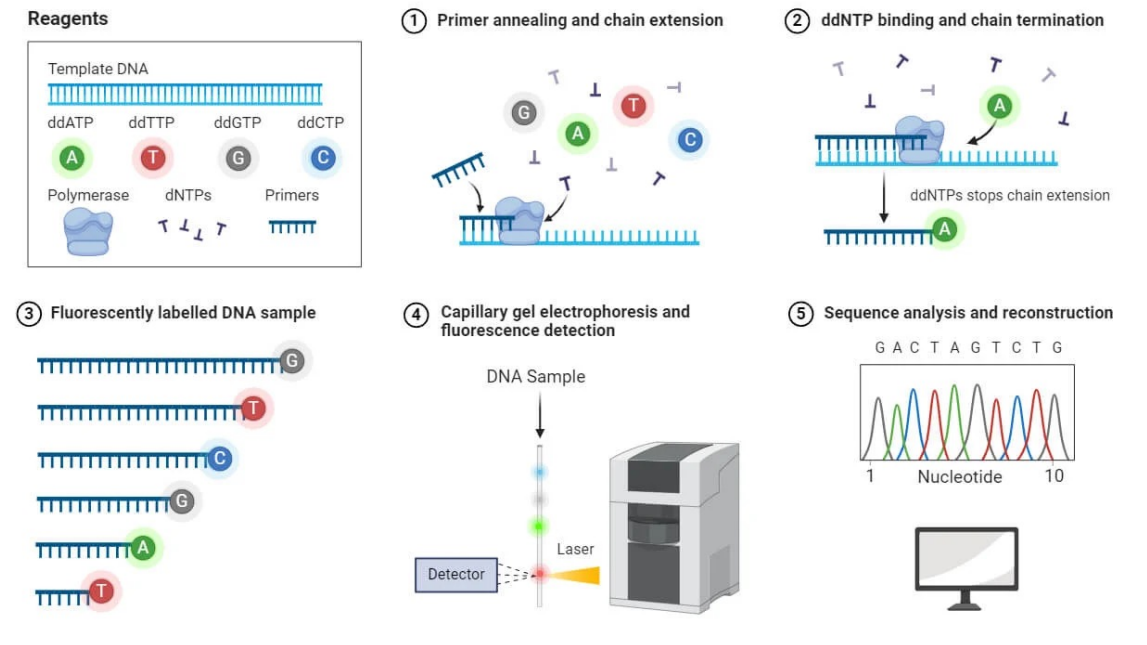


Regions that are highly conserved +
Regions that are variable =

Gene Ideal for molecular amplification and bacterial identification

ISOLATE IDENTIFICATION BY SANGER SEQUENCING

- Cultivated isolates unable to be identified to desired level
- PCR amplification of region of interest (e.g. 16S, ITS, DI/D2, RPO-beta, etc.)
- Cleanup of PCR Reaction
- Addition of new polymerase, dNTP, and fluorescently labeled, ddNTP → elongation terminators
- Fragments are separated by capillary electrophoresis with single nucleotide resolution
- Software reads electropherogram to generate sequence



SANGER SEQUENCING FOR IDENTIFICATION

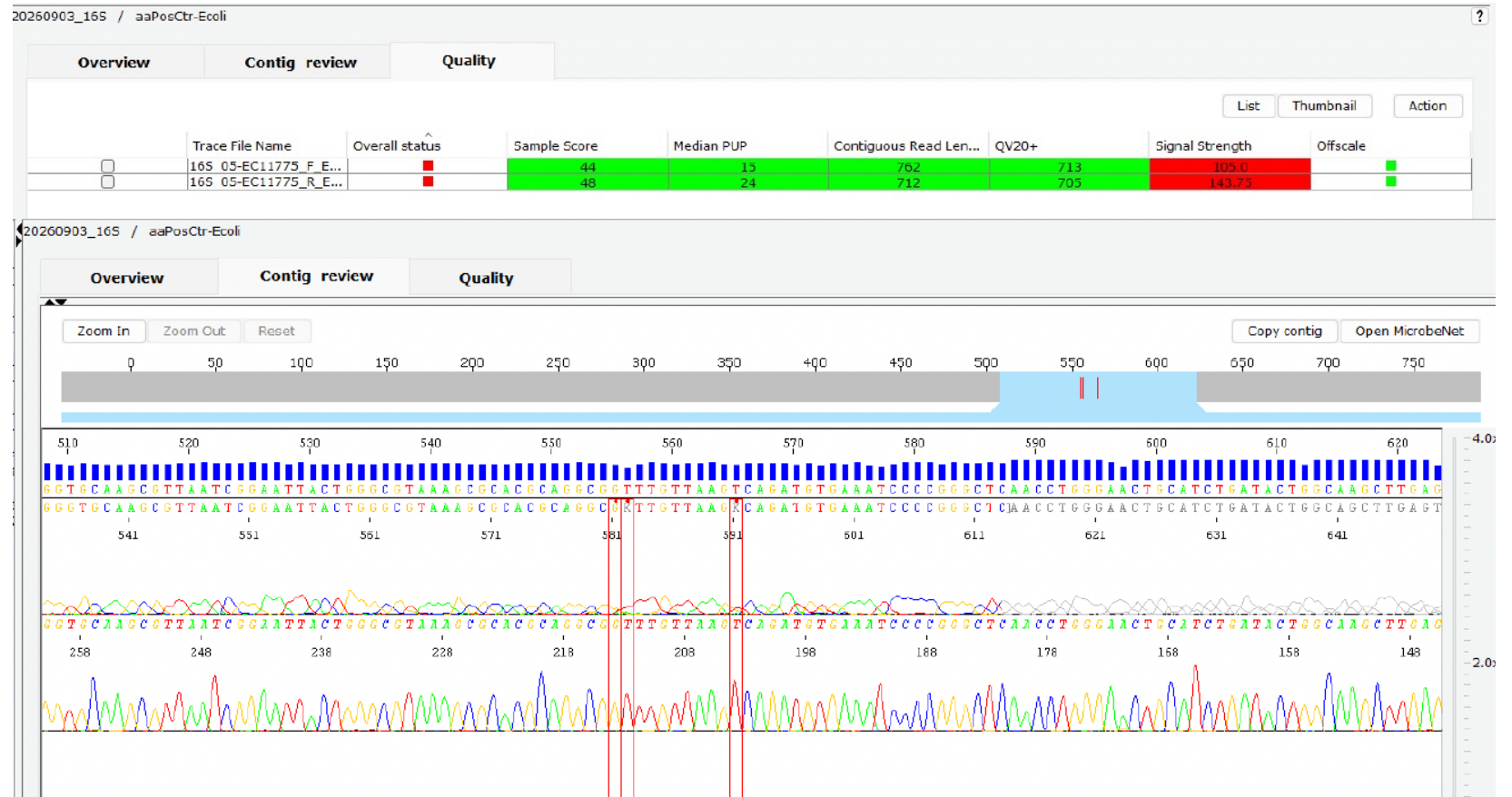
Typically forward and reverse reactions sequenced to provide more high quality coverage

Quality metrics typically look at “quality” of base calls and summarize for the sequence generated

Once sequence generated, need to compare to database for identification

Pros and cons of different databases

Correlation with known microbiology and clinical is important if possible



DATABASE SELECTION

Positive Control NCBI

Job Title: **Nucleotide Sequence**

RID: [9KJTNB1N014](#) Search expires on 09-05 03:32 am [Download All](#) ▼

Program: BLASTN [Citation](#) ▼

Database: rRNA_typerstrains/16S_ribosomal_RNA [See details](#) ▼

Query ID: lcl|Query_2426515

Description: None

Molecule type: dna

Query Length: 752

Other reports: [Distance tree of results](#) [MSA viewer](#) [?](#)

Filter Results

Organism: only top 20 will appear ☐ exclude

Type common name, binomial, taxid or group name

[+ Add organism](#)

Percent Identity: to E value: to Query Coverage: to

[Filter](#) [Reset](#)

Descriptions Graphic Summary Alignments Taxonomy

Sequences producing significant alignments Download ▼ Select columns ▼ Show 100 ▼ [?](#)

☒ select all 100 sequences selected [GenBank](#) [Graphics](#) [Distance tree of results](#) [MSA Viewer](#)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, complete sequence	Escherichia fergusonii AT...	1369	1369	100%	0.0	99.47%	1542	NR_074902.1
Shigella flexneri strain ATCC 29903 16S ribosomal RNA, partial sequence	Shigella flexneri	1369	1369	100%	0.0	99.47%	1488	NR_026331.1
Shigella dysenteriae strain ATCC 13313 16S ribosomal RNA, partial sequence	Shigella dysenteriae	1367	1367	100%	0.0	99.47%	1487	NR_026332.1
Escherichia coli strain NBRC 102203 16S ribosomal RNA, partial sequence	Escherichia coli	1367	1367	100%	0.0	99.34%	1467	NR_114042.1
Shigella sonnei strain CECT 4887 16S ribosomal RNA, partial sequence	Shigella sonnei	1363	1363	100%	0.0	99.34%	1530	NR_104826.1
Escherichia fergusonii ATCC 35469 16S ribosomal RNA, partial sequence	Escherichia fergusonii AT...	1356	1356	100%	0.0	99.20%	1473	NR_027549.1
Escherichia coli strain U 5/41 16S ribosomal RNA, partial sequence	Escherichia coli	1354	1354	100%	0.0	99.07%	1450	NR_024570.1
Escherichia fergusonii strain NBRC 102419 16S ribosomal RNA, partial sequence	Escherichia fergusonii	1358	1358	100%	0.0	98.94%	1467	NR_114079.1
Escherichia marmotae strain HT073016 16S ribosomal RNA, partial sequence	Escherichia marmotae	1341	1341	100%	0.0	98.80%	1504	NR_136472.1
Shigella boydii strain P288 16S ribosomal RNA, partial sequence	Shigella boydii	1341	1341	100%	0.0	98.80%	1515	NR_104901.1
Pseudoescherichia vulneris strain ATCC 33821 16S ribosomal RNA, partial sequence	Pseudoescherichia vulneris	1338	1338	100%	0.0	98.67%	1443	NR_119109.1
Escherichia albertii strain Albert 19982 16S ribosomal RNA, partial sequence	Escherichia albertii	1308	1308	100%	0.0	98.01%	1494	NR_025569.1
Pseudocitrobacter vendiensis strain CPO20170097 16S ribosomal RNA, complete sequence	Pseudocitrobacter vendi...	1291	1291	100%	0.0	97.61%	1542	NR_180316.1

- Public v. commercial
- Curated v. wild-west
- Updated (or maybe not if you don't like nomenclature changes)
- Can be considered a professional interpretation (Part B) service
- Most of the time, is straightforward; sometimes it is not.

CLSI MM18 IS AN EXCELLENT GUIDE



[**Read Sample**](#)

Standard Document

Second Edition

Molecular Diagnostics

CLSI MM18

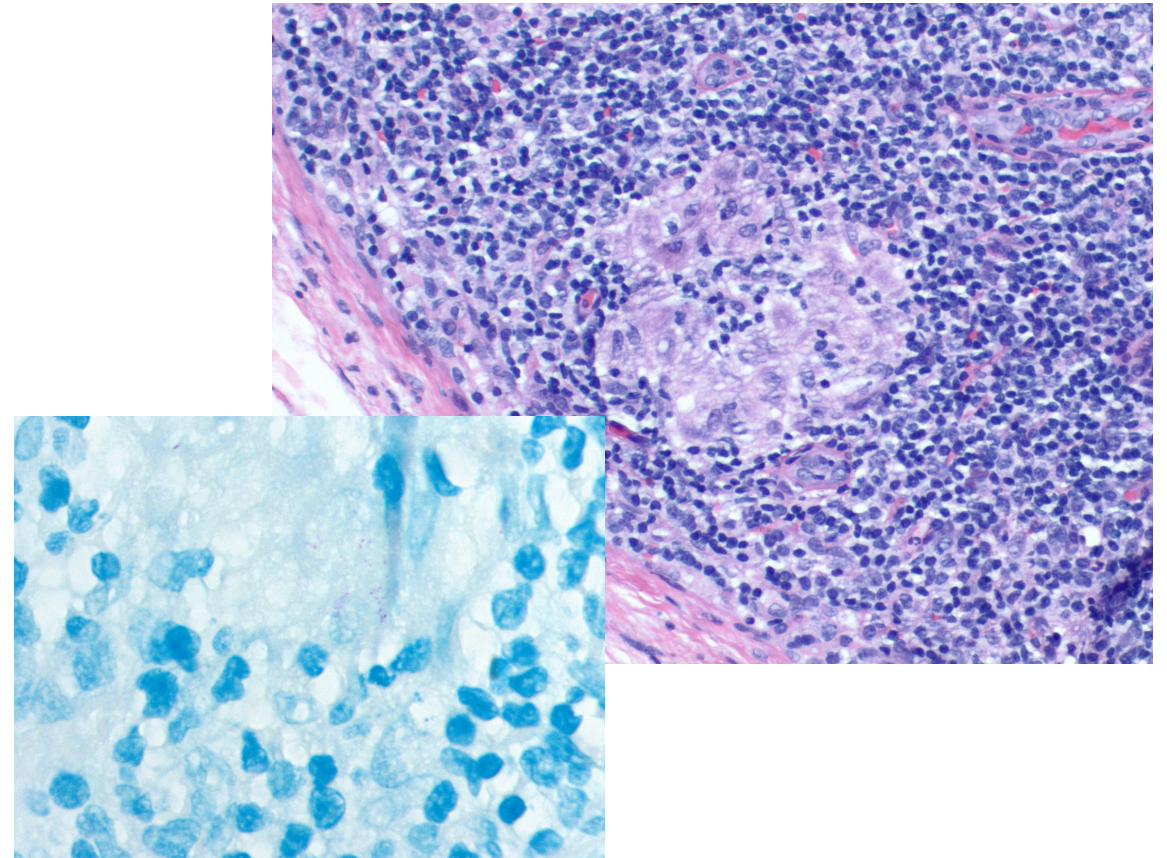
Interpretive Criteria for Identification of Bacteria and Fungi by Targeted DNA Sequencing

This guideline provides the most up-to-date information for microbial classification using targeted DNA sequencing, with a particular focus on interpreting and reporting the results.

This edition of the document was corrected in November 2018 and November 2022. Read the full correction notices [**here**](#) and [**here**](#), and learn more about our corrections process [**here**](#).

A CASE

- A 59-year-old man was admitted with multiple annular erythematous plaques and polyarthralgia involving his bilateral wrists, bilateral ankles, right knee and right shoulder.
- lymphadenopathy was present, and he was diagnosed with B-cell LPD
- Multiple biopsies were taken of cutaneous lesions and lymph nodes with granulomas and AFB seen.
- All were culture negative and M.tb PCR negative
- Tissue sequencing at UW identified *Mycobacterium leprae* DNA from an unpreserved skin biopsy with primers to 16S ribosomal RNA and the rpoB gene.



METHODS AVAILABLE FOR MICROBE IDENTIFICATION IN TISSUE

Method	Advantages	Disadvantages
Traditional Culture	• Sensitive for appropriate organisms • Obtain isolate for ID and AST if needed • Obtain isolate for storage	• Potentially recovers environmental orgs • No histologic correlation alone • Some organisms non-viable or non-cultivable • Some orgs take weeks to grow
Histology	• Can assess tissue reaction / invasion • Readily available with good TAT	• Morphology alone inadequate for identification • Infrequently encountered • Reduced sensitivity
IHC	• Works well for available Abs	• Limited number of Abs for IHC
Molecular	• Allows identification for most organisms	• Send-out with TAT and cost • Method to method variability • DNA degradation in formalin

Ideally multiple, complimentary methods are used

TISSUE SEQUENCING METHODS

	Pros	Cons
Sanger Sequencing	Relatively Low Error Rates (~0.1%)	Expensive
	Long sequence reads (up to 1000 bp)	Not truly high-throughput
	Much better than any alternatives (until recently)	Needs separate DNA isolation and/or amplification
	Excellent for sequencing limited numbers of samples	Labor intensive; finicky
		May need multilocus sequencing for some organisms
		Cannot resolve mixed sequences
Next-Gen Sequencing	Cheap, Easy, Fast (if you have the instrument and expertise)	Smaller sequence reads (100 bp to 500 bp)
	Can be quantitative	Higher error rates (depending on method)
	Designed to use unamplified DNA as input	Computationally complex data analysis
	NGS is not a single method or technique; tremendous complexity in “NGS” label	

HISTOLOGY V. CULTURE FOR FUNGI

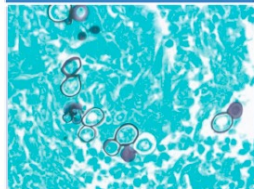
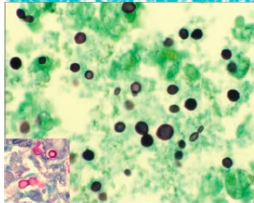
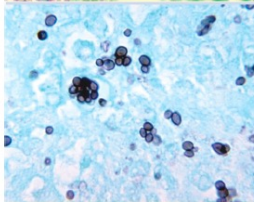
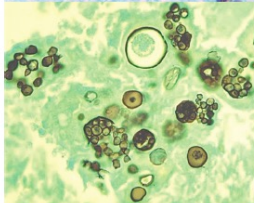
Table 1
Presence of Septate Mold in Tissue or BAL or BW Specimens by Histologic and Cytologic Examination and by Culture

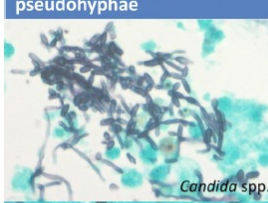
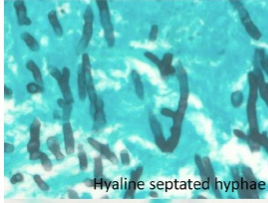
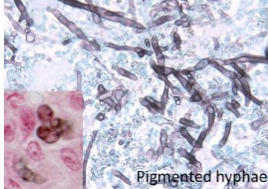
Specimen Type	No. of Specimens	Positive Histocytologic Findings	Positive Culture and Histocytologic Findings	Positive Culture, Negative Histocytologic Findings
Autopsy tissue	23	23	12	0
Surgical or biopsy tissue	1,683	30	9	17
BM, FL, LN, and NL	669	0	0	2
Soft tissue lesion	174	3	0	2
Lung mass lesion	178	5	0	2
Abscess	308	8	1	7
All other, excluding skin	354	14	8	4
BAL or BW	1,185	48	28	68

BAL, bronchoalveolar lavage; BM, bone marrow; BW, bronchial wash; FL, sterile fluid aspirate; LN, lymph node; NL, nonlung fine-needle aspirate.

- Discordance (or absence) of fungal growth and histology is one of the most common tissue questions we get in clin micro
- Tissue based sequencing is one possible resolution

CHALLENGES WITH HISTOLOGIC DX OF FUNGI

Morphology of yeasts	Description, diagnosis and comment
	Description: Broad-based budding yeasts (10-15 microns in size). Diagnosis: Broad-based budding yeasts. Comment: The morphology is consistent with <i>Blastomyces dermatitidis</i>; however, endospores of <i>Coccidioides</i> spp., <i>Candida</i> spp, <i>Histoplasma</i>, <i>Cryptococcus</i>, and <i>Aspergillus</i> conidia can be confused histologically.
	Description: Narrow-based budding yeasts (4- 10 microns in size) with a thick mucicarmine positive capsule and positive with melanin stains (Fontana Masson). Diagnosis: Narrow-based budding yeasts with mucicarmine positive capsule. Comment: The morphology is consistent with <i>Cryptococcus</i> spp.; however, <i>Candida</i> spp. and <i>Histoplasma</i> can be confused histologically.
	Description: Small yeasts (2-4 microns in size) with narrow based budding grouped in clusters inside macrophages. Diagnosis: Small yeasts with narrow based budding. Comment: The morphology is consistent with <i>Histoplasma capsulatum</i>; however, small variant of <i>B. dermatitidis</i>, capsule deficient <i>Cryptococcus</i>, endospores of <i>Coccidioides</i> spp., <i>Pneumocystis jirovecii</i>, <i>Penicillium marneffeii</i>, and <i>Candida glabrata</i> can be confused histologically.
	Description: Spherules with multiple endospores (10 to 100 microns in size). Diagnosis: Spherules with multiple endospores. Comment: The morphology is consistent with <i>Coccidioides</i> spp.; however, <i>Blastomyces</i>, <i>Histoplasma</i>, <i>Candida</i>, <i>Pneumocystis</i> and other yeasts can be confused histologically when no spherules are present. In addition, <i>Rhinosporidium seeberi</i> has sporangia with endospores which are larger.

Morphology of hyphae and pseudohyphae	Description, diagnosis and comment
	Description: Small yeasts (3- 5 microns in size) intermingled with pseudohyphae and hyphae. Diagnosis: Yeasts with pseudohyphae. Comment: The morphology is consistent with <i>Candida</i> spp.; however, <i>Aspergillus</i> spp. and other hyaline fungi can be confused histologically.
	Description: Non-pigmented (hyaline), septated hyphae with acute angle branching. Diagnosis: Non-pigmented (hyaline), septated hyphae. Comment: The morphology is consistent with <i>Aspergillus</i> spp., <i>Fusarium</i> spp., <i>Scedosporium</i> spp., <i>Trichoderma</i> spp., <i>Paecilomyces</i> spp. and others. Mucorales genera can sometimes have this morphology.
	Description: Non-pigmented (hyaline), pauciseptate ribbon-like hyphae with right angle branching. Diagnosis: Non-pigmented (hyaline), pauciseptate hyphae. Comment: The morphology is consistent with <i>Mucorales</i> genera; however, <i>Aspergillus</i> spp. and other septated hyaline hyphae can sometimes have this morphology.
	Description: Pigmented irregular hyphae and yeast-like structures both with septations. Diagnosis: Pigmented yeasts and hyphae with septations. Comment: The morphology is consistent with dematiaceous fungi including: <i>Madurella</i> spp, <i>Fonsecaea</i> spp, <i>Cladophialophora</i> spp, <i>Exophiala</i> spp, <i>Curvularia</i> spp, <i>Bipolaris</i> spp, and others.

CLINICAL MICROBIOLOGY REVIEWS, Apr. 2011, p. 247-280
 0893-8512/11/\$12.00 doi:10.1128/CMR.00053-10
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Vol. 24, No. 2

Histopathologic Diagnosis of Fungal Infections in the 21st Century

Jeannette Guarner^{1*} and Mary E. Brandt²

Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia,¹ and Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia²

CORRELATION OF DIRECT EXAMINATION + SEQUENCING

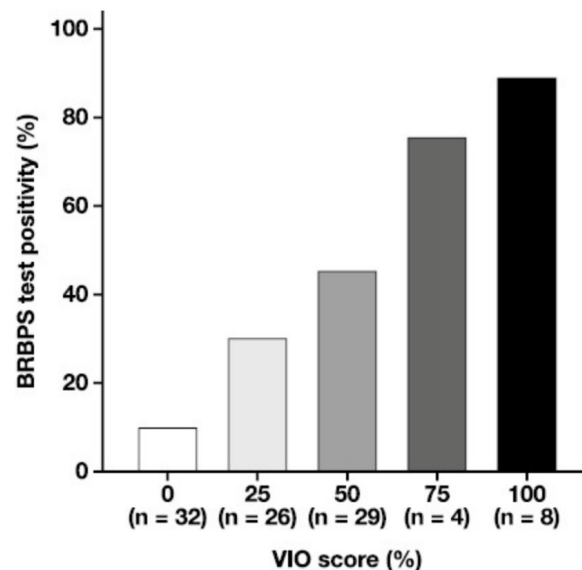


FIGURE 1 Broad-range bacterial polymerase chain reaction with sequencing (BRBPS) positivity according to visualized inflammation or organism (VIO) score categories.

> [Am J Clin Pathol.](#) 2023 Nov 2;160(5):477-482. doi: 10.1093/ajcp/aqad074.

Development of a scoring system to identify high-yield specimens for bacterial broad-range 16S rRNA gene PCR with sequencing at a tertiary care medical center

Rebekah E Dumm¹, Laurel J Glaser², Kyle G Rodino²

Affiliations + expand
PMID: 37458096 DOI: [10.1093/ajcp/aqad074](#)

TABLE 3 Clinical Utilization of BRBPS Results by Specimen VIO Rank^a

[Open in new tab](#)

BRBPS results	VIO score				
	0%	25%	50%	75%	100%
Positive result	10 (3/29)	30 (6/20)	45 (9/20)	75 (3/4)	88 (7/8)
Considered pathogen	67 (2/3)	67 (4/6)	89 (8/9)	100 (3/3)	100 (7/7)
Considered contaminant	33 (1/3)	33 (2/6)	11 (1/9)	0 (0/3)	0 (0/7)
Antimicrobial treatment change	17 (5/29)	20 (4/20)	50 (10/20)	0 (0/4)	50 (4/8)
Positive result	n = 3	n = 6	n = 9	n = 3	n = 7
Start appropriate treatment	0 (0/2)	0 (0/4)	0 (0/8)	0 (0/3)	14 (1/7)
Target treatment	33 (1/3)	33 (2/6)	78 (7/9) ^b	0 (0/3)	43 (3/7)
No change in treatment	67 (2/3)	67 (4/6)	22 (2/9)	100 (3/3)	43 (3/7) ^c
Negative result	n = 26	n = 14	n = 11	n = 1	n = 1
De-escalate treatment	11 (3/26)	0 (0/14)	27 (3/11)	0 (0/1)	0 (0/1)
Discontinue treatment	4 (1/26)	21 (3/14)	0 (0/11)	0 (0/1)	0 (0/1)
No change in treatment	85 (22/26)	79 (11/14)	73 (8/11)	100 (1/1) ^d	100 (1/1) ^d

CONSIDERATIONS FOR TISSUE SEQUENCING

- Highest yield in specimens with visualized organisms
- Fresh tissue is typically superior to FFPE, but organisms present in FFPE / HE stain may not be present in microbiology sample
- Factors I consider when reviewing send-out requests:
 - Organisms seen in tissue → Send
 - Heart valve tissue → Send
 - Specimen quality including WBC
 - Antibiotic Pre-Treatment
 - Clinical Impact of 7 to 14 day TAT
- Tissue sequencing can be an important adjunct method when organisms are visualized in tissue but cultures were not obtained or do not grow.
- There is tremendous variability in tissue sequencing protocols and a limited number of clinical labs offer testing
- As with all things, clinical correlation is required to incorporate all available results for patient care



NEXT GENERATION SEQUENCING INCLUDING METAGENOMICS



SEQUENCING APPLICATIONS

Application	Specimen	Question answered	Typical platform
Amplicon (16S/ITS)	Pure culture isolate	ID when conventional methods fail	Sanger
Amplicon (16S/ITS)	Sterile Fluid / Tissue	"Name this organism when culture fails / isn't performed"	Sanger, ONT, Illumina
Primary Sample Metagenomics (mNGS)	CSF, BAL, sterile fluid	"Unbiased — what pathogen is in this sample?"	Illumina, ONT
Plasma microbial cfDNA	Blood plasma	"Non-invasive broad pathogen screen"	Illumina
Outbreak genomics	Multiple isolates	"Are these cases the same strain?"	Illumina, ONT, PacBio

NEXT GENERATION SEQUENCING (NGS): OVERVIEW & CORE CONCEPTS

- What is NGS?
 - Massively parallel sequencing: millions to billions of DNA fragments sequenced simultaneously
 - Also called 'second-generation sequencing' (in contrast to Sanger = first-gen)
 - Fundamental shift: from single-target to genome-wide analysis
- Key NGS Concepts:
 - Read Depth / Coverage: Number of times each base is sequenced (e.g., 100x = each base read ~100 times)
 - Read Length: Short-read NGS typically 75-300 bp; longer reads with newer technologies
 - Library Preparation: Targeted panels vs. whole genome vs. metagenomics
 - Sequencing-by-Synthesis (SBS): Most common NGS chemistry (Illumina)
 - Clonal Amplification: Fragments amplified before sequencing to increase signal

NEXT GENERATION SEQUENCING

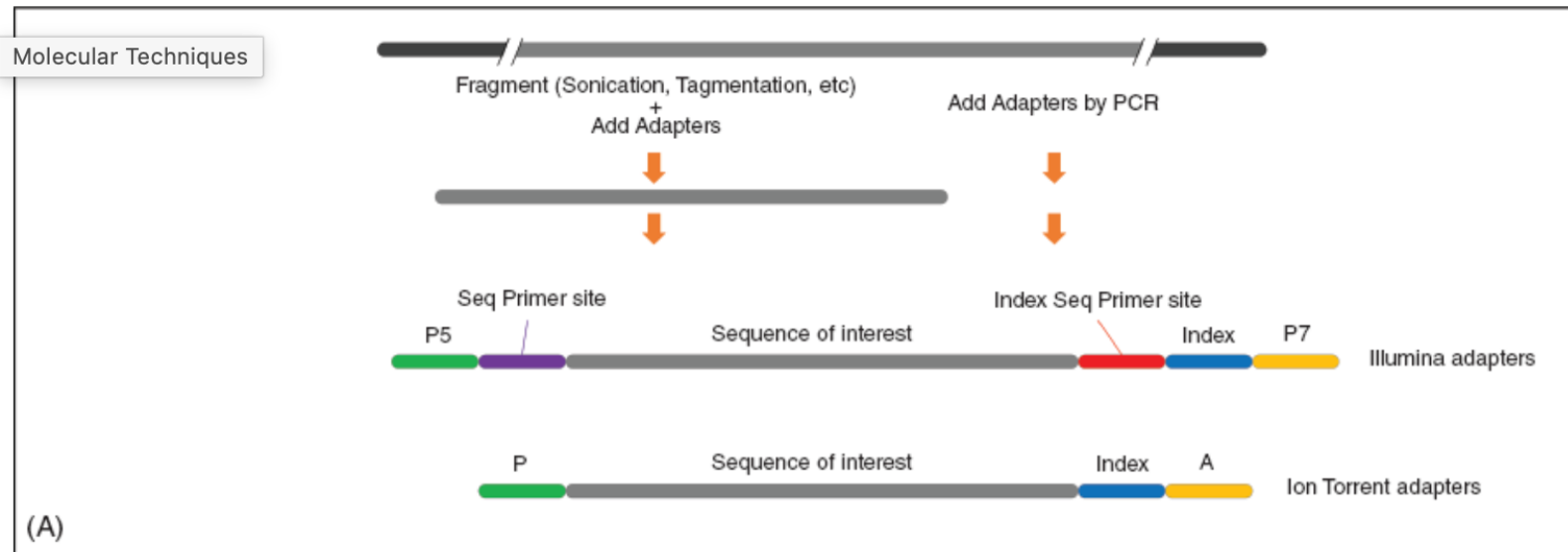
- Multiple levels of complexity contained in NGS
- Variations by:
 - Method (short read, long read)
 - Manufacturer (illumina, ion torrent, PacBio, Oxford Nanopore)
 - Input (PCR amplicons, whole organism, all DNA or DNA/RNA)
 - Analysis pipelines
 - Many other method specific variations
- Most common applications in Clin Micro are:
 - Sequencing of 16S (or comparable) PCR amplicons
 - WGS of recovered organisms for strain typing or identification
 - Metagenomics (mNGS) from cell-free specimens
- Many NGS applications are highly-parallel generating large amounts of data in single run

SEQUENCE METHOD ANALYTIC COMPARISONS

Feature	Sanger	Illumina	Ion Torrent	Oxford Nanopore
Chemistry	Chain termination	Reversible-terminator SBS	Semiconductor H ⁺	Nanopore current
Read length	400–900 bp	2×150–300 bp	200–600 bp	~13–23 kb (>1 Mb)
Raw accuracy	99.999%	≥99.9%	~99%	~99% simplex / 99.9% duplex
Dominant error	Mixed	Substitutions	Homopolymer indels	Indels
Max output/run	~0.3 Mb	up to 6 Tb	up to 50 Gb	up to 15 Tb
Turnaround	2–4 h	13–56 h	2–19 h	Minutes–72 h (real-time)
Amplification	PCR	Bridge PCR	Emulsion PCR	None
Instrument cost	~\$100k	\$100k–\$850k	\$50k–\$150k	\$1k–\$160k

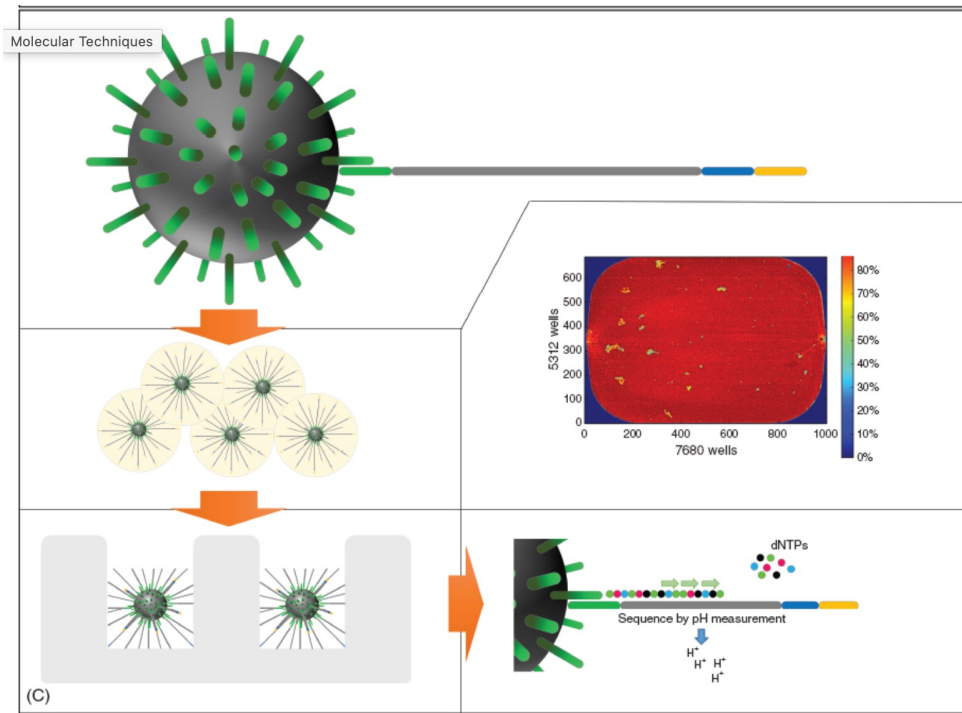
ADAPTERS ENABLE UNBIASED SEQUENCING

- Adapters are added during processing to allow for indexing / bar-coding of samples for later deconvolution
- Can be added to amplified products or fragmented DNA
- Enables generic primers to initiate sequencing reactions
- Enables physical capture of sequence of interest

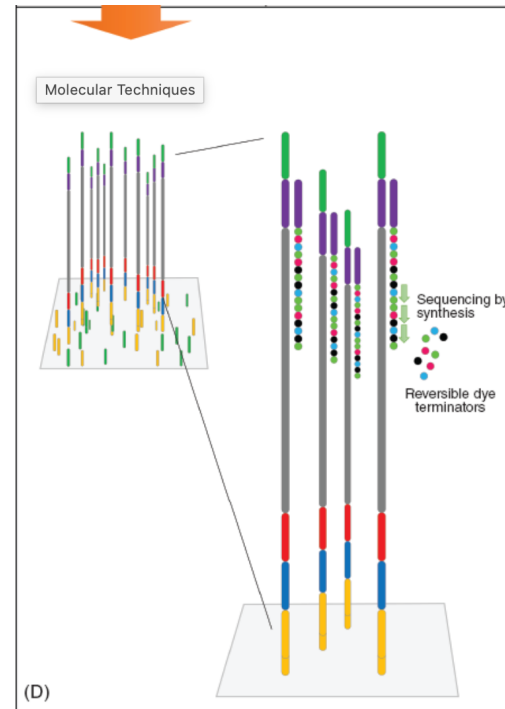


UNDERLYING SCHEMA OF COMMON NGS METHODS

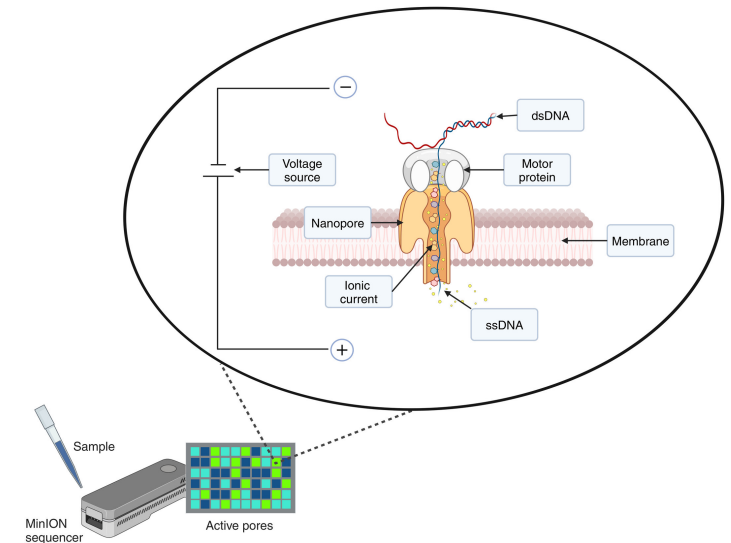
Ion Torrent



Illumina Sequencing by Synthesis



Oxford Nanopore



Intl Journal of Cancer, Volume: 155, Issue: 12,
Pages: 2129-2140, First published: 19 July
2024, DOI: (10.1002/ijc.35100)

NEXT GENERATION SEQUENCING

- Multiple levels of complexity contained in NGS
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- Most common applications in Clin Micro are:
 - Sequencing of 16S (or comparable) PCR amplicons incl. COVID
 - WGS of recovered organisms for strain typing
 - Metagenomics (mNGS) from cell-free specimens



ILLUMINA WGS NGS METHOD OVERVIEW

DNA Extraction:

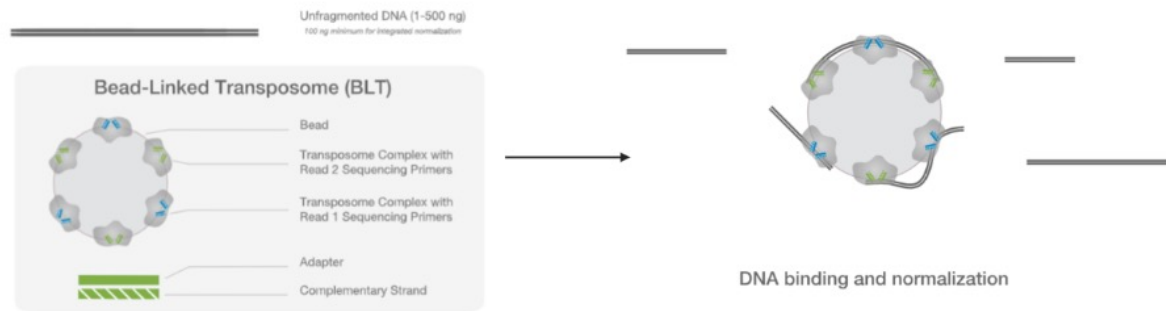
- Genomic DNA is extracted from cultured bacteria
- DNA input requirement for the tagmentation step is 100-500 ng



Could also start with PCR Amplicons (e.g. I6S)

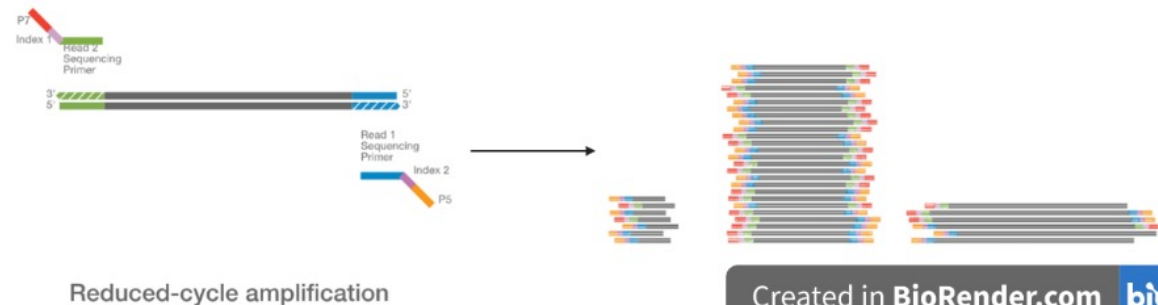
DNA Tagmentation and Normalization:

- Large genomic DNA is fragmented by the transposase
- Illumina Read 1 and Read 2 adapters attached
- Each library is normalized by the limiting number of transposase on the beads



Amplification and indexing of library:

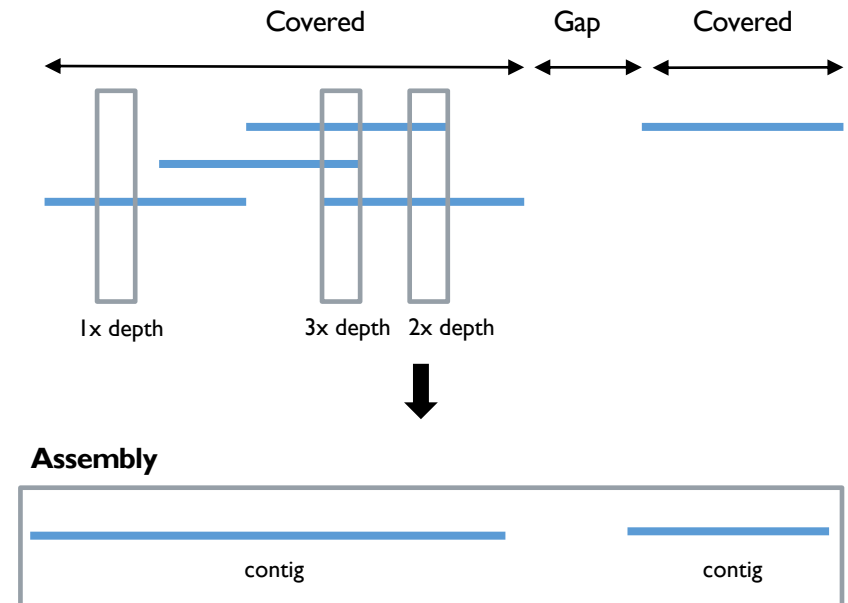
- Each library is amplified and uniquely indexed
- Once indexed, all libraries are pooled in one sequencing run



Created in BioRender.com

NGS ANALYSIS AND ASSEMBLY

- Data analysis involves assembling a “pipeline” of different tools (some home developed)
- Significant computational time is required for most NGS work
- Assembly (SPADES, MEGAHIT, or other assemblers)
 - Amplicons are tiled based on overlapping ends
 - A consensus assembly is formed
 - Assembly contains multiple contigs of various lengths
 - Small pathogens: Aim for 50-100X depth
- Quality Assessment of the Assembly using QUAST
 - Compare the assembly against a reference genome
 - Genome size, % GC, N50, L50, etc.



- **Highly parallel sequencing allows identification of microbes from mixed specimens including microbiome characterization**
- **16S PCR amplicons typical input in these cases**

LOTS OF FUN QC FOR ILLUMINA OUTPUT



Multiqc QC Reports

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2025-03-26, 13:29 UTC based on data in: `/nextflow/work/d1/b050a81921bf12118e234f3461a34f`

	M Clusters	Yield (Mb)	% Clusters	% Yield	Bases (Mb) ≥ Q30 (PF)	% Bases ≥ Q30 (PF)	% Perfect Index
R1_escherichia_coli	3.3	985.4	2.3%	2.3%	857.9	87.1%	97.0%
R1_escherichia_coli	3.5	1 034.3	2.4%	2.4%	902.9	87.3%	95.4%
R1_staphylococcus_aureus	2.8	823.5	1.9%	1.9%	740.2	89.9%	97.5%
R1_escherichia_coli	2.5	750.6	1.7%	1.7%	653.1	87.0%	95.5%
R1_enterococcus_faecalis	3.6	1 080.4	2.5%	2.5%	959.9	88.8%	97.5%
R1_enterococcus_faecium	2.9	857.5	2.0%	2.0%	756.7	88.2%	97.9%
R1_enterococcus_faecium	2.3	699.8	1.6%	1.6%	624.3	89.2%	97.3%
R1_escherichia_coli	3.5	1 039.5	2.4%	2.4%	904.6	87.0%	96.8%
R1_escherichia_coli	3.1	923.0	2.1%	2.1%	800.7	86.7%	97.6%
R1_enterococcus_faecium	3.6	1 085.7	2.5%	2.5%	963.3	88.7%	96.7%
R1_enterococcus_faecium	3.6	1 061.6	2.4%	2.4%	942.3	88.8%	97.8%

Multiqc QC Reports

[General Stats](#)

[FastQC](#)

[Sequence Counts](#)

[Sequence Quality Histograms](#)

[Per Sequence Quality Scores](#)

[Per Base Sequence Content](#)

[Per Sequence GC Content](#)

[Per Base N Content](#)

[Sequence Length Distribution](#)

[Sequence Duplication Levels](#)

[Overrepresented sequences](#)

[Adapter Content](#)

[Status Checks](#)

[ABYSS Genomic Assembly: N50](#)

[ABYSS Genomic Assembly: Total Length](#)

[Prokka](#)

[Cutadapt](#)

[Filtered Reads](#)

[Trimmed Sequence Lengths \(3'\)](#)

[bclconvert](#)

[Sample Statistics](#)

[Lane Statistics](#)

[Clusters by lane](#)

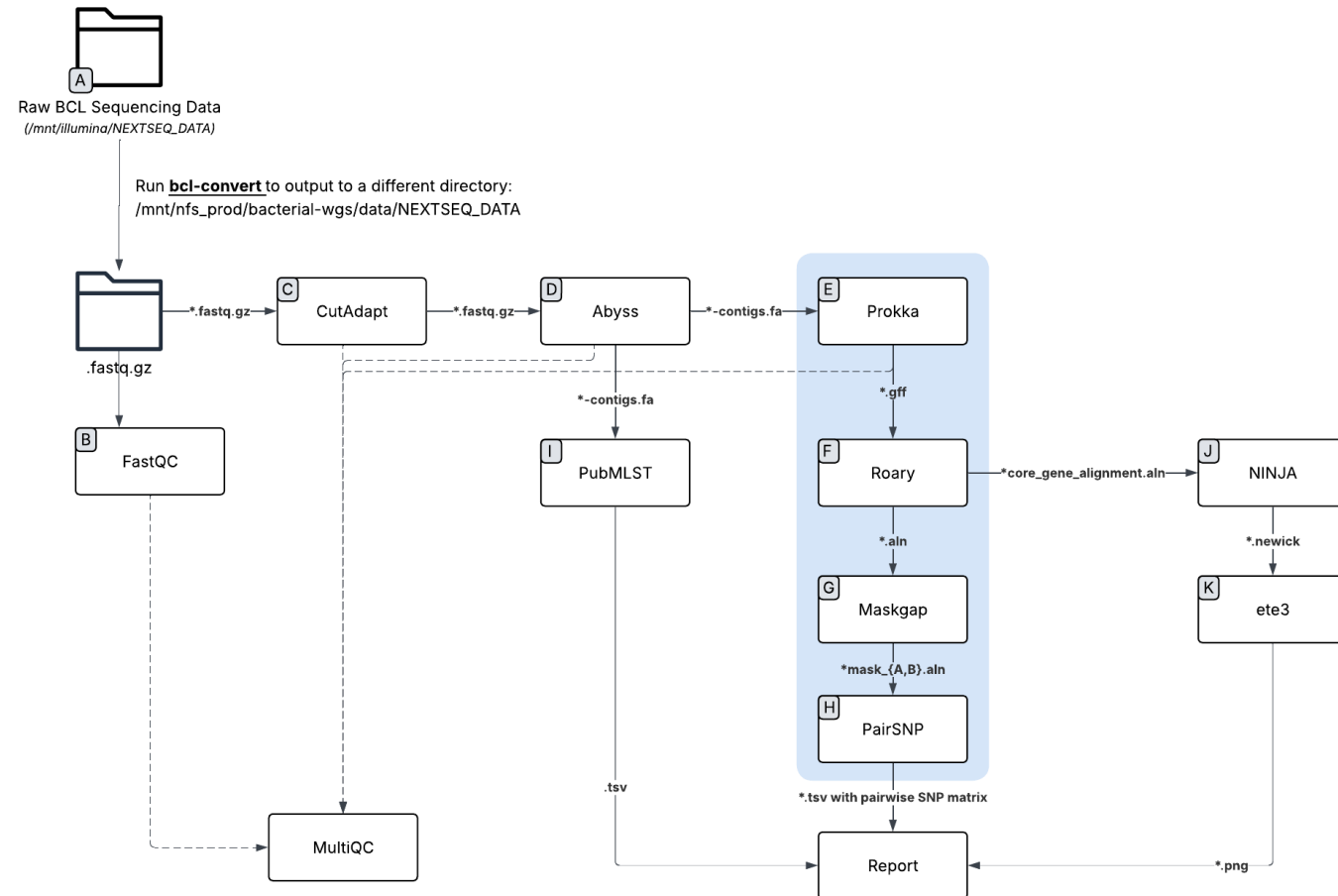
[Clusters by sample](#)

[Undetermined barcodes by lane](#)

NGS CHALLENGES

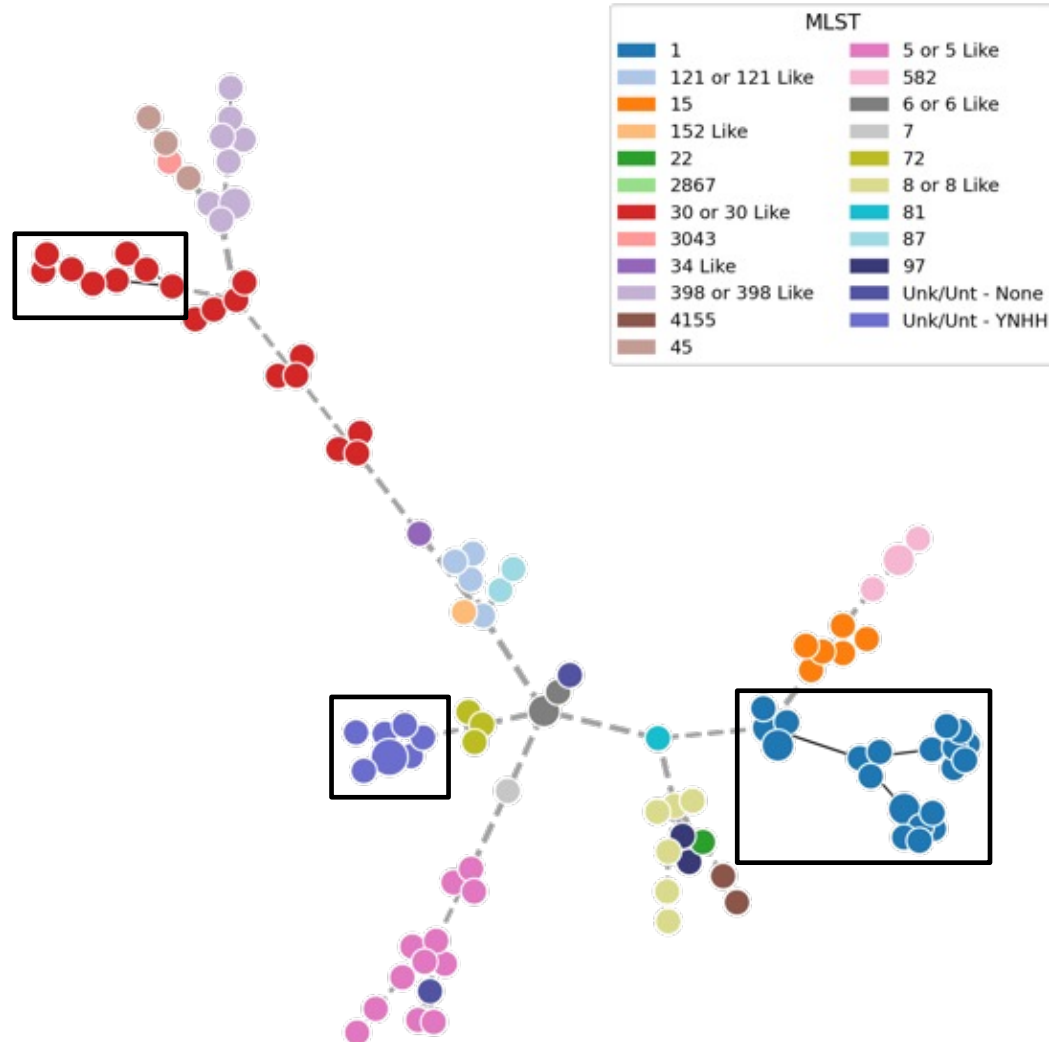
- “Dry Lab” is as essential as “Wet Lab”
- Cost-per-base is going down, but entirely ignores labor and other consumables
- Both dry lab and wet lab are not remotely standardized → lab developed pipelines and lab developed tests
- Some things are becoming more off the shelf, but NGS remains very specialized with dedicated teams
- Generation of sequence is just the start, depending on application need bespoke / custom / unique workflows and pipelines for analysis

SCHEMATIC OF WHOLE GENOME SEQUENCING AND DATA ANALYSIS



cgMLST Minimum Spanning Tree — NICU MSSA Isolates
 Branch lengths logarithmically scaled for distances >5 alleles | Dashed edges = distance >5 alleles | Shared nodes = 0 allele distance

a) Colored by MLST



b) Colored by Cluster

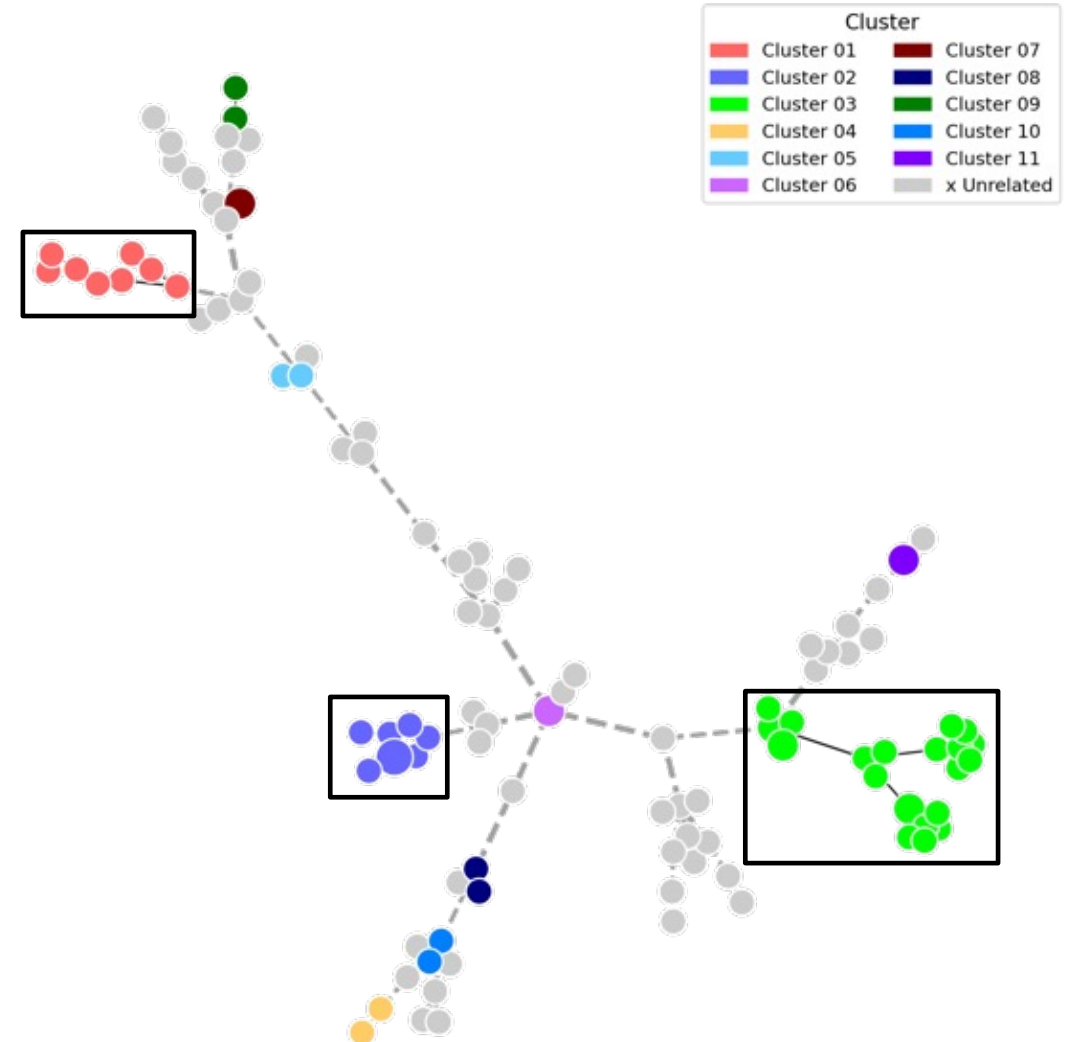


Figure 2. Minimum spanning tree organized by a) MLST or b) Cluster.

OVERALL SUMMARY

- Infectious disease diagnosis requires correlation among all available methods, none of which are perfect
- Culture, histology, NAAT, and sequencing all have a role to play
- Newer sequencing and mNGS methods offer great promise, but comparative clinical utility has not been established in the face of great costs
- Dissemination of standardized methods may improve availability of these methods

SEQUENCING APPLICATIONS

If you need to...	Best method	Why
Identify a culture/MALDI-negative isolate	I6S / ITS (Sanger or ONT full-length)	Species-level ID
Predict resistance from an isolate	WGS (Illumina ± long read)	Mature for TB, Others
Investigate a suspected outbreak	WGS + cgMLST/SNP	Highest resolution, cost-saving
Diagnose culture-negative sterile-site infection	mNGS (CSF/fluid)	Unbiased, antibiotic-independent
Non-invasive broad pathogen screen	Plasma mcfDNA	No procedure needed

WHAT IS METAGENOMICS?

- *Shotgun-sequence all nucleic acid in a specimen (CSF, BAL, tissue, sterile fluid)*
- *Classify reads against microbial databases — **hypothesis-free** detection of bacteria, viruses, fungi, parasites*
- **Overall performance (meta-analysis, 20 studies):** pooled sensitivity 75%, specificity 68%
- **Workflow challenges:**
 - >95–99% of reads are **human** — host depletion and deep sequencing are required
 - Environmental DNA must be controlled for in assay processes

PLASMA CELL FREE METAGENOMICS

- **Concept:** extract and sequence short microbial DNA fragments circulating in plasma; non-invasive; quantified in molecules/μL (MPM). Commercially the **Karius Test** (~1,250-taxon database, TAT ~26 h).
- **Validation:** 93.7% agreement with blood culture in 350 sepsis-alert patients; more etiologic determinations than all microbiological testing combined
- **Real-world impact is variable:**
 - Positive clinical impact 7–53% across studies
 - Highest in **culture-negative endocarditis**
 - Suspected **fastidious/zoonotic/vector-borne** pathogens.
- **Invasive fungal disease:**
 - Overall sensitivity ~48%, specificity 100%;
 - Better for **mucormycosis (100%)** than aspergillosis (~46%)[118].
- **Commensal DNA can appear in bloodstream of up to 20% of patients → False Positive**

METAGENOMICS IN CLINICAL MICRO

nature
microbiology

ARTICLES

<https://doi.org/10.1038/s41564-018-0349-6>

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease

Timothy A. Blauwkamp^{1,3*}, Simone Thair^{2,3}, Michael J. Rosen¹, Lily Blair¹, Martin S. Lindner¹, Igor D. Vilfan¹, Trupti Kawli¹, Fred C. Christians¹, Shivkumar Venkatasubrahmanyam¹, Gregory D. Wall¹, Anita Cheung¹, Zoë N. Rogers¹, Galit Meshulam-Simon¹, Liza Huijse¹, Sanjeev Balakrishnan¹, James V. Quinn², Desiree Hollemon¹, David K. Hong¹, Marla Lay Vaughn¹, Mickey Kertesz¹, Sivan Bercovici¹, Judith C. Wilber^{1,3} and Samuel Yang^{2,3}

Clinical Metagenomic Sequencing for Diagnosis of Meningitis and Encephalitis

M.R. Wilson, H.A. Sample, K.C. Zorn, S. Arevalo, G. Yu, J. Neuhaus, S. Federman, D. Stryke, B. Briggs, C. Langelier, A. Berger, V. Douglas, S.A. Josephson, F.C. Chow, B.D. Fulton, J.L. DeRisi, J.M. Gelfand, S.N. Naccache, J. Bender, J. Dien Bard, J. Murkey, M. Carlson, P.M. Vespa, T. Vijayan, P.R. Allyn, S. Campeau, R.M. Humphries, J.D. Klausner, C.D. Ganzon, F. Memar, N.A. Ocampo, L.L. Zimmermann, S.H. Cohen, C.R. Polage, R.L. DeBiasi, B. Haller, R. Dallas, G. Maron, R. Hayden, K. Messacar, S.R. Dominguez, S. Miller, and C.Y. Chiu

- Karius© = cell free plasma DNA metagenomics
- Unbiased sequence of DNA fragments in plasma
- “Liquid biopsy” for infectious diseases

- UCSF CSF metagenomics
- Unbiased sequences of RNA and DNA fragments in CSF
- Now for-profit Delve Biosciences

CSF MNGS 7 YEAR UPDATE

nature medicine



Article

<https://doi.org/10.1038/s41591-024-03275-1>

Seven-year performance of a clinical metagenomic next-generation sequencing test for diagnosis of central nervous system infections

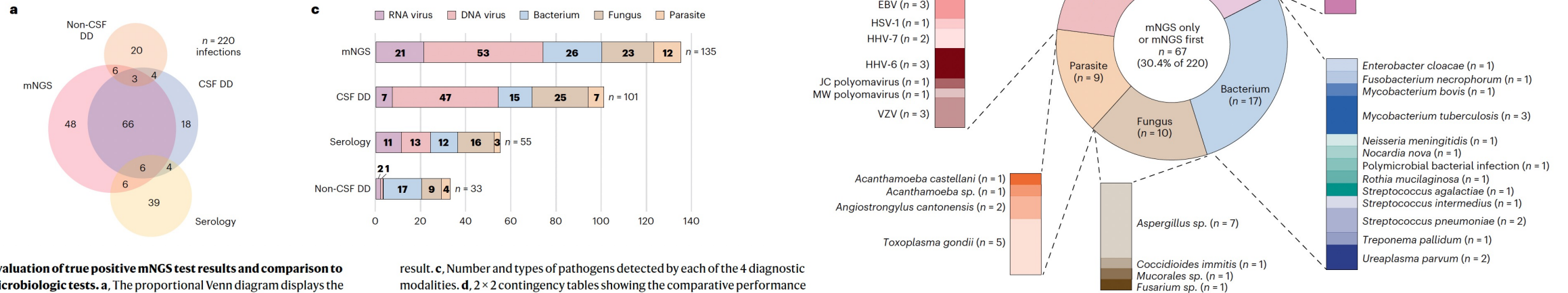


Fig. 3 | Evaluation of true positive mNGS test results and comparison to other microbiologic tests. a, The proportional Venn diagram displays the overlap among four modalities (mNGS, CSF direct detection, non-CSF direct detection and serologic testing) in diagnosis of CNS infections. **b**, Pathogens detected by mNGS testing only ($n = 48$) or mNGS testing first ($n = 19$). Among the 19 pathogens that were detected by mNGS testing first, 11 pathogens were detected by another microbiologic test run in parallel, whereas 8 were detected by another test, but only for orthogonal confirmation of the initial mNGS positive

result. **c**, Number and types of pathogens detected by each of the 4 diagnostic modalities. **d**, 2×2 contingency tables showing the comparative performance of mNGS testing compared to other diagnostic modalities. The P value is based on comparison between mNGS and a conventional testing modality using the two-sided McNemar's test. CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; LCMV, lymphocytic choriomeningitis virus; Neg, negative; NPV, negative predictive value; Pos, positive; PPV, positive predictive value; VZV, varicella-zoster virus.

METAGENOMICS IN CLINICAL MICRO

Advantages	Disadvantages
Detection of biopsy refractory lesions	Cost -- \$2500 per specimen (Hospital eats on inpatients)
Not dependent on “expert clinician” → comprehensive	Negative does not “rule out” infection → over interpretation risk
TAT	May miss infx requiring serologic Dx
Strong anecdotal	Not suitable for samples with lots of human DNA
	DNA Only (pcfDNA)
	No comprehensive AST
	No localizing information (pcfDNA)

Peers are all struggling to figure this out

Culture negative endocarditis and biopsy refractory tissue lesions are most common uses for plasma cfDNA

Kingdom-crossing meningitis / encephalitis differential in immunocompromised for CSF

CHALLENGES WITH METAGENOMICS

- **Contamination / false positives:** 68.6% of false-positive microbes in one QA study came from wet-lab processes; reagent and environmental "kitome" contamination is pervasive in low-biomass samples.
- **Serology Gap:** Serology still preferred for some pathogens
- **Host DNA background** limits sensitivity, especially in high-cellularity CSF and respiratory specimens.
- **No reliable AST from metagenomics:** very major error rate up to 24% for AMR prediction from metagenomic data.
- **Cost:** ~\$500–2,000 per mNGS test.
- **Standardization gap:** Wide variation in application and degree of stewardship and consultation

UNIVERSITY OF NEBRASKA NGS GUIDELINES ARE GREAT

- <https://blog.unmc.edu/infectious-disease/2024/09/19/clinical-guidance-for-next-generation-sequencing-of-infectious-diseases-qa-from-the-antimicrobial-stewardship-team>
- **Other Guidelines to Consider:**
 - 2025 AHA BCNE statement (consider plasma/whole-blood metagenomics after 72 h negative cultures)
 - 2023 Duke-ISCVID criteria (sequencing detection of *C. burnetii*, *Bartonella*, *T. whipplei* = major criterion)
 - ASTCT 2025 (mcfDNA for suspected non-Aspergillus molds)



Clinical Guidance on the Use of Next Generation Sequencing (NGS) Tests for Infectious Diseases

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PRACTICAL STUFF



CONSIDERATIONS FOR NEW ASSAYS

- Are these new analytes / organisms / assays for your lab?
 - Do you have baseline positivity? Ordering patterns? Banked validation material?
- Is this a new test system for you?
 - Is the instrument interfaced? Are there known issues with the instrument? Do you have enough staff trained on the instrument?
- Is there a change in sample collection kit / device / media?
 - Can you support two systems? Will you reject? Will you modify IFU? How will you swap out old kits?
- Do you hope to get reimbursed for this testing?
 - Are billing codes set up? Does your business plan reflect reality? Do you need / have a business plan?
- Are there important stewardship considerations for pre-analytic factors?
 - Will ordering take off now in house? Is there a possibility for miss / over ordering? Under ordering?
- Are there important reporting considerations for post-analytic factors?
 - Do you need to correlate with ASP? Any other specialists?

RESOURCES FOR VERIFICATION / VALIDATION GUIDANCE

Resources / Guidance

- CAP / Accreditation Checklist
- Institution specific guidelines
- New York State CLEP Checklist – Even if not NYS lab, good guidance
- Package inserts of related, FDA-approved assays
- ASM Press – Clin Micro Procedures Handbook; Manual of Molecular Microbiology

Specimen Sources

1. Characterized internal clinical samples
2. Phone a friend
3. State lab
4. Purchase from reference labs
5. Commercially prepared samples
6. Contrived samples from cultured organisms or procured QC material

VERIFICATION = COMPARABLE TO (MFR) ESTABLISHED PERFORMANCE SPECS

CLIA

§ 493.1253 Standard: Establishment and verification of performance specifications.

- (a) **Applicability.** Laboratories are not required to verify or establish performance specifications for any test system used by the laboratory before April 24, 2003.
- (b)
 - (1) **Verification of performance specifications.** Each laboratory that introduces an unmodified, FDA-cleared or approved test system must do the following before reporting patient test results:
 - (i) Demonstrate that it can obtain performance specifications comparable to those established by the manufacturer for the following performance characteristics:
 - (A) Accuracy.
 - (B) Precision.
 - (C) Reportable range of test results for the test system.
 - (ii) Verify that the manufacturer's reference intervals (normal values) are appropriate for the laboratory's patient population.

CAP

****REVISED** 12/26/2024**

COM.40300 Verification of Test Performance Specifications - FDA-cleared/approved Tests

Phase II



Prior to clinical use of each unmodified FDA-cleared or approved test, the laboratory has performed a verification study and prepared a written assessment of each of the following test method performance specifications, as applicable, using a sufficient number of characterized samples:

- 1. Analytical accuracy**
- 2. Analytical precision**
- 3. Reportable range**

If I follow the instructions, then I get the expected results.

VALIDATION = ESTABLISHING PERFORMANCE SPECS

CLIA

(2) **Establishment of performance specifications.** Each laboratory that modifies an FDA-cleared or approved test system, or introduces a test system not subject to FDA clearance or approval (including methods developed in-house and standardized methods such as text book procedures), or uses a test system in which performance specifications are not provided by the manufacturer must, before reporting patient test results, establish for each test system the performance specifications for the following performance characteristics, as applicable:

- (i) Accuracy.
- (ii) Precision.
- (iii) Analytical sensitivity.
- (iv) Analytical specificity to include interfering substances.
- (v) Reportable range of test results for the test system.
- (vi) Reference intervals (normal values).
- (vii) Any other performance characteristic required for test performance.

CAP

****REVISED** 12/26/2024**

COM.40350 Validation of Test Performance Specifications - Modified FDA-cleared/ approved Tests and LDTs

Phase II



Prior to clinical use of each modified FDA-cleared or approved test and laboratory-developed tests (LDTs), the laboratory has performed a validation study and prepared a written assessment of each of the following test method performance specifications, as applicable, using a sufficient number of characterized samples:

- Analytical accuracy
- Analytical precision
- Reportable range
- Analytical sensitivity (lower detection limit)
- Analytical specificity
- Any other performance characteristic required to ensure analytical test performance

HOW MANY AND WHICH ONES?

MIC.64960 Validation or Verification Studies - Specimen Selection

Phase II



Validation or verification studies were performed with an adequate number and representative (reasonable) distribution of samples for each type of specimen (eg, blood, fresh/frozen tissue, paraffin-embedded tissue).

NOTE: The number of specimens to be included in a verification study for each specimen type is to be determined by the laboratory director. Verification studies must include the following:

- Evaluation of adequate numbers of positive and negative specimens across the specimen types to be used in the assay (eg, urine, genital swabs, rectal swabs, pharyngeal swabs), even if they are obtained with the same collection device*
- Evaluation of local specimens representing the strains or genotypes, as appropriate, if geographic variations are known or expected in the strains or genotype of organisms tested*

HOW MANY AND WHAT KIND - NYS

Accuracy

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- rep

Guidance: Specimen characteristics for accuracy verification:

- Accuracy should be verified by conducting a randomized, blinded validation study where the assay results are compared to those of a gold standard or FDA or CLEP approved assay, or results of spiked clinical samples that are compared to predicted results based on the spiking values.
- If multiple sub-types or genotypes are available for the organism being assayed, please include an example of each type, or a representative range of subtypes. As many different specimens and different strains, types, or clinical isolates as possible should be used in spiking studies (especially for specimen matrices that are routinely tested and pathogens that are routinely isolated in the laboratory). If your laboratory has difficulty in obtaining sufficient samples of a particular specimen type, or any other problems in fulfilling the validation requirements, please contact CLEP for guidance.
- The samples should be authentic clinical specimens, but spiked samples are acceptable when clinical specimens are not available. If spiked samples are used, at least 10 samples should be close to the LOD (maximum, 10-fold above that level). If authentic clinical samples are used, at least 10 of the 30 positives should be weak positives, if the methods used allow for this determination. Include multiple different strains and specimens.
- For quantitative assays, please provide data across the full range of concentrations likely to be encountered in clinical samples.
- Any discrepant results need to be explained.
- Multiplex assays (especially those with multiple sample types) can present a difficult situation for the design of a validation study, since it can be a large and expensive endeavor to assay 40 samples (30 positive and 10 negative) for each analyte in each specimen type. Samples can be spiked with multiple organisms, so as to reduce the number of samples required. CLEP may be contacted for guidance prior to the submission of a package with multiplex analysis having four or more simultaneous targets.

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KEY PRINCIPLES FOR EVALUATING VALIDATIONS / VERIFICATIONS

- Patient specimens are preferred to contrived specimens. Contrived specimens should match actual specimens as closely as possible.
- Multiple concentrations across the range of detection should be used, but be mindful of including too many specimens at or below the LOD that may not repeat (esp. after freeze-thaw).
 - (I like to run challenge specimens near LOD if we have an existing assay for baseline that I don't count in accuracy to better understand comparison)
- Intra-run precision is hard to assess on random-access platforms, but efforts should be made to verify that the same specimen yields the same result across time.
- If appropriate, some samples can pull “double duty” for verifying accuracy, precision, and/or reportable range if verification study designed appropriately.
- Samples positive for one pathogen are typically negative for other pathogens → They can count

WHAT ARE MODIFICATIONS?

▼ Modifications to Approved Assays



Modifications are changes to the intended use and/or the testing procedure for FDA-approved, NYS-approved assays, and legacy assays. Modifications to an assay that utilizes a standard method that incorporates a newer technology/method are considered to be a new LDT and not a modification. A full method validation submission would be required.

Modifications that change the intended use include a change to the specimen type; the type of analysis (e.g., qualitative vs. quantitative); the purpose of the assay (e.g., screening, diagnosis, prognosis, monitoring, and /or confirmation); or the target population(s) as specified in the NYS approval letter or FDA-approved package insert.

In clinical and forensic toxicology, adding further drug targets to a previously approved assay using the same matrix (e.g., urine or oral fluid), extraction and injection parameters (e.g. “dilute and shoot” in negative mode; “dilute and shoot” in positive mode, or solid-phase extraction) is considered a modification. The introduction of a new specimen matrix requires a comprehensive LDT method validation submission.

CHALLENGES WITH ASSAY MODIFICATIONS

****REVISED** 12/09/2025**

COM.40250 Manufacturer's Instructions

Phase II



The laboratory follows manufacturer's instructions for all test systems or provides validation records if the test has been modified.

NOTE: Following manufacturer's instructions (or CAP checklist requirements if they are more stringent) includes performing quality control, calibration, calibration verification, and related functions as applicable to the scope of testing. Reagents, fluids, and disposable materials supplied by the laboratory must meet the specifications in the instructions.

If the laboratory modifies manufacturer's instructions, the test is no longer FDA-cleared/approved, and the modification(s) must be validated by the laboratory. This requirement also applies to laboratories not subject to US regulations for tests approved by an internationally recognized regulatory authority that are modified by the laboratory.

Changes in the specimen type or collection device are examples of common modifications (see "modification of manufacturer's instructions" in the Definition of Terms). Additional requirements for validation/verification may be found in the discipline-specific checklists.

For waived and moderately complex tests, if manufacturer instructions are modified, requirements for high complexity testing apply.

§ 493.1459 Condition: Laboratories performing high complexity testing; general supervisor.

The laboratory must have one or more general supervisors who are qualified under § 493.1461 of this subpart to provide general supervision in accordance with § 493.1463 of this subpart.

§ 493.1461 Standard: General supervisor qualifications.

The laboratory must have one or more general supervisors who, under the direction of the laboratory director and supervision of the technical supervisor, provides day-to-day supervision of testing personnel and reporting of test results. In the absence of the director and technical supervisor, the general supervisor must be responsible for the proper performance of all laboratory procedures and reporting of test results.

- Assessing the adequacy for analytic sensitivity studies for seemingly minor assay modifications (e.g. change in transport media) remains a challenge. *Full LOD studies might be burdensome, but are not wrong.*
- Changing specimen types or patient populations may not affect analytic sensitivity, but overall clinical sensitivity might be affected. Laboratory validations are not well suited to assess this, but laboratories must validate clinical claims.

LETS REVIEW AN IFU

Alinity m

HSV 1 & 2 / VZV AMP Kit

Created May 2024

REF 09N61-095
53-608319/R1

CUSTOMER SERVICE: 1-800-553-7042
CUSTOMER SERVICE INTERNATIONAL:
CALL YOUR ABBOTT REPRESENTATIVE

Instructions must be carefully followed. Reliability of assay results cannot be guaranteed if there are any deviations from these instructions.

NAME

Alinity m HSV 1 & 2 / VZV Assay

- Alinity m HSV 1 & 2 / VZV AMP Kit (09N61-095)
- Alinity m HSV 1 & 2 / VZV CTRL Kit (09N61-085)

INTENDED USE

The Alinity m HSV 1 & 2 / VZV assay is an in vitro real-time polymerase chain reaction (PCR) assay for the qualitative detection and differentiation of Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2) and Varicella Zoster Virus (VZV) DNA from clinician-collected cutaneous or mucocutaneous lesion swab specimens from symptomatic patients suspected of active herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus infection. The Alinity m HSV 1 & 2 / VZV assay is intended to aid in the diagnosis of herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus active cutaneous or mucocutaneous infections. Negative results do not preclude herpes simplex virus type 1, herpes simplex virus type 2 or varicella-zoster virus infections and should not be used as the sole basis for diagnosis, treatment or other management decisions.

The Alinity m HSV 1 & 2 / VZV assay is not intended for use with cerebrospinal fluid (CSF) or to aid in the diagnosis of HSV or VZV infections of the central nervous system (CNS). The Alinity m HSV 1 & 2 / VZV assay is not intended for use in prenatal screening.

SUMMARY AND EXPLANATION OF THE TEST

HSV-1 and HSV-2: HSV-1 and HSV-2 are large double-stranded DNA viruses of the family *Herpesviridae*. HSV-1 and HSV-2 are genetically and antigenically distinct forms of HSV. Typically, HSV-1 is transmitted through

HSV 1 & 2 / VZV
REF 09N61-095
53-608319/R1

III

R_x
ONLY

IVD

The Alinity m System is designed to be a random access analyzer that can perform the Alinity m HSV 1 & 2 / VZV assay in parallel with other Alinity m assays on the same instrument.

HSV-1, HSV-2, and VZV DNA from specimens are extracted automatically onboard the Alinity m System using the Alinity m Sample Prep Kit 2, Alinity m Lysis Solution, and Alinity m Diluent Solution. The Alinity m System employs magnetic microparticle technology to facilitate nucleic acid capture, wash and elution. The resulting purified nucleic acids are then combined with the liquid unit-dose activation reagent, liquid unit-dose amplification reagents, and Alinity m Vapor Barrier Solution, and transferred by the instrument to an amplification/detection module for PCR amplification, and real-time fluorescence detection.

Assay controls are tested at or above an established minimum frequency to help ensure that instrument and reagent performance remain satisfactory. During each control event, a negative control and a positive control are processed through sample preparation and PCR procedures that are identical to those used for specimens. Assay controls are used to demonstrate proper sample processing and assay validity.

The Alinity m HSV 1 & 2 / VZV amplification reagents include primers and probes that amplify and detect an exogenous internal control (containing plasmid DNA). Amplification and detection of the internal control demonstrates proper sample processing. The internal control is used to demonstrate assay validity.

The possibility of nucleic acid contamination on the Alinity m System is minimized because:

- Aerosol barrier pipette tips are used for all pipetting. The pipette tips are discarded after use.
- PCR amplification and detection is carried out automatically in a sealed reaction vessel.
- Disposal of the reaction vessel is performed automatically by the Alinity m System.

For additional information on system and assay technology, refer to the Alinity m System Operations Manual, Section 3.

INTENDED USE

The Alinity m HSV 1 & 2 / VZV assay is an in vitro real-time polymerase chain reaction (PCR) assay for the qualitative detection and differentiation of Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2) and Varicella Zoster Virus (VZV) DNA from clinician-collected cutaneous or mucocutaneous lesion swab specimens from symptomatic patients suspected of active herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus infection. The Alinity m HSV 1 & 2 / VZV assay is intended to aid in the diagnosis of herpes simplex virus 1, herpes simplex virus 2 and/or varicella-zoster virus active cutaneous or mucocutaneous infections. Negative results do not preclude herpes simplex virus type 1, herpes simplex virus type 2 or varicella-zoster virus infections and should not be used as the sole basis for diagnosis, treatment or other management decisions.

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SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

Specimen Collection

Lesion swab specimens, including cutaneous and mucocutaneous, collected in commercially available viral transport media (VTM) (Copan UTM®, BD UVT, Remel M4RT®) can be used with the Alinity m HSV 1 & 2 / VZV assay on the Alinity m System.

Specimen Types

Swab specimens from lesions, including cutaneous and mucocutaneous, can be used with the Alinity m HSV 1 & 2 / VZV assay on the Alinity m System.

The Alinity m System does not provide the capability to verify specimen types. It is the responsibility of the operator to use the correct specimen types in the assay.

Specimen Storage

Specimen Type	Temperature	Maximum Storage Time ^a
Lesion swab specimens	15°C to 25°C	4 days
including cutaneous and	2°C to 8°C	14 days
mucocutaneous	-25°C to -15°C	90 days ^b
	-70°C or colder	90 days ^b

^a From date of collection.

^b Avoid more than 3 freeze / thaw cycles.

SPECIFIC PERFORMANCE CHARACTERISTICS

Limit of Detection (Analytical Sensitivity)

The limit of detection (LoD) for HSV-1, HSV-2, and VZV was determined by testing dilutions of HSV-1, HSV-2 or VZV cultured virus spiked in pooled negative clinical lesion swab specimens. For each analyte, a total of 72 replicates of each panel member were tested across 3 Alinity m HSV 1 & 2 / VZV AMP Kit lots and 3 Alinity m Systems (one lot per system) over multiple days. Probit analysis of the data determined that the concentration detected with 95% probability (LoD by Probit) for HSV-1, HSV-2, and VZV was 5.90 TCID₅₀/mL, 2.07 TCID₅₀/mL, and 0.055 TCID₅₀/mL respectively. Refer to Table 1.

Table 1. Limit of Detection

Analyte	Strain	LoD (TCID ₅₀ /mL)
HSV-1	MacIntyre	5.90
HSV-2	MS	2.07
VZV	Ellen	0.055

Analytical Specificity - Cross-Reactivity and Microbial Interference

A total of 55 potential cross-reacting microorganisms (viruses, bacteria, and fungi) that are commonly encountered in lesion swab specimens were tested with Alinity m HSV 1 & 2 / VZV assay to assess analytical specificity. The microorganisms were tested at 10^5 Units/mL for viruses and fungi, and 10^6 Units/mL for bacteria or the highest titer available. The microorganisms are listed in **Table 3**. Each cross-reactive microorganism was also tested in the presence of 3X LoD HSV-1, HSV-2, VZV viruses or negative matrix. No cross-reactivity was observed in the presence of these potential cross-reactive microorganisms. In addition, all positive samples reported positive results for HSV-1, HSV-2, and VZV in the presence of these microorganisms.

Table 3. Cross-Reacting and Interfering Microorganisms Tested

<i>Acinetobacter calcoaceticus</i>	Herpes Virus 6A strain GS
<i>Acinetobacter lwoffii</i>	Herpes Virus 6B strain Z29
<i>Actinomyces israelii</i>	HIV-1
Adenovirus type 1	HPV 16
Adenovirus type 7	HPV 18
<i>Bacteroides fragilis</i>	<i>Kingella kingae</i>
BK virus	<i>Klebsiella pneumoniae</i>
<i>Bordetella pertussis</i>	<i>Listeria monocytogenes</i>
<i>Campylobacter jejuni</i>	<i>Mobiluncus mulieris</i>
<i>Candida albicans</i>	<i>Moraxella catarrhalis</i>
<i>Chlamydia pneumoniae</i>	<i>Mycobacterium tuberculosis</i> ^a
<i>Chlamydia trachomatis</i> serovar D	<i>Neisseria gonorrhoeae</i>
<i>Chlamydia trachomatis</i> serovar I	<i>Neisseria meningitidis</i>
<i>Clostridioides difficile</i>	<i>Proteus mirabilis</i>
<i>Clostridium perfringens</i>	<i>Proteus vulgaris</i>
<i>Cryptococcus neoformans</i>	<i>Pseudomonas aeruginosa</i>
<i>Cutibacterium acnes</i>	<i>Staphylococcus aureus</i>
Cytomegalovirus	<i>Staphylococcus epidermidis</i>
<i>Enterobacter cloacae</i>	<i>Staphylococcus saprophyticus</i>
<i>Enterococcus faecium</i>	<i>Streptococcus agalactiae</i>
<i>Enterococcus faecalis</i>	<i>Streptococcus mitis</i>
Epstein-Barr virus	<i>Streptococcus mutans</i>
<i>Escherichia coli</i>	<i>Streptococcus pneumoniae</i>
<i>Fusobacterium nucleatum</i>	<i>Streptococcus pyogenes</i>
<i>Haemophilus ducreyi</i>	<i>Streptococcus salivarius</i>
<i>Haemophilus influenzae</i>	<i>Treponema pallidum</i>
Hepatitis B virus	<i>Toxoplasma gondii</i>
Hepatitis C virus	

^a *Mycobacterium tuberculosis* genomic DNA was used as target.

Interfering Substances

The performance of the Alinity m HSV 1 & 2 / VZV assay was evaluated with potentially interfering endogenous and exogenous substances that may be present in cutaneous or mucocutaneous lesion specimens. A panel composed of 24 substances listed in **Table 4** was tested in the absence or presence of HSV-1, HSV-2, or VZV (MacIntyre strain, MS strain, Ellen strain, respectively) at 3X LoD in the Alinity m HSV 1 & 2 / VZV assay. There was no evidence of interference (false positive or false negative results) caused by the substances tested at the concentrations shown below in **Table 4**.

Table 4. Interfering Substances Tested	
Substance	Test Level
Blood (human)	5% v/v
Leukocytes	10 ⁶ cells/mL
Mucin	0.3% v/v
Urine	5% v/v
Feces	0.1% w/v
Human Serum Albumin	1% w/v
Saliva	4% v/v
Seminal Fluid	5% v/v
K-Y Jelly (Personal Lubricant)	2.5% w/v
Vaginal Contraceptive Gel	2.5% w/v
Monistat (Miconazole Nitrate Vaginal Cream)	3% w/v
Preparation H (Hemorrhoidal Ointment)	2.5% w/v
Abreva (Cold Sore Cream)	2.5% w/v
Acyclovir	2.5% w/v
Vagisil (Anti-Itch Cream)	0.25% w/v
Vagicare (Anti-Itch Cream)	2.5% w/v
Feminine Wash (Douche)	2.5% w/v
Denavir (Anti-viral cream)	2.5% w/v
Feminine Deodorant Spray	2.5% w/v
Lip Balm	3% w/v
Summer's Eve (Body Powder)	2.5% w/v
Toothpaste	2.5% w/v
Casein protein	0.7% w/v
Mouthwash	3% v/v

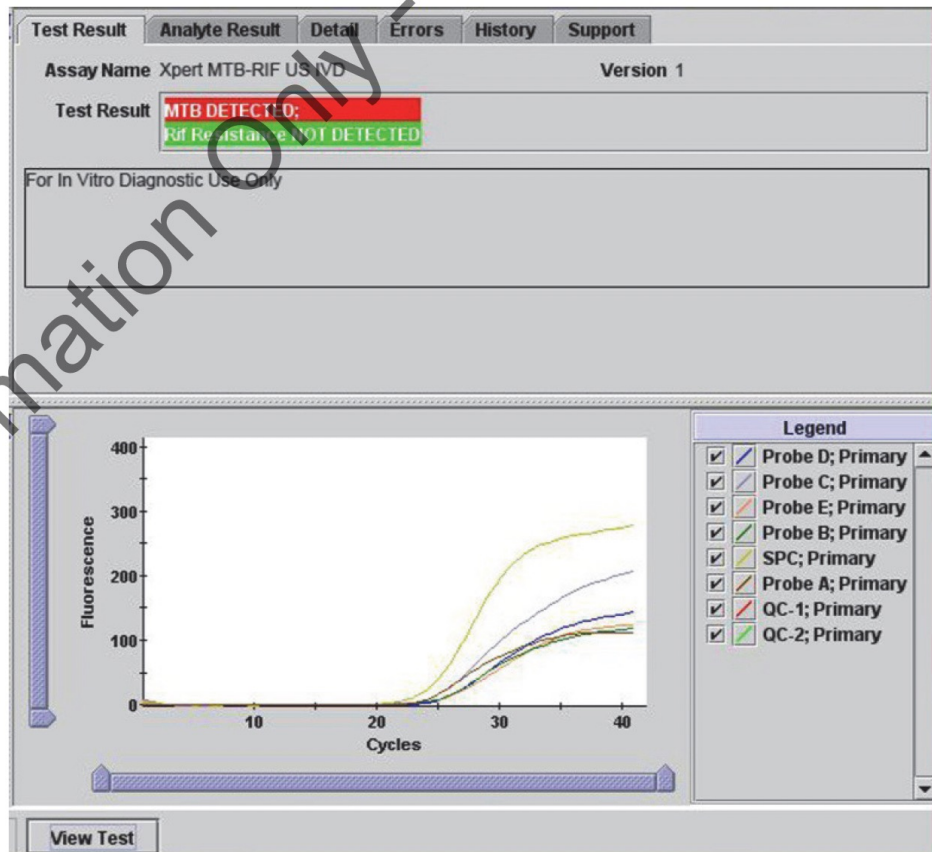
CASE I – QUESTION FALSE POSITIVE

- Provider calls the lab asking about potential false positive NAAT result.
 - “Flu A PCR was positive, but the patient doesn’t have any flu symptoms and now he is on isolation.”
- What can we do to investigate?
 - Look at the sample results
 - Look at the run
 - Look at QC / Contam Monitoring
 - Any known issues with assay?
 - Is this seasonally appropriate?
- Sources of false positive:
 - Carryover
 - Contamination
 - Non-specific reactivity / Cross reactivity
 - Probe degradation
- If available, these should have a higher Ct (lower burden) but not definitive
- Could re-run the sample (but a low positive might not repeat)
- Could recommend a new sample
- Could recommend “clinical correlation”

CASE 2 – REPEAT M.TB PCR TESTING

- “I’m worried a resistant M.tb sub-population is emerging while on therapy. Can we repeat the Xpert M.tb / RIF PCR?”
 - Or
- “I ordered a repeat Xpert M.tb / RIF PCR on my patient. RIF resistance was originally NOT detected, but now it says RIF resistant and we are running around with our hair on fire. What’s up with that?”
- Lets go to Xpert IFU to review

M.TB RIF ASSAY IS CLEVER ASSAY



- Multiple probes anneal to RPO-beta locus for identification of M.tb
- Lack of binding of probes with specific characteristics (probe, relative Ct value) implies mutations associated with rifampin resistance present
- At low-levels (as in patients being treated), assays perform oddly and non-uniformly potentially giving rise to false results
- ***In this case, that is false rifampin resistance calls***

CLINICAL CLAIMS - FDA

COM.40625 Clinical Claims Validation - FDA-cleared/approved Tests

Phase II



For FDA-cleared/approved tests, the laboratory validates clinical claims not included in the manufacturer's instructions.

NOTE: For laboratories not subject to US regulations, this requirement also applies to tests that are approved by an internationally recognized regulatory authority.

A clinical claim is a communication from the laboratory to its users (including but not limited to clinicians and patients) regarding a test's sensitivity and specificity, predictive values for a disease or condition, clinical usefulness, cost-effectiveness or clinical utility.

To adequately support a clinical claim, the laboratory must perform a clinical validation study, unless the clinical validity of the test is documented in peer-reviewed literature or textbooks. The clinical validation study must include at least 20 samples and must include both positive and negative samples. If the laboratory uses fewer samples, the laboratory director or designee meeting CAP director qualifications must record the criteria used to determine the appropriateness of the sample size.

Evidence of Compliance:

- ✓ Records of clinical studies performed by the laboratory **OR** peer-reviewed literature that reasonably substantiates all claims made by the laboratory about a test

REFERENCES

- 1) Clinical and Laboratory Standards Institute (CLSI). *A Framework for Using CLSI Documents to Evaluate Medical Laboratory Test Methods*. 3rd ed. CLSI report EP19. Clinical and Laboratory Standards Institute; 2022.

CASE 3 – BCID / CULTURE + AST DISCORDANCE

- “The BCID showed E.coli that was CTX-M negative, but the E.coli was ceftriaxone resistant.”
- ”The BCID showed MRSA, but the culture shows MSSA + CoNS.”
- “The BCID showed VRE E.faecalis, but the culture shows VSE E.faecalis.”
- What can we do?
 - Review AST results
 - Review purity plates and primary plates – different morphologies?
 - Retest the bottle?
 - Sub the bottle to selective agar?
- How far down the rabbit hole do you want to go?
- What is biologically plausible?
 - For E.coli
 - ~5% of our ceftriaxone resistant E.coli are CMY+ (plasmid AmpC) so would be CTX-M positive
 - But, would look at AST pattern for wonkiness and potentially repeat if something seemed off
 - For MSSA + CoNS
 - This is a thing that happens, but would review for multiple morphologies
 - For double Enterococcus
 - Would review for multiple morphologies

MOLECULAR AMR LINKAGE TO ORGANISM

MIC.65630 Antimicrobial Resistance Markers by Molecular Analysis - Clinical Validity Phase I



Detection of genotypic antimicrobial resistance markers (eg, *vanA*, *mecA*, *bla_{KPC}*, etc.) is linked or attributed to a corresponding organism in the final laboratory report when molecular analysis is performed directly on patient specimens (eg, urine or respiratory specimens) or in blood culture broth. This can be accomplished through molecular detection of targets identifying the corresponding organism or by detection of resistance markers from organisms recovered in concurrent culture.

NOTE 1: The intent of this requirement is to ensure that detection of antimicrobial resistance has clinical validity. Ideally, genotypic antimicrobial resistance results will be correlated with other available results, such as identification of isolates in culture and their phenotypic AST results, with the goal of aiding in the assessment of clinical significance of such findings. There may be instances where the organism is not recovered in culture (eg, due to lack of viability) or confirmation by culture-based methods may not be possible; however, efforts should be taken to link the antimicrobial resistance gene to the organism. Resistance markers should only be reported when present with compatible/appropriate organisms.

NOTE 2: This requirement does not include either screening tests (eg, rectal swab for carbapenemase gene detection) or surveillance tests (eg, testing performed to monitor for a community- or population-level occurrence and establish incidence or prevalence of that occurrence, if results do not go back to individual patients).



OPEN UP TO QUESTIONS / SCENARIOS FROM THE FLOOR

REIMBURSEMENT BRIEFLY

- Reimbursement is typically very opaque to laboratory
- Reimbursement for an individual test may vary by insurer, insurance plan, associated diagnoses
- Reasons for denial may vary and not be entirely consistent even if you get the data to review
- In general, smaller panels (lower analyte CPT Codes) are more likely to get reimbursed while larger panels are less likely to be reimbursed
- Proper coding by ordering providers is essential, but concerns about institutionally encouraged up-coding may prevent embedded guidance (at least at our place)
- Increasing availability of flex-panels is encouraging

GI PANEL CPT CODES AS EXAMPLE

Billing and Coding: Gastrointestinal Multiplex Nucleic Acid Amplification

A56642

Group 1 (2 Codes)

Group 1 Paragraph

Note: Providers are reminded to refer to the long descriptors of the CPT code.

Group 1 Codes

Code	Description
87505	Nfct agent detection gi
87506	ladna-dna/rna probe tq 6-11

Group 2 (1 Code)

Group 2 Paragraph

Note: Providers are reminded to refer to the long descriptors of the CPT code.

Group 2 Codes

Code	Description
87507	ladna-dna/rna probe tq 12-25

Group 1 (1 Code)

Group 1 Paragraph

It is the provider's responsibility to select codes carried out to the highest level of specificity and selected from the ICD-10-CM code book appropriate to the year in which the service is rendered for the claim(s) submitted.

The following ICD-10-CM code supports medical necessity and provides coverage for CPT codes: **87505** and **87506**.

Group 1 Codes

Code	Description
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Group 2 (1 Code)

Group 2 Paragraph

It is the provider's responsibility to select codes carried out to the highest level of specificity and selected from the ICD-10-CM code book appropriate to the year in which the service is rendered for the claim(s) submitted.

The following ICD-10-CM code supports medical necessity and provides coverage for CPT code **87507**.

Group 2 Codes

Code	Description
R19.7*	Diarrhea, unspecified

Group 2 Medical Necessity ICD-10-CM Codes Asterisk Explanation

*Note: Dual Diagnosis: When reporting ICD-10-CM code R19.7, one of the immunosuppression diagnosis codes listed in Table 3 below must also be reported.

TRYING TO TIE IT ALL TOGETHER

- Molecular diagnostics have revolutionized the diagnosis of infectious diseases
 - For some disease states they have entirely replaced conventional methods OR are the only method available
 - For other disease states they remain complementary to traditional microbiologic methods
- Commercial options for NAAT continue to expand and the vast majority of NAAT that most of us perform are likely commercial esp. PCR methods
- Sequencing based methods are becoming more common, but are most often sent out
 - Stewardship and triaging specimens is essential as these methods have limitations that not all understand and are expensive
- There are common questions for implementing molecular microbiology assays, but good resources are available