

# Today's Featured Speaker

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# Molecular Diagnostics at Point of Care

When will we get there, and where is 'there' anyway?

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# Learning Objectives

- Participants should be able to:
  - Describe the basic work-flow of molecular diagnostic testing.
  - Describe some major amplification and detection methods
  - Distinguish between real-time and non-real-time molecular methods
  - Recognize the properties of an analyte that make it a candidate for molecular testing e.g. discrete, single or small # of targets, present in samples...do an easier-harder kind of organization.
  - Recognize emerging molecular diagnostic platforms that may be usable at point-of-care
  - Describe unique quality issues in molecular diagnostics which impact their use at point of care e.g. contamination, inhibition, sampling

# Outline

- What is molecular diagnostics, exactly?
- Why would we want to do POC molecular testing?
- Molecular diagnostics technologies.
- What analytes make sense?
- What platforms are out there?
- What unique quality issues will there be?
- Thinking about the future.

# What is Molecular Diagnostics?

- Analysis of DNA or RNA for diagnostic purposes. Molecular diagnostics have found widespread application with the advent of *amplification methods* (PCR and related approaches).
- Huge scope
  - From single-target molecular detection of pathogens...
  - To pharmacogenomic analysis of metabolism genes for drug dosing...
  - To whole genome sequencing for disease susceptibility and God knows what all.

# Molecular Diagnostic Testing

•Specimen

•DNA / RNA Extraction

•Amplification of Target

•Detection of amplified target

•Interpretation and Clinical Use

# Why Amplify?

## ○ *Sensitivity*

- can detect small numbers of organisms
- can even detect dead or damaged organisms
- can detect unculturable organisms

## ○ *Speed*

- 4-48 hour turnaround
- inoculum independence

# Why Amplify, continued

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## ○ Targets

- Test for things there's no other way to test
- Uncultivable bugs
- Genetics
  - Pharmacogenomics
  - Prenatal testing
  - Hypercoagulability, etc.
- Oncology
  - Hematologic malignancies
    - Diagnostic markers
    - Minimal residual disease



# Why Not Amplify?

- Clinical significance?
- Technical problems
  - Contamination
  - Inhibition
- Cost
- COST
- CO\$T

# Specimen Sources

- Blood/Serum
  - heme and hemelike compounds strongly inhibit
  - pathogens in low concentrations
  - anticoagulants (heparin, EDTA, citrate) inhibit
  - serum proteases can be inactivated by heating
- Urine
  - amorphous salts during storage make purification difficult
  - urinary inhibitors vary widely
- CSF
  - spun pellets often contain high inhibitor concentrations
- Sputum
  - can contain huge amounts of DNA (up to 14 mg/ml)
- Stool
  - the most difficult specimen
  - many inhibitors, large background of bacterial and cellular DNA



# Extraction



## ○ DNA/RNA Extraction

- Depends on:
- Specimen source (blood, CSF, stool)
- Target organism (human tumor, CMV, M. tuberculosis)
- Target nucleic acid (DNA, RNA)

## ○ Increasing automation

- Magnetic or other separation methods.
- **REQUIRED** for POC

# Amplification

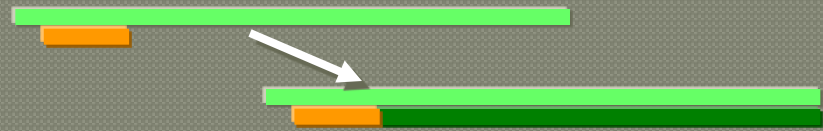


- Nucleic Acid Amplification means taking a small number of targets and copying a specific region many, many times.
- NAAT, NAT, etc; commonly-used abbreviations
- PCR is the most common amplification scheme, but there are others!

# Amplification Enzymology

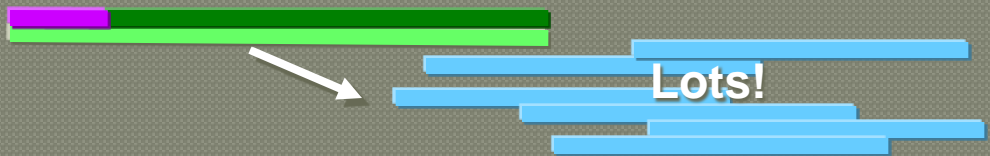
- *DNA polymerase*

- makes DNA from ssDNA, requires priming



- *RNA polymerase*

- makes RNA from dsDNA, requires specific start site



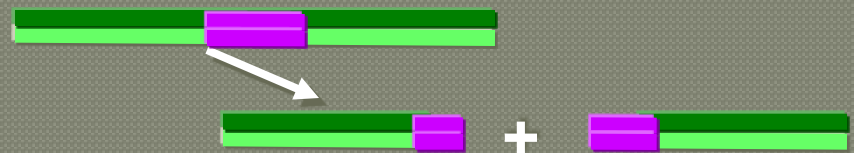
- *Reverse transcriptase*

- makes DNA from RNA, requires priming

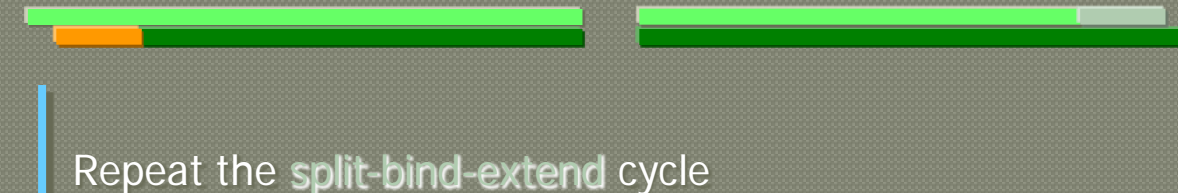
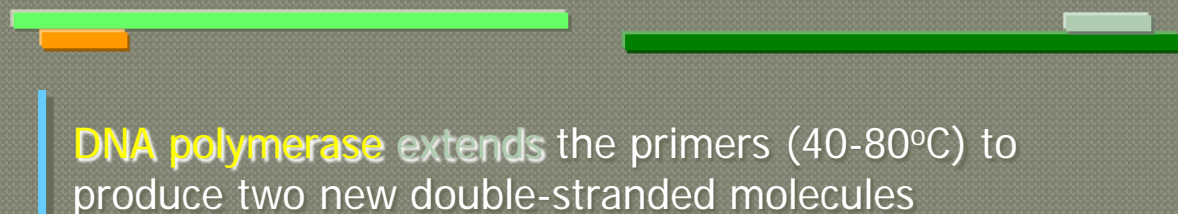
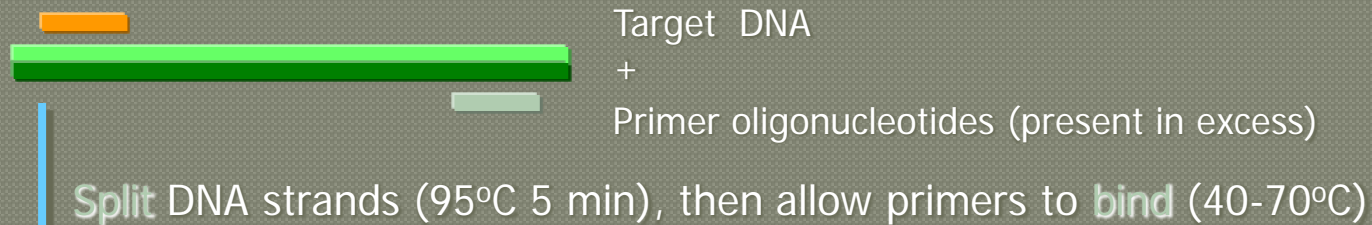


- *Restriction endonucleases*

- cut DNA in a sequence specific manner

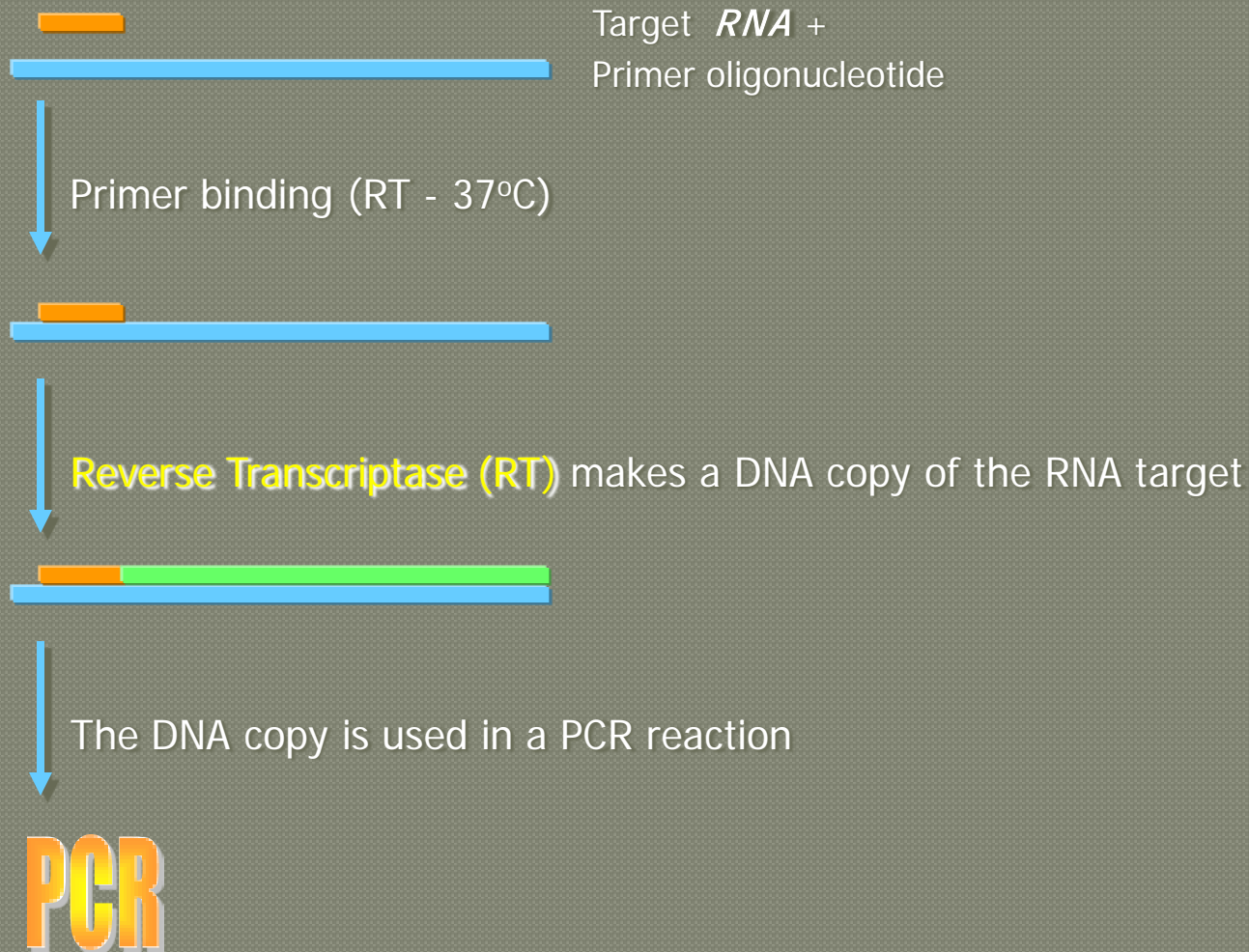


# Polymerase Chain Reaction (PCR)



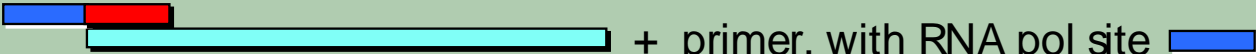

This 'short product' amplifies exponentially in subsequent split-bind-extend cycles, driven by the temperature changes in a 'thermal cycler'.

# Reverse Transcriptase PCR (RT-PCR)



# Transcription-Mediated Amplification

Target= RNA or single-stranded DNA

 + primer, with RNA pol site 

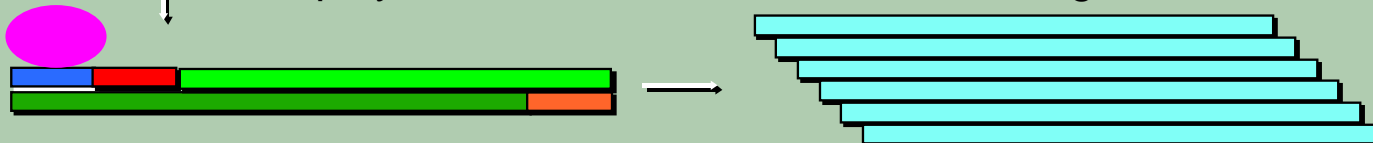
↓ reverse transcriptase makes DNA from the RNA



↓ split strands (95°C 5 min), then anneal second primer, which is extended by the reverse transcriptase



↓ RNA polymerase transcribes 10-1,000 new target RNAs

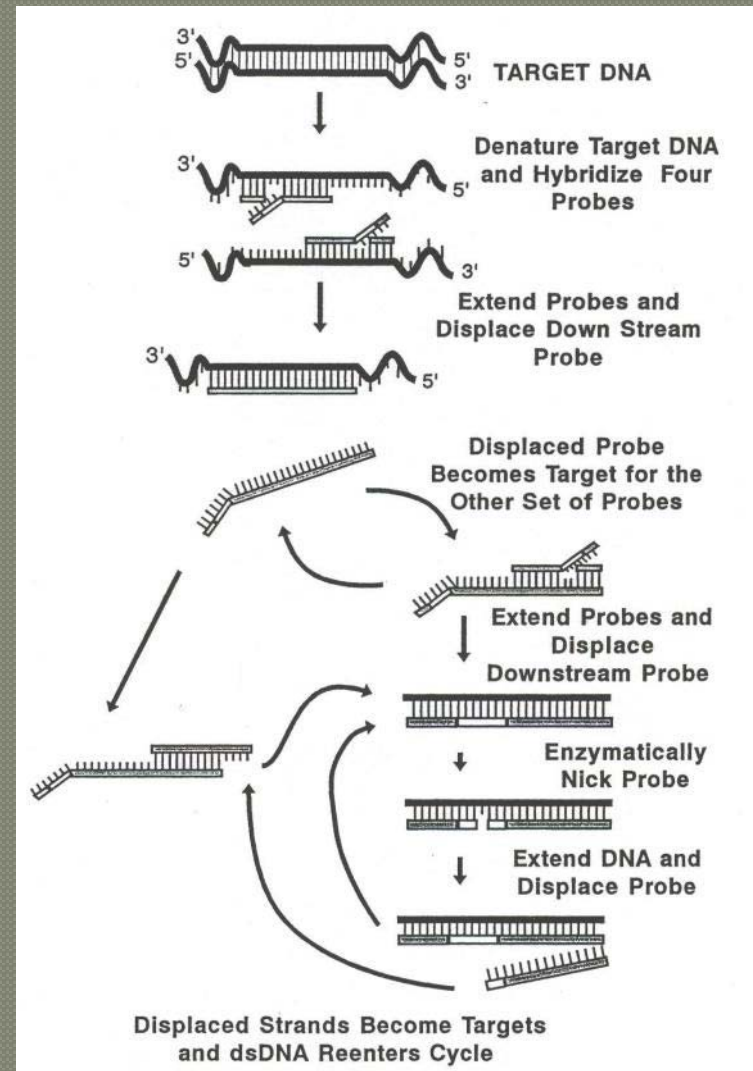


*A small number of cycles can produce a 10<sup>6</sup> fold amplification*



# Strand-displacement Amplification

- Complex
- But it works





# Detecting PCR Products



- Gel electrophoresis ( $\pm$  Southern blotting)
- Enzyme-linked assays
- Hybridization  
Protection/chemiluminescent assay
- A multitude of formats available, to serve market and technical needs

# Real-Time PCR

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

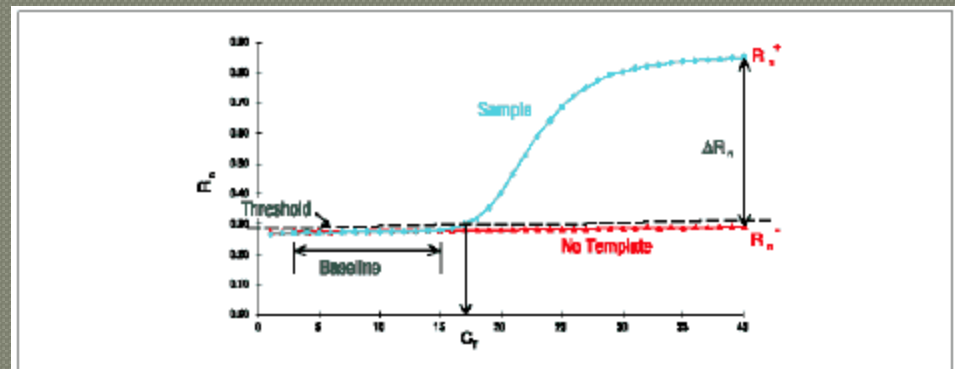


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNARNA Real-Time Quantitative PCR bulletin).

# Real-Time PCR Instruments

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

# Real-time PCR Chemistries

## ● Essential Fluorescence Chemistry

- Shorter wavelength=higher energy
- Activation with high-energy light, usually UV
- Emission at a lower energy, usually visible
- Different fluorochromes have different (and hopefully distinguishable) activation and emission wavelengths.

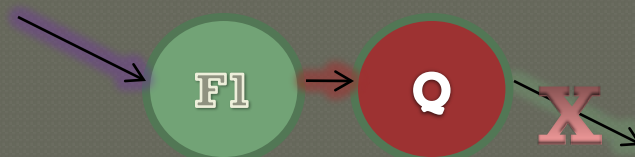


- The more fluorochromes a real-time instrument can detect, the more 'channels' it is described as having, and the more targets can be detected.

# Quenching

## ○ Quenching

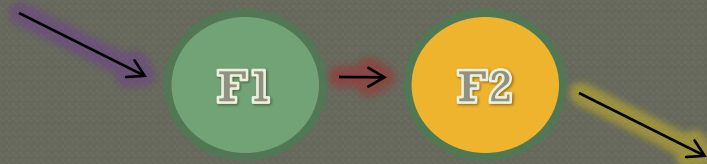
- Fluorescence occurs when a photon bumps an electron to a higher energy level, then another photon is emitted when it drops back to ground state.
- Some compounds ('quenchers') suck up that energy before it can be reemitted, 'quenching' the fluorescence.



- This is distance dependant; the closer the molecules are the more efficient the quenching.

# Fluorescence Resonance Energy Transfer (FRET)

- A second fluorochrome can suck up the energy from the activated fluorochrome and re-emit it at its emission frequency.

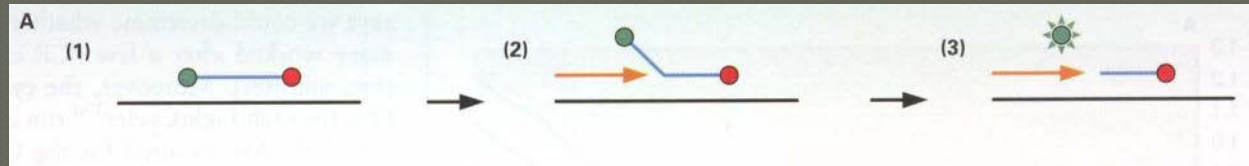


- This is distance dependant; the closer the molecules are the more efficient the energy transfer.

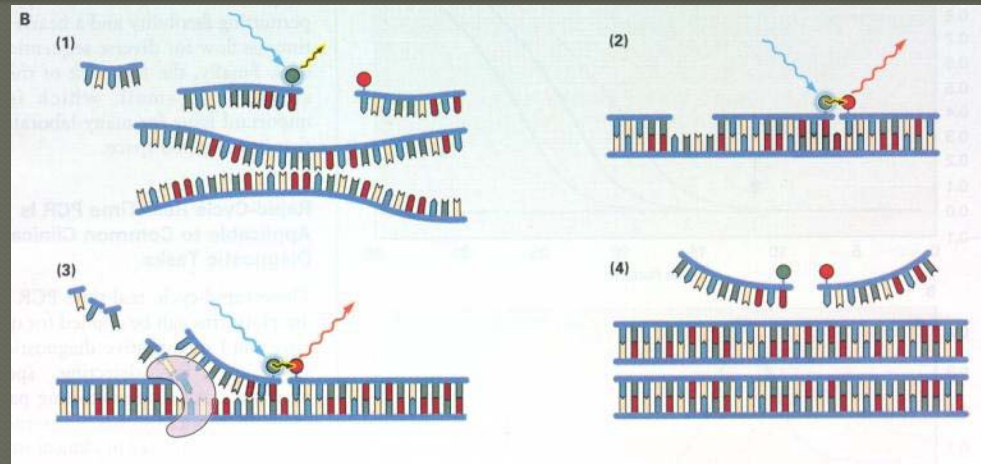


# Real-Time Detection Schemes

- Taqman Probes

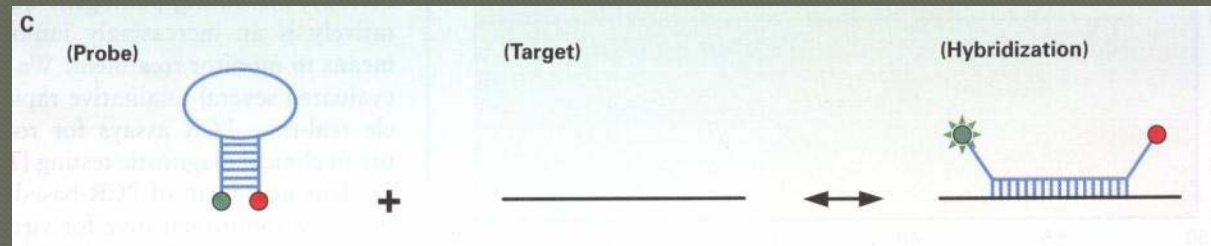


- FRET Probes



- Molecular Beacons

- Several others



# Contamination!

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- **What happens** when you make  $10^6$  copies of a single short sequence in a 100ml reaction?
  - You end up with  $10^4$  copies/ul
  - What happens when you pop the top off a microcentrifuge tube?
    - ...or pipet anything
    - ...or vortex anything
    - ...or...
- **You create aerosols**
  - Droplet nuclei with diameters from 1-10  $\mu\text{m}$  persist for hours/days
  - Each droplet nucleus contains amplified DNA
  - Each amplified molecule can initiate a new amplification reaction

# Ways to Prevent Contamination

- *Meticulous technique*

- Hoods, UV, bleach, physical separation of work areas

- *Assay design*

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

- *Chemical and Physical Inactivation*

- UNG

# POC Molecular Diagnostics

## ● Infectious Disease

- Outpatient POC
  - GC / *Chlamydia*
  - Group A strep
  - HIV / HCV viral load
- Acute-care POC – Lab vs POC
  - Respiratory pathogens
  - CNS pathogens
- Nosocomial / Screening
  - MRSA / VRE
  - *C. difficile*
- Biopreparedness
  - Military development and applications
- Diseases of Under-resourced populations
  - Tuberculosis incl drug-resistance

## ● Others

- Pharmacogenetics
- Hypercoagulability
- Other genetic diseases
- Oncology
  - Lower priority for POC
  - Large number of diseases
  - Solid tumors – need tissue
  - Generally easier follow-up.
- NOTE: the ones in pink actually exist in some form (mostly pre-approval). The rest are guesses.

# Thinking about the Targets

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- Single targets are easier than multiples
  - Even single targets may require multiple primers and probes due to polymorphisms
    - One MRSA test uses 7 primers and 5 probes!
- Genetic targets are easier than microbes
  - Easier to get large amounts
  - Easier extractions
- Qualitative tests are easier than quantitative
  - *Chlamydia* vs. HIV viral load; bcr-abl for diagnosis vs for disease monitoring.

# Why Molecular? Rapid flu versus Other Methods

Influenza A Rapid Test Performance¶					
Rapid Test¶	Sens%¶	Spec%¶	Compared With¶	Comments¶	Reference¶
Directigen ¶	58.8¶	99.2¶	Molecular¶	A&B performance combined¶	Liao et al JCM 47(3):527-32, 2009 Mar¶
3M-QuickVue ¶ BinaxNow¶	75¶ 73¶ 55¶	98¶ 99.5¶ 100¶	Culture¶	Archived specimens¶	Dale et al JCM 46(11):3804-7, 2008 Nov¶
BinaxNow¶	53¶	¶	RT-PCR¶	2 of 237 samples were flu B pos by RT-PCR but flu A by NOW. ¶	Landry et al JCV. 43(2):148-51, 2008 Oct¶
BinaxNow¶	61¶	100¶	RT-PCR¶	DFA was 81% sensitive¶	Rahman et al Diag Micro Infect Dis 62(2):162-6, 2008 Oct¶
RemelXpect-¶ BinaxNow¶	47.7¶ 78.3¶	98.7¶ 98¶	Culture¶	20.3/99.8 Flu B-¶ 35.9/99.9 Flu B¶	Cruz et al JCV 41(2):143-7, 2008 Feb¶
BinaxNow¶	52¶	¶	RT-PCR¶	70% in days 1-3 of disease¶	Nilsson et al Inf Cont & Hosp Epi 29(2):177-9, 2008 Feb¶
Directigen ¶	42¶	96¶	Culture¶	¶	Rahman et al Diag Micro Infect Dis 58(4):413-8, 2007 Aug¶
BinaxNow-¶ Directigen-¶ QuickVue¶	73¶ 69¶ 67¶	99¶ 100¶ 100¶	RT-PCR¶	Sensitivity only 30% vs flu B for all¶	Hurt et al JCV 39(2):132-5, 2007 Jun¶
Quickvue¶	85¶	97¶	RT-PCR¶	¶	Mehlmann et al JCM 45(4):1234-7, 2007 Apr.¶
Directigen + Quickvue + BinaxNOW¶	63¶	97¶	RT-PCR¶	Data pooled from all rapids; ¶	Grijvala et al Pediatrics. 119(1):e6-11, 2007 Jan¶

Convenience sample of recent literature; selected by Medline search + fit to single page

# What Will Be First?

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- Things that're easy
  - MRSA, already on GeneExpert
- Things that're hot
  - Influenza
- Things where existing tests perform poorly
  - Respiratory viruses in general
  - Group A strep
  - Group B strep

# Staphylococcus aureus

- Major nosocomial and community-acquired pathogen
  - Responsible for >20% of bacteremia in US/Canada
  - Transmissible nosocomially and in the community.
- Gram-positive cocci in clusters
- Sepsis, pneumonia, skin, wound, and soft tissue infections, osteomyelitis, UTI, endocarditis, etc.





# Multiple Staphylococcal Skin Abscesses

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# Foot Ulcer in a Diabetic Patient

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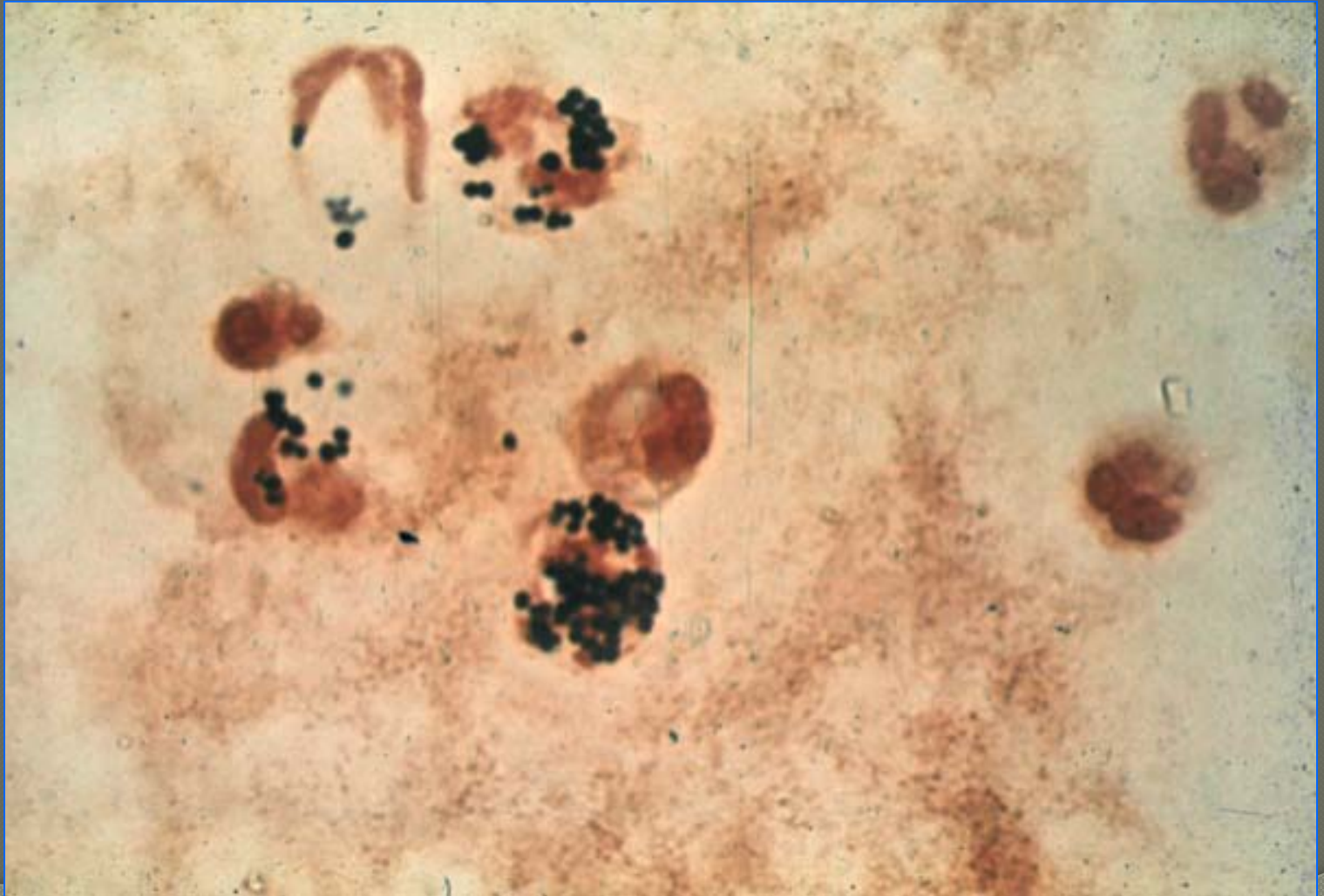


# Staphylococcal Osteomyelitis and Drainage from Fistulae



Staphylococcal  
botryomycosis

# Gram stained material from fistula



# Resistance in *Staphylococcus aureus*

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- Antibiotic resistance -- lots
  - Methicillin (oxacillin) resistance
    - Nosocomial
    - Community-acquired
  - Emerging resistance to vancomycin and other drugs used to treat MRSA.

# MRSA

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- First described in 1961; first penicillinase-resistant semisynthetic menicillin introduced in 1960.
- Acquisition of the *mecA* gene.
  - Codes for altered PBP; PBP2a
  - Variable expression
- Steadily increasing in nosocomial populations
  - Multi-resistant
- Community-acquired strains
  - Tend to be non-multi-resistant
  - Outbreak and sporadic
  - Skin & soft tissue infections

# Detecting MRSA

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- In clinical specimens

- Gram stain and culture
- PCR test for skin and soft tissue infections
- PCR for rapid ID in positive blood cultures

- SURVEILLANCE

- Increasing interest in detecting colonization
  - Primary site: anterior nares
  - Also axilla and other skin sites
- Increasing data that detection & isolation of colonized patients can decrease infections
- Specialized culture methods: 24-48h
- Molecular testing: 1-2h

# Molecular Methods for MRSA

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- Sensitive and specific
- Rapid
- Relatively expensive
- Some are simple enough for POC use
  - None waived yet
  - Useful for rapid placement in surveillance
- Wound and soft-tissue infections
- (Blood culture rapid assessment)



# What Will a Molecular POC Test Look Like?

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- Automated, fully integrated
  - Sample preparation
  - Amplification and detection
  - Reproducibility
  - Reliability
  - Such systems are emerging
- Quality need not be compromised for POC molecular tests
  - **Unlike** most of the antigen tests versus lab-based methods

# The GeneExpert – Almost There!!!

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- Self-contained molecular platform
  - Based on Smartcycler hardware
- Comparatively simple to operate
  - FDA-approved as a *moderate complexity* method.
    - Surveillance nasal swabs
    - Skin-soft tissue infections
    - Blood culture ID
  - In development as *FDA waived* method.

# GeneExpert Testing

- Self-contained extraction / amplification / analysis
- Sample collected from nares on a swab
- Swab broken into extraction vial, vortexed, added to cartridge
- Reagents added
- Place cartridge in instrument, result in 60 min



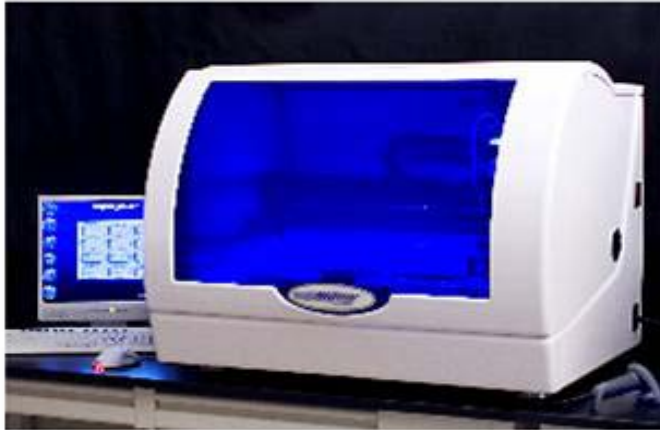
# Handylab 'Lab on a Chip'

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- Small, low sample volumes, rapid analysis; combine
  - Fluid actuation
  - Sample pretreatment
  - Sample separation
  - Signal amplification
  - Signal detection
- To be distributed by BD

# Handylab Systems

**Jaguar**



- Complete Automation
- Random Access
- Microfluidics
- Unitized Room Temperature Reagents
- Rapid Detection
- Contamination Control
- Open Menu for Laboratory Developed Tests

**Raider**



- Automated Amplification & Detection
- 12-15 Minute Real Time PCR
- Random Access
- Microfluidics
- Touch Screen
- Contamination Control

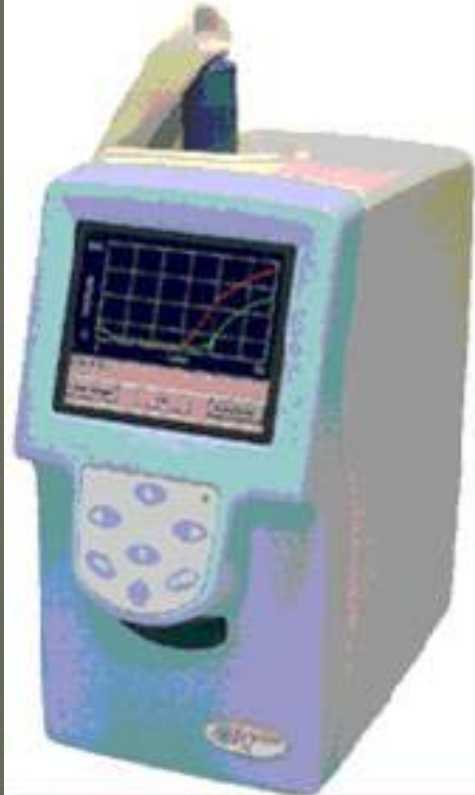
- 1-24 Samples
- 4  $\mu$ l sample size
- 45-90 minutes
- Swabs, urine, plasma, csf, blood

# Enigma Diagnostics FL and ML



## ● **Key features:**

- Fully automated real-time PCR system
- Rapid test (30 ~ 45 minutes to result)
- Multi-sample and scalable accepts swabs and liquids (e.g. urine, blood plasma)
- Integrated sample preparation and analysis
- Low system price
- Small footprint (no specialist skills or cold storage requirements)
- Miniaturized multi-well thermal cycling
- Freeze dried reagents
- Microfluid sample prep



## Liat™ Analyzer Advantages

- Rapid sample-to-result automation with quantitative analysis
- Completely closed system eliminates contamination and allows nucleic acid testing in any setting
- Flexible platform is adaptable to various assays and analytes

- Flexible tube divided into sealed segments

# BioSeeq Plus Smiths Detection



- Handheld, fully field portable LATE PCR technology
- Supports up to 6 independent simultaneous assays
- Detects trace levels of specific BWA agents as low as 100 organisms
- Identifies biological warfare agents such as Anthrax, Tularemia, Plague and Pan-Orthopox
- Results in approximately 65 minutes
- Rechargeable lithium-ion battery pack

## Specifications

Size	30.5 x 18 x 7.5 cm (12 x 7 x 3 in.)
Weight	Detector (including battery pack) 3.0 kg (6.6 lbs) Battery pack 0.47kg (1.04 lbs)
Power	External: 12Vdc, via an ac adaptor Battery: Re-chargeable Lithium-Ion battery pack -can run up to 10 hours on a single charge
Communications	RS232. Can also be converted to USB with the addition of a commercially available serial connector
Capacity	Up to 6 independant simultaneously assays
Consumables	Self contained single use sample cartridge device PCR based training consumables available.
Typical Test Time	LATE PCR results in approximately 65 minutes depending on environmental conditions



# Idaho Technologies Filmarray



- Automated Protocol; start the run & walk away
- Integrated Sample Preparation
- Automated analysis of results
- Results in less than an hour
- Microarray of up to 120 targets

# What to think about

- All the usual QC and QA, plus:
- Interferences
  - Extraction efficiency
  - **Inhibition** by:
    - Blood
    - DNA
  - Internal amplification / extraction controls
- Contamination
  - Extraordinarily sensitive methods
  - **Specimen** cross-contamination
    - Native material transferred from a positive to a negative specimen
    - Collection devices
    - Ports, racks, hands
  - **Amplicon** contamination
    - From amplified material
    - How well is the product contained?
    - Waste disposal
  - Carry-over studies

# Future Developments

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- Technological advances
  - performance
  - speed
  - footprint
- Expanded test menus
  - quantitative assays
- Resource limited settings

# Recommendation

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- “Point-of-care testing, especially those analyses that are conducted at the patient’s bedside, in a physician’s office, or in a clinic, is a growing trend in health care, and clinical microbiology professionals should prepare for this future reality. Clinical microbiologists must ensure that the individuals who perform point-of-care testing understand how to interpret the results. Clinical microbiologists should be called upon to help select the assay targets, advise on test formats, and participate in clinical trials.”
- From “Clinical Microbiology in the 21<sup>st</sup> Century: Keeping the Pace”. American Academy of Microbiology, 2008. Available on-line at: <http://www.asm.org/academy/index.asp?bid=58445>

# Acknowledgements

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- ◉ Molecular platform slides courtesy Dr. Roger Klein.