Molecular Diagnostics at Point of Care

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

Sheldon Campbell M.D., Ph.D.

Pathology and Laboratory Medicine, VA Connecticut

Department of Laboratory Medicine, Yale School of Medicine

Learning Objectives

- Participants should be able to:
  - Describe the basic work-flow of molecular diagnostic testing.
  - Describe some major amplification and detection methods.
  - Recognize the properties of analytes that make them candidates for molecular testing.
  - Recognize emerging molecular diagnostic platforms that may be usable at point-of-care.
  - Assess platforms for influenza testing in the context of POCT.
  - Describe unique quality issues in molecular diagnostics which impact their use at point of care.
  - Recognize Campbell's Laws of POCT and their implications for the future of molecular methods.

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 whatall.

Molecular Diagnostic Testing

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

Poll questions 1-3

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

- 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

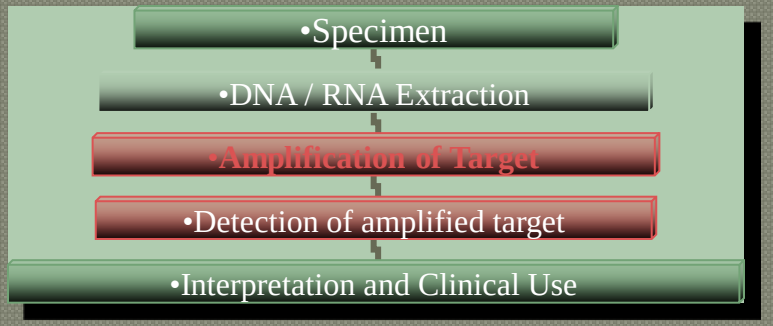
Why Not Amplify?

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

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

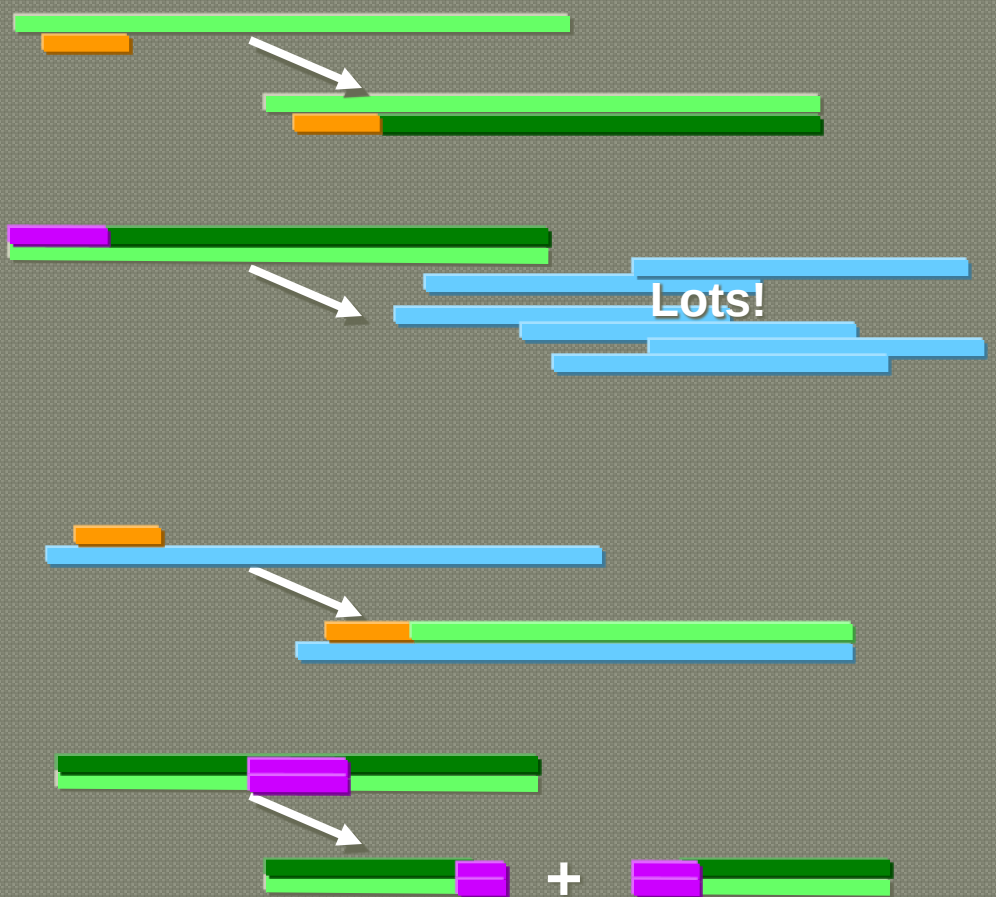
Amplification



- 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)

Target DNA
+
Primer oligonucleotides (present in excess)

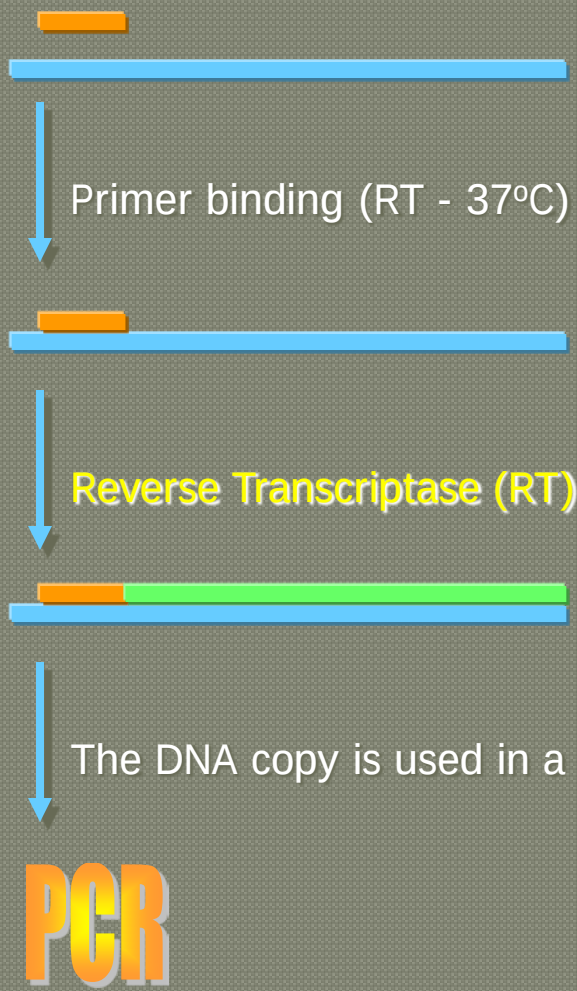
Split DNA strands (95°C 5 min), then allow primers to bind (40-70°C)

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

Repeat the split-bind-extend cycle

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

Reverse Transcriptase PCR (RT-PCR)



Target *RNA* +
Primer oligonucleotide

Primer binding (RT - 37°C)

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

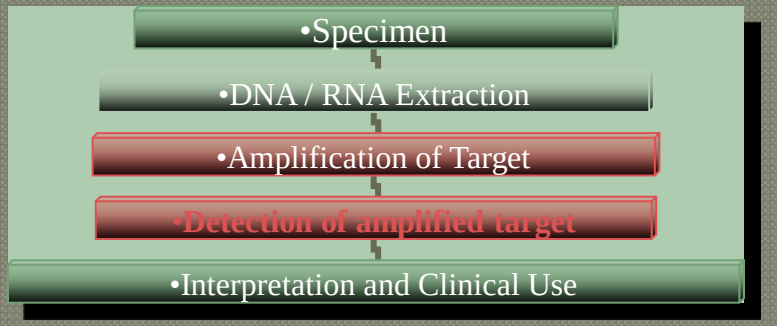
The DNA copy is used in a PCR reaction

PCR

Other Amplification Methods

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

Detecting PCR Products



- Gel electrophoresis (± 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

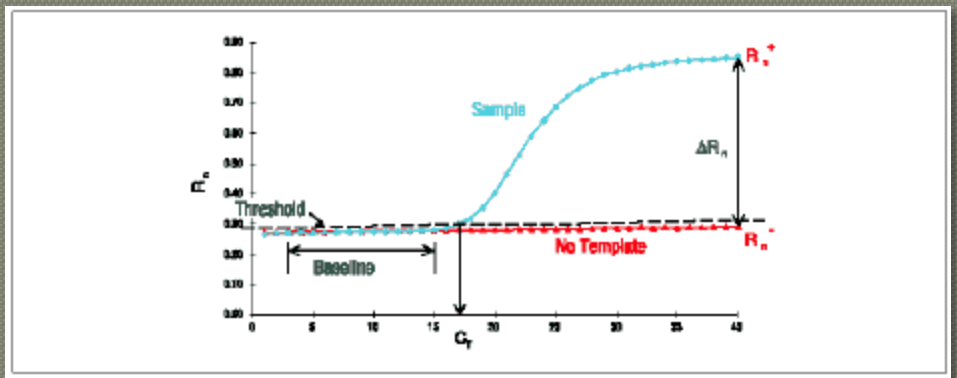


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNA/RNA Real-Time Quantitative PCR bulletin).

Real-Time PCR Instruments

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

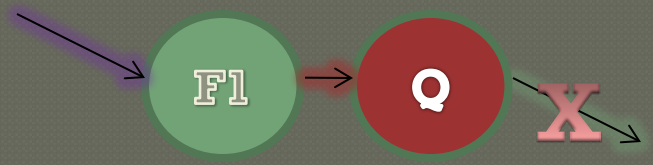
Real-time PCR Chemistries

- 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

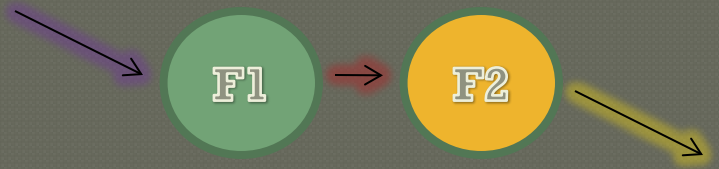
- Quenching
  - Fluorescence occurs when a photon bumps an electron to a higher energy level, then another photon is emitted when it drops back to ground state.
  - Some compounds ('quenchers') suck up that energy before it can be reemitted, 'quenching' the fluorescence.



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

Fluorescence Resonance Energy Transfer (FRET)

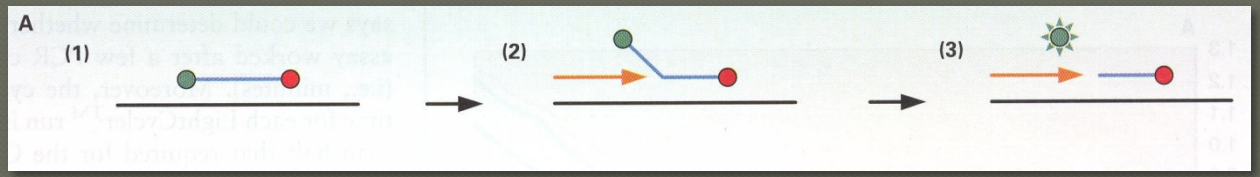
- A second fluorochrome can suck up the energy from the activated fluorochrome and re-emit it at its emission frequency.



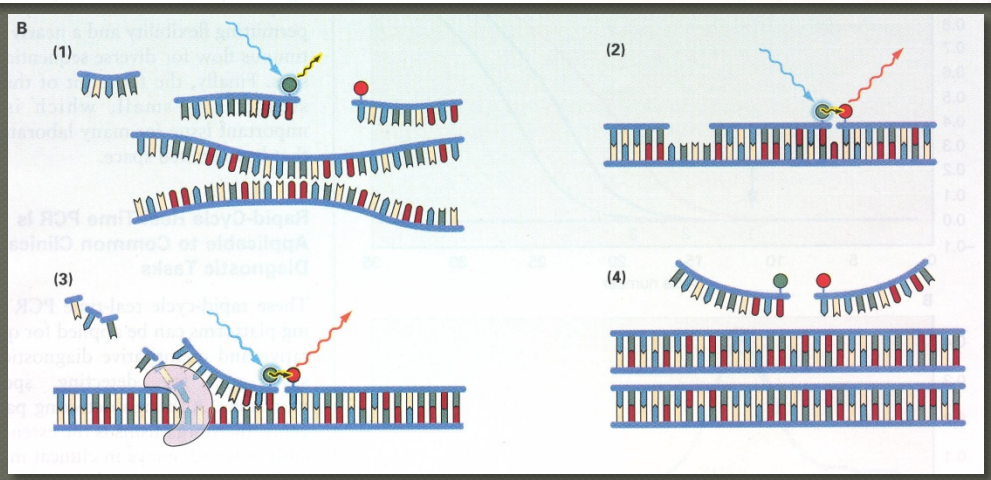
- This is distance dependant; the closer the molecules are the more efficient the energy transfer.

Real-Time Detection Schemes

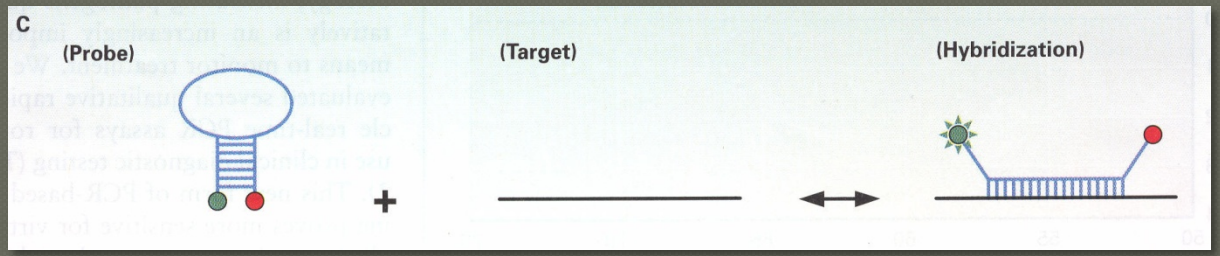
- Taqman Probes



- FRET Probes



- Molecular Beacons
- Several others



Contamination!

- 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 μm 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*

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.

What Will Be First?

- Things that're easy
  - MRSA, already on GeneExpert (arguably the first simple molecular platform)
- Things that're hot
  - Influenza and other respiratory viruses
- Things where existing tests perform poorly
  - Respiratory viruses in general
  - Group A strep
  - Group B strep
- Things for hard-to-reach populations
  - *Chlamydia* and gonorrhoea
  - Tuberculosis and other diseases in poor parts of the world.

What Will a Molecular POC Test Look Like?

- 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

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

Molecular Testing for Influenza

- Real-time methods can provide result in ~1h or so.
- Molecular methods as a class exceed culture in sensitivity (probably due to viral loss in transport)
- Detection properties do vary from system to system – do your homework!
- Moderately to very expensive equipment
- Moderate to high complexity (no CLIA-waived tests yet).

- Now clearly the 'gold standard'
- Information sources:
  - http://www.cdc.gov/flu/pdf/professionals/diagnosis/table1-molecular-assays.pdf
  - CAP Website for some price information
  - Manufacturer's web sites and PubMed for pictures, workflow and other information.

FDA-approved Molecular Influenza Tests

- Cepheid Xpert Flu Assay
- eSensor Respiratory Viral Panel
- FilmArray Respiratory Panel
- Ibis PLEX-ID Flu (seems to be off the market)
- Iquum LIAT Influenza A/B Assay
- Prodesse PROFLU and PROFAST
- Quidel Molecular Influenza A+B Assay
- Qiagen Artus Influenza A/B Rotor-gene RT-PCR kit
- Simplexa Flu A/B & RSV and Flu A/B & RSV Direct and Influenza A H1N1 (2009)
- Verigene Respiratory Virus Nucleic Acid Test and RV+ Test
- X-TAG Respiratory Viral Panel and RVP-FAST

More on the way!!

Cepheid Xpert Flu Assay

- From Cepheid
- Detects Flu A and B; discriminates 2009 H1N1.
- Approved for nasopharyngeal swabs, nasal aspirates, and nasal washes.
- Moderately complex
- List price ~$50/cartridge, instruments $24,900–$174,400 depending on capacity
- Sample to answer ~1h

Xpert Flu Workflow

1

Transfer 300µl of prepared sample into the large hole

2

Dispense binding reagent into small hole

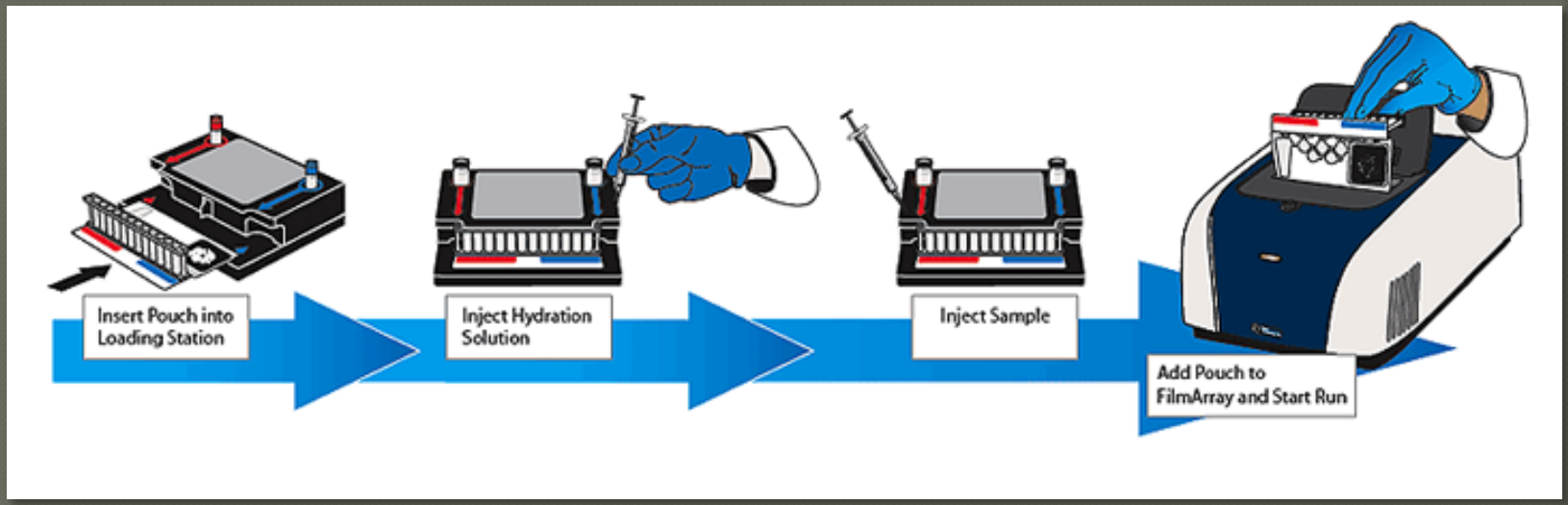
3

Insert cartridge and start assay

FilmArray Respiratory Panel

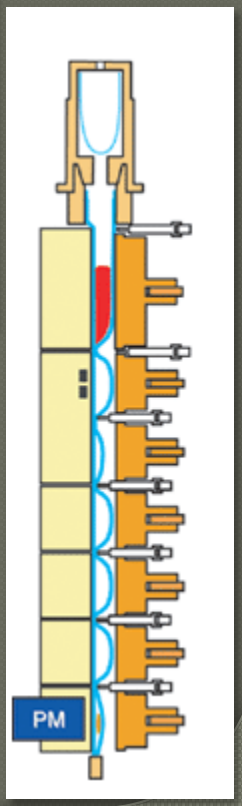
- From: Biofire, in the process of being acquired by BioMerieux
- Detects: Influenza A and B (discriminates H1, H3, 2009 H1) Respiratory Syncytial Virus, Parainfluenza 1, 2, 3 and 4 virus, Human Metapneumovirus, Rhinovirus/Enterovirus, Adenovirus, 4 Coronavirus variants, *Bordetella pertussis*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*
- Approved for NP swabs
- Moderately complex
- List price: $129/sample; instruments $39,500 each
- Sample to answer ~1h

Filmarray Workflow

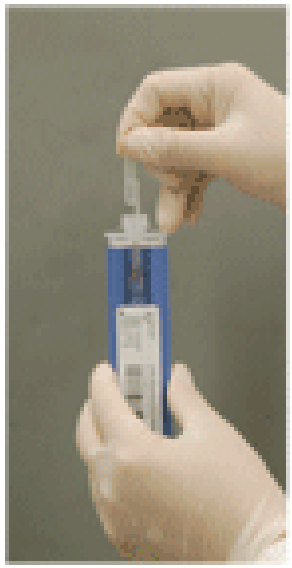


Iquum LIAT Influenza A/B Assay

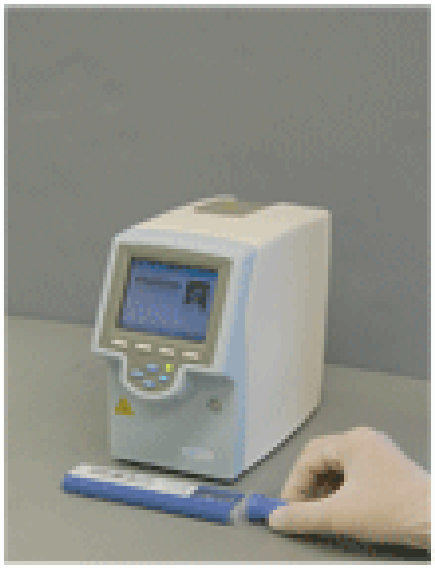
- From Iquum (recently acquired by Roche); LIAT stands for Lab-In-A-Tube
- Detects Influenza A&B
- Approved for NP swabs
- Moderately complex
- List price N/A
- Sample to answer .5h



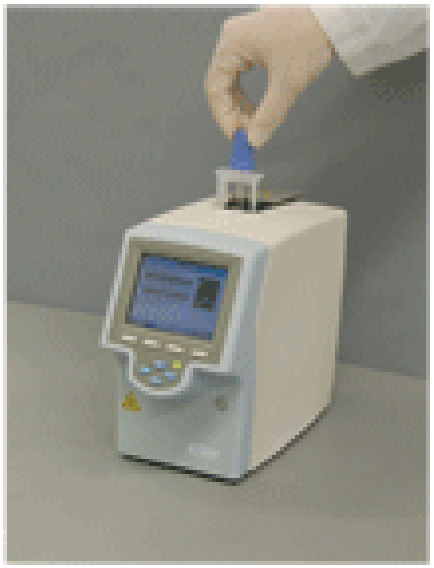
LIAT Workflow



STEP 1.
Add sample



STEP 2.
Scan barcode



STEP 3.
Insert tube

Done!
Results in 20 minutes

Simplexa Flu A/B & RSV and Flu A/B & RSV Direct and Influenza A H1N1 (2009)

- From Focus Diagnostics / 3M
- Detects Influenza A&B and RSV; a separate test discriminates 2009 H1N1
- Approved for NP Swabs
- Highly complex (Direct version is Moderately complex)
- List price: $49 reagents, requires Focus/3M Cycler
- Sample to answer ~4h, ~2h for Direct

Verigene Respiratory Virus Nucleic Acid Test and RV+ Test

- From Nanosphere
- Detects Influenza A & B, RSV A&B, Plus version discriminates H1, H3, and 2009 H1N1
- Approved for NP swabs
- Moderately complex
- List price $70 reagents, instruments N/A
- Sample to answer 3.5h

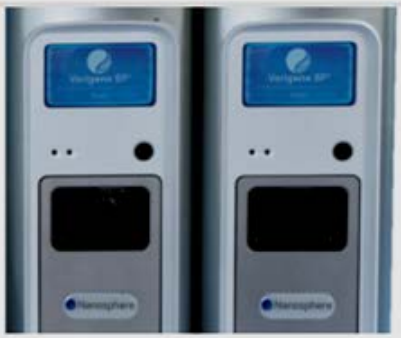
Verigene RV / RV+ Workflow

STEP 1

Load Test Cartridge, test consumables, and sample into Processor *SP*

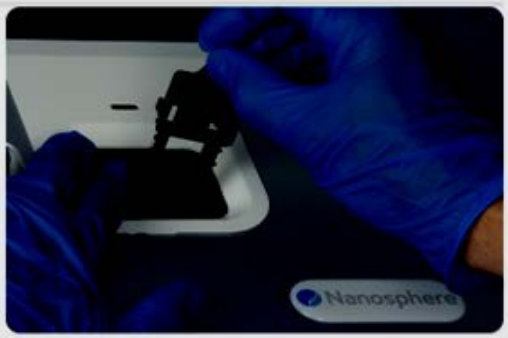
STEP 2

Automated sample preparation and test processing on Processor *SP*



STEP 3

Place slide from Test Cartridge in Verigene Reader for results



Not All Molecular Tests Are The Same

- Numerous, rather confusing studies; I picked one simple example.
- Don't take this as a comprehensive assessment of both assays; neither performed as well as the authors' homebrew RT-PCR.

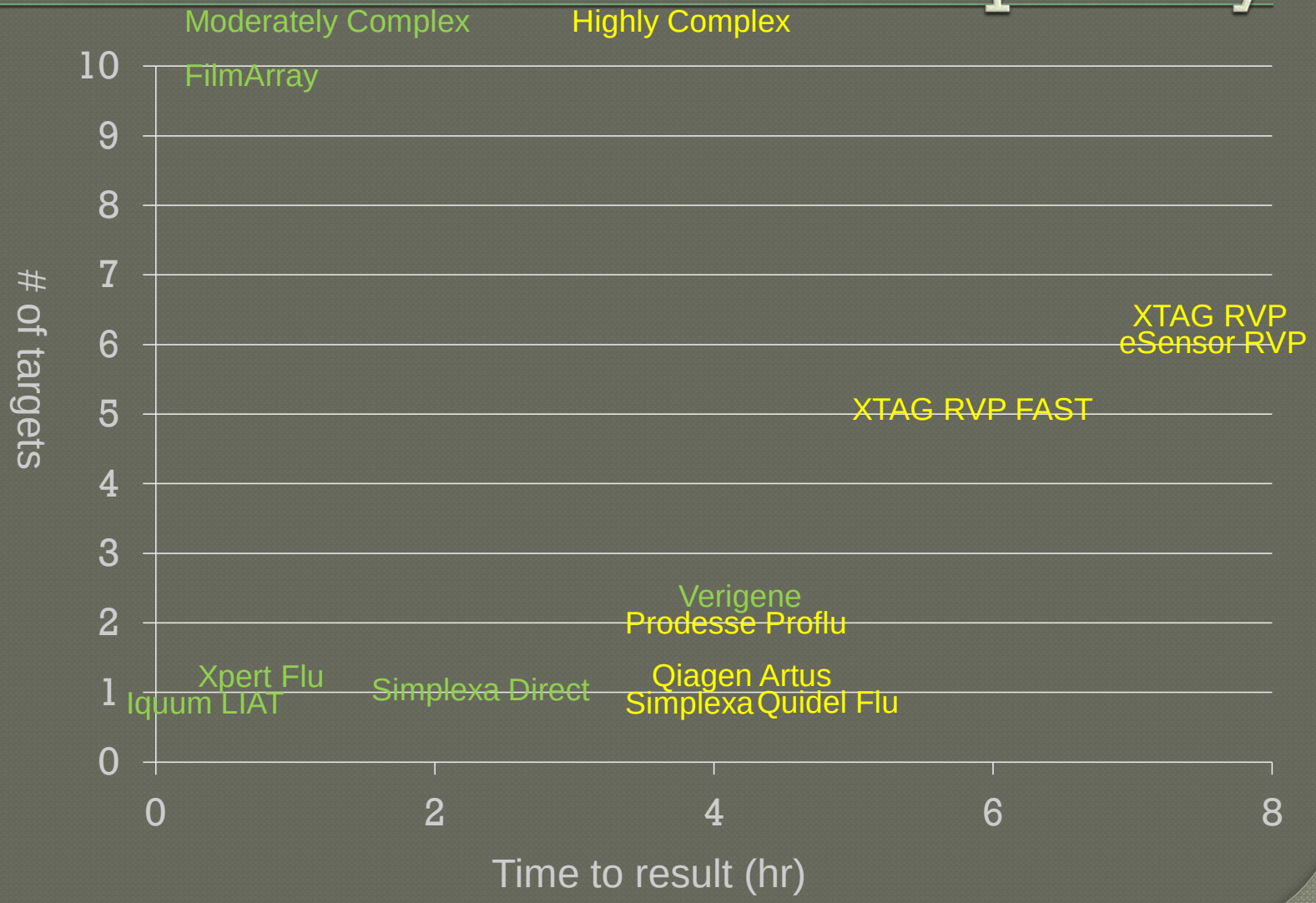
TABLE 1

Sensitivity of the Verigene RV+ test and the Simplexa Flu A/B & RSV kit by virus (n = 350)

Test	% Sensitivity for[a]:		
	Influenza A virus	Influenza B virus	RSV
Verigene RV+	96.6 (56/58)	100 (21/21)	100 (93/93)
Simplexa	82.8 (48/58)	76.2 (16/21)	94.6 (88/93)

Comparative Evaluation of the Nanosphere Verigene RV+ Assay and the Simplexa Flu A/B & RSV Kit for Detection of Influenza and Respiratory Syncytial Viruses; Kevin Alby, Elena B. Popowitch and Melissa B. Miller, J. Clin. Microbiol. January 2013 vol. 51 no. 1 352-353

Speed and Multiplexing and Complexity



Parsing Throughput

- The lower-complexity tests can typically test just one sample per module; throughput is then limited by number of modules and time per test.
  - Unfortunately, flu testing tends to be low-volume except during the season, when the volume expands hugely.
- Higher-complexity tests often done in batches of 24 or more depending on the number of targets and the capacity of the real-time instrument; potential for higher throughput.
- Economies of scale can make higher-complexity tests have less labor per sample if done in high volume.

Parsing Cost

- Cost per test depends on reagent + instrumentation + labor.
  - How many single-test modules do you need?
- Make sure to count in instrumentation for extraction, if needed.
- Reimbursement is a moving target; ask an expert.
- Potential for savings elsewhere in the system, if your bean-counters are sophisticated.

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

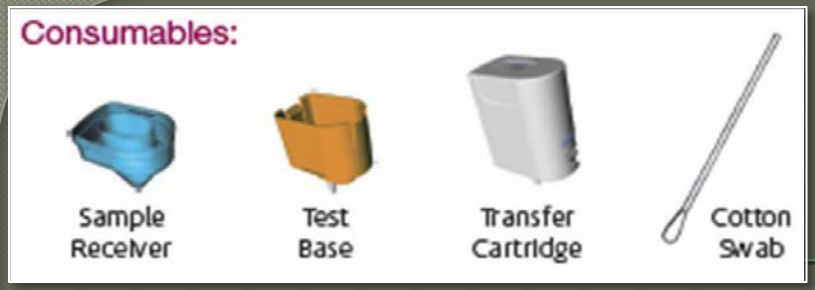
When Will They Be Waived?

- 1,200 hours per waiver application
- FDA expects each manufacturer will spend 2,800 hours creating and maintaining the record of the application
- $350,000 = total operating and maintenance cost associated with a waiver application (specimen collection, lab supplies, reference testing, shipping, instructional materials, study oversight)

Federal Register, vol. **78**, April 19, 2013.

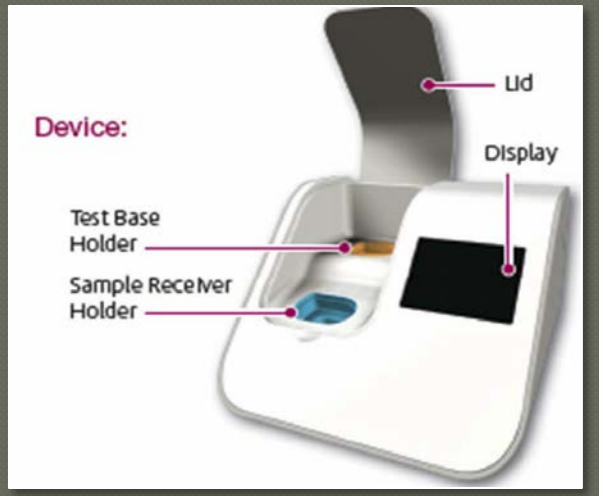
Alere I Influenza A and B

- Recently approved (6/16/2014)
- CLIA Waived; 15 min to result



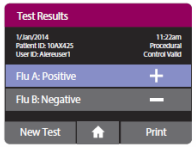
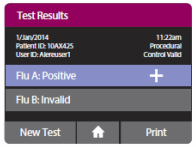
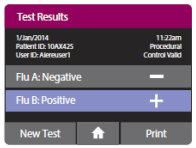
Alere I Workflow

- Bring supplies to room temperature.
- Put test base and sample receiver on instrument; allow to warm.
- Place swab in sample receiver, mix.
- Apply transfer cartridge to sample receiver.
- Move transfer cartridge to test base.
- Close lid; test runs 10 minutes.



RESULT INTERPRETATION

When the test is complete, the results are clearly displayed on the instrument screen. An individual result for both influenza A and influenza B will be provided.

Instrument Display	Interpretation of Results and Follow-up Actions
	**Flu A Viral RNA Detected; Flu B Viral RNA Not Detected.** This result does not rule out co-infections with other pathogens or identify any specific influenza A virus subtype.
	**Flu A Viral RNA Detected; The presence or absence of Flu B Viral RNA cannot be determined.** This result does not rule out co-infections with other pathogens or identify any specific influenza A virus subtype.
	**Flu B Viral RNA Detected; Flu A Viral RNA Not Detected.** This result does not rule out co-infections with other pathogens or identify any specific influenza B virus lineage.

Future Developments

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

Where are we going?

- I've thought about this a lot.
- Derived Campbell's Laws of POCT
- Two Laws, with inpatient and outpatient corollaries
  - Feedback encouraged.

Campbell's First Law of POCT

- Nobody ever went into Nursing because they wanted to do lab tests.
  - I can't document this with a literature citation, but it has high face-validity.
  - Anecdotally, our nurses/docs have hated glucose monitoring (still done but loathed), ER troponins (tried, failed), and rapid HIV (tried, failed).

Campbell's Second Law of POCT

- No POC test is easier than checking one more box on the laboratory order form.
  - Waived tests are easy, but much, much harder than checking one more box on a form you already filled out.
  - A lot of simple, rapid tests end up being *done in the lab*.

Campbell's Laws Example: Primary Care HIV Testing

- **June 8, 2010: Provider A:** "Sheldon, has rapid testing been considered to prevent this problem? Would this be feasible? Might allow us to expand testing to highest yield sites (i.e. the ER)…"
- **July-October 2010:** Set up program, created templated progress notes, ordered kits, trained 20+ Primary Care providers to do rapid HIV tests.
- **October 2010-January 2011:** Number of rapid HIV tests performed: **1**
- **January 2011: Provider B:** "Even though I am one of the biggest proponents, I have only done one, and that was for another provider who didn't know how to do it. I don't see people clamoring to do the test. I'm interested in Provider A's thoughts."
- **Response, Provider A:** "We have had very little use in <our clinic>. I think that it's so easy to send the pt for bloodwork that there is not much demand."
- **January 7, 2011, POCC:** "Next week I will be coming around to the Primary Care areas to collect the HIV kits. Please have them easily accessible. Thank you and have a pleasant weekend."

Campbell's Laws: Inpatient Corollaries

- An inpatient POC test is useful only if:
  - The time for transport to the lab for THAT SINGLE ANALYTE significantly and negatively impacts care, OR
  - The test is performed on an easily-obtained sample (e.g. fingerstick blood) more frequently than routine blood draws are obtained.

Campbell's Laws: Outpatient Corollaries

- An outpatient POC test is useful only if:
  - The test result is available during the patient visit AND a decision can be made or action taken on the basis of it without waiting for other lab results, OR
  - If you can make money doing it.

Campbell's Outreach / Developing-World Corollaries

- Sometime's there's no lab-order form.
- Sometimes there's no nurse.
- Sometimes there's no refrigeration, power, or lights.
- Campbell's Laws should not be applied outside of a healthcare environment where the basic terms apply.

Recommendation

- "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 21st Century: Keeping the Pace". American Academy of Microbiology, 2008. Available on-line at: http://www.asm.org/academy/index.asp?bid=58445

Acknowledgements

- FDA waiver requirements from a slide provided by Dr. Barbara Robinson-Dunn.