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.
  - 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

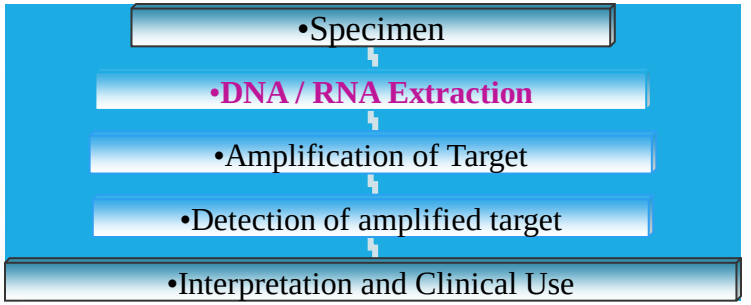
Why Amplify, continued

- 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?

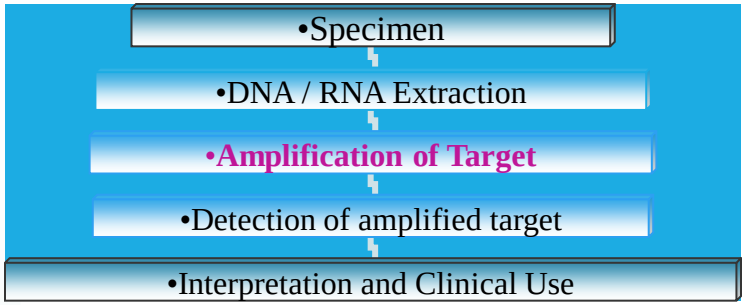
- Clinical significance?
- Technical problems
  - Contamination
  - Inhibition
- Cost
- COST
- CO$T

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

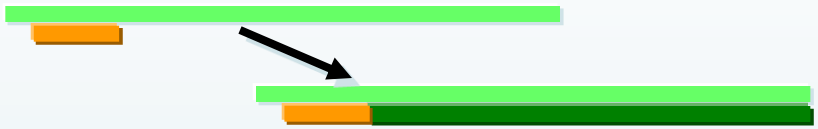
Amplification



- 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!

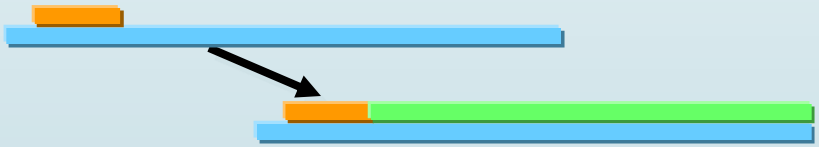
Amplification Enzymology

- DNA polymerase
  - makes DNA from ssDNA, requires priming

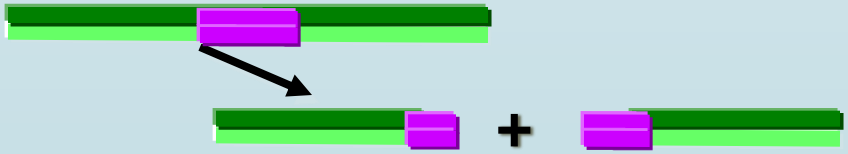


- RNA polymerase
  - makes RNA from dsDNA, requires specific start site

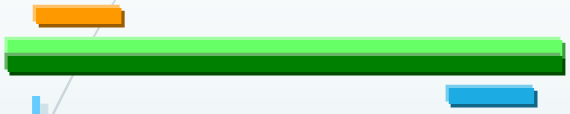
- Reverse transcriptase
  - makes DNA from RNA, requires priming



- Restriction endonucleases
  - cut DNA in a sequence specific manner



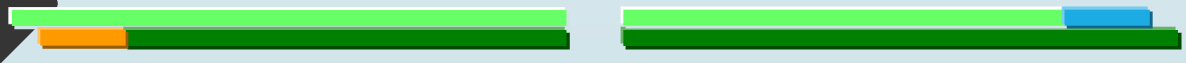
Polymerase Chain Reaction (PCR)



Target DNA
+
Primer oligonucleotides (present in excess)

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

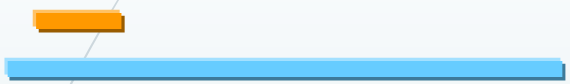
DNA polymerase extends the primers (40-80°C) to produce two new double-stranded molecules



Repeat the split-bind-extend cycle

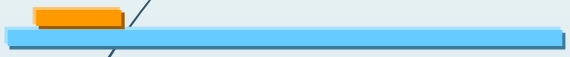
This 'short product' amplifies exponentially in subsequent split-bind-extend cycles, driven by the temperature changes in a 'thermal cycler'.

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

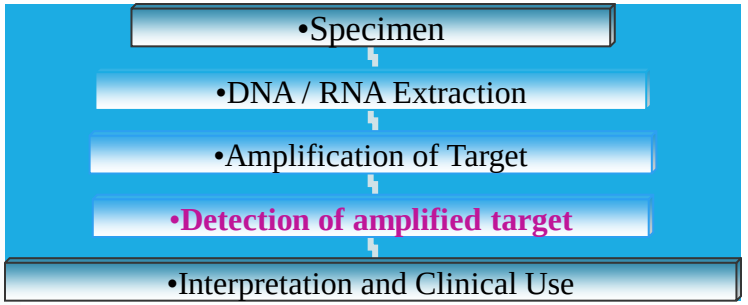
The DNA copy is used in a PCR reaction

PCR

Other Amplification Methods

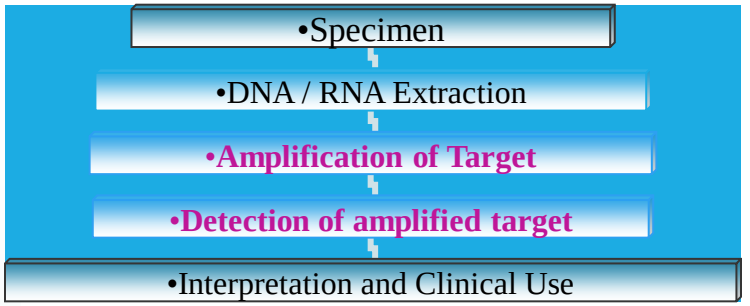
- PCR isn't all there is!
  - Transcription-mediated amplification (TMA)
  - Loop-mediated isothermal AMPlification (LAMP)
  - Others
  - Isothermal technologies decrease the complexity of the instrument required.

Detecting PCR Products in the Old Days



- 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.
- Frequently use PCR for amplification
  - Robust
  - Off-patent

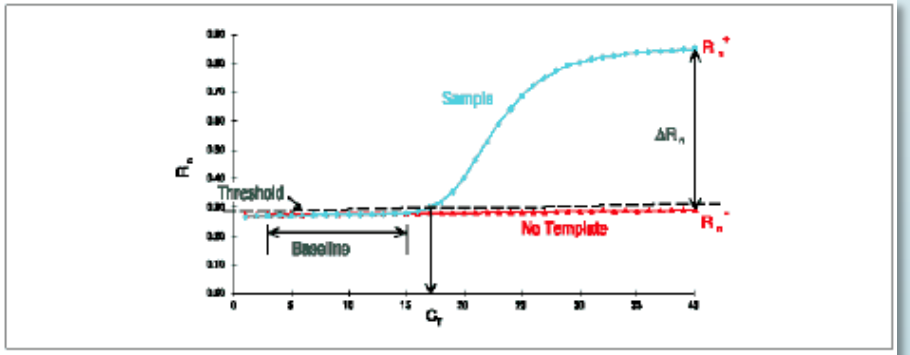


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNA/RNA Real-Time Quantitative PCR bulletin).

Real-Time PCR Instruments

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

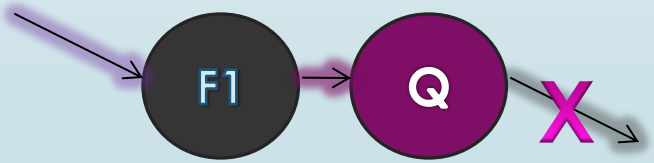
Real-time PCR Chemistries

- 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

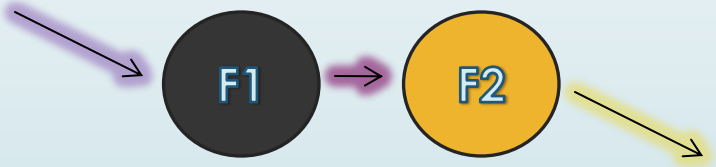
- Quenching
  - Fluorescence occurs when a photon bumps an electron to a higher energy level, then another photon is emitted when it drops back to ground state.
  - Some compounds ('quenchers') suck up that energy before it can be reemitted, 'quenching' the fluorescence.



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

Fluorescence Resonance Energy Transfer (FRET)

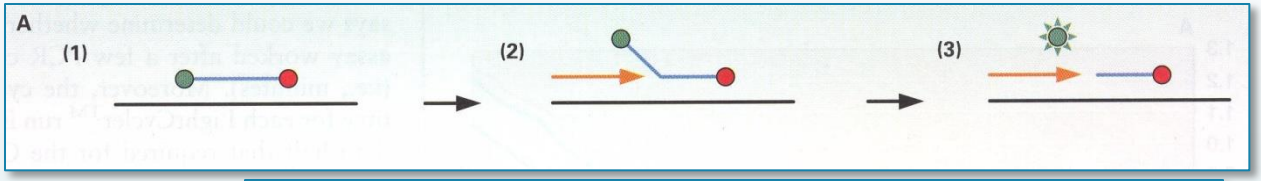
- A second fluorochrome can suck up the energy from the activated fluorochrome and re-emit it at its emission frequency.



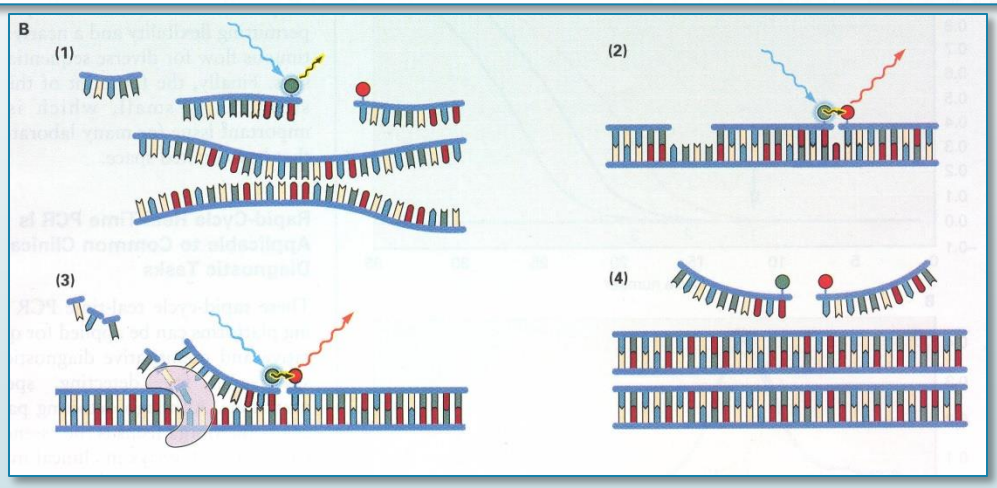
- This is distance dependant; the closer the molecules are the more efficient the energy transfer.

Real-Time Detection Schemes

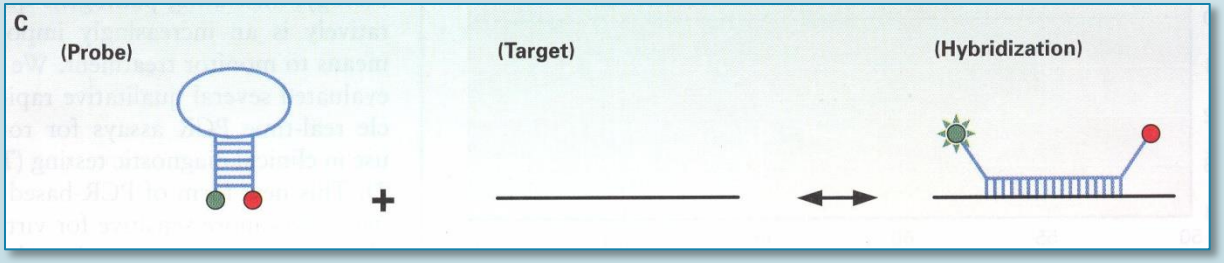
- Taqman Probes



- 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
    - GI pathogens
  - 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 FDA-approved form of moderate complexity or waived. The rest are in active development.

What's 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

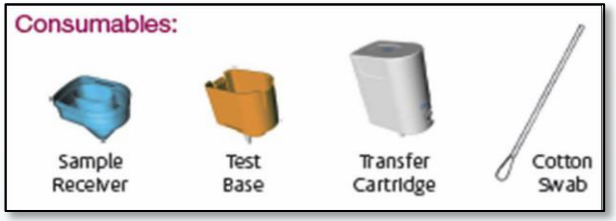
Molecular Testing for Influenza

- Real-time methods can provide result in <1h.
- 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
- Multiple methods of waived to high complexity.
- 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.

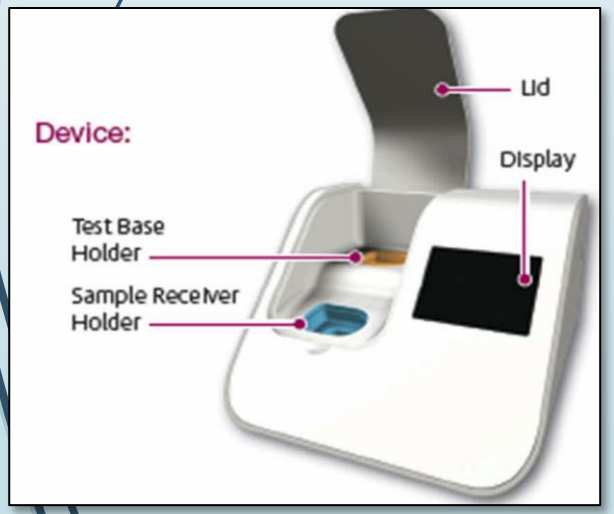
FDA-approved Molecular Influenza Tests

- Waived complexity
  - Alere i Influenza A and B
  - Roche LIAT Influenza A/B Assay
- Moderate or High complexity.
  - Cepheid Xpert Flu Assay
  - eSensor Respiratory Viral Panel
  - FilmArray Respiratory Panel
  - 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

Alere I Influenza A&B

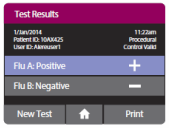
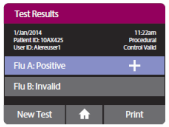
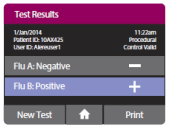


- CLIA-waived
  - Bring supplies to room temperature.
  - Put test base and sample receiver on instrument; allow to warm.
  - Place swab in sample receiver, mix.
  - Apply transfer cartridge to sample receiver.
  - Move transfer cartridge to test base.
  - Close lid; test runs 10 minutes.



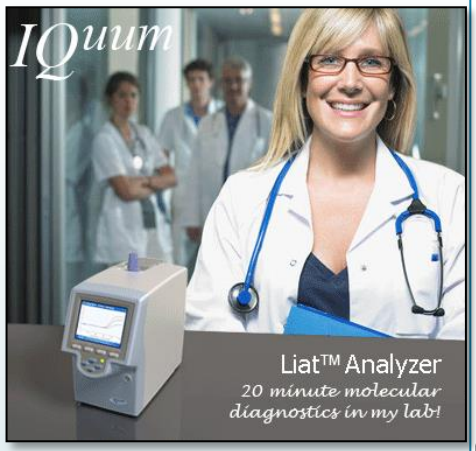
RESULT INTERPRETATION

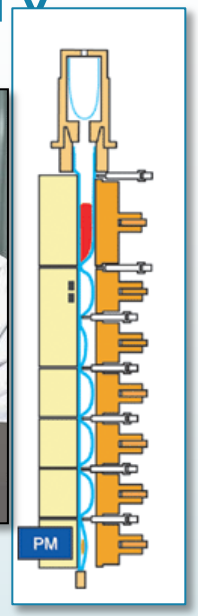
When the test is complete, the results are clearly displayed on the instrument screen. An individual result for both influenza A and influenza B will be provided.

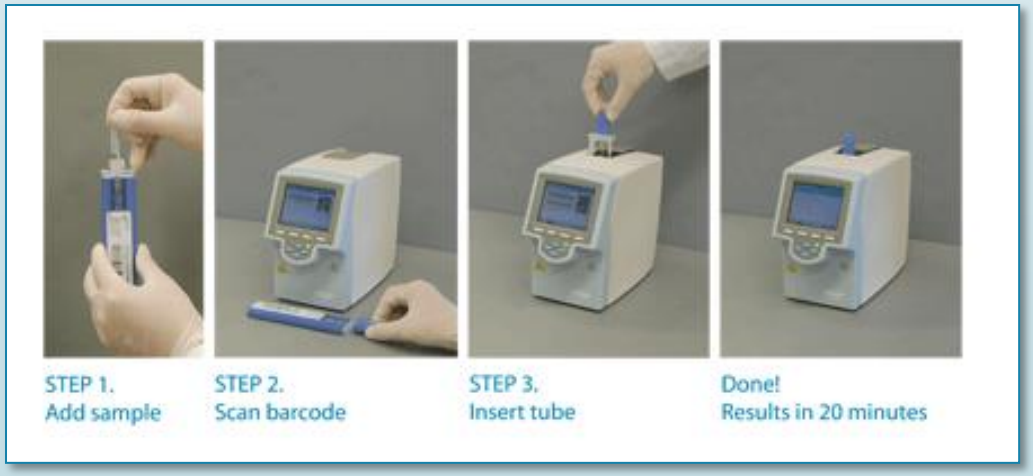
Instrument Display	Interpretation of Results and Follow-up Actions
	**Flu A Viral RNA Detected; Flu B Viral RNA Not Detected.** This result does not rule out co-infections with other pathogens or identify any specific influenza A virus subtype.
	**Flu A Viral RNA Detected; The presence or absence of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

Roche LIAT Influenza A/B Assay

- CLIA waived
- LIAT stands for Lab-In-A-Tube
- Detects Influenza A&B
- Sample to answer .5h



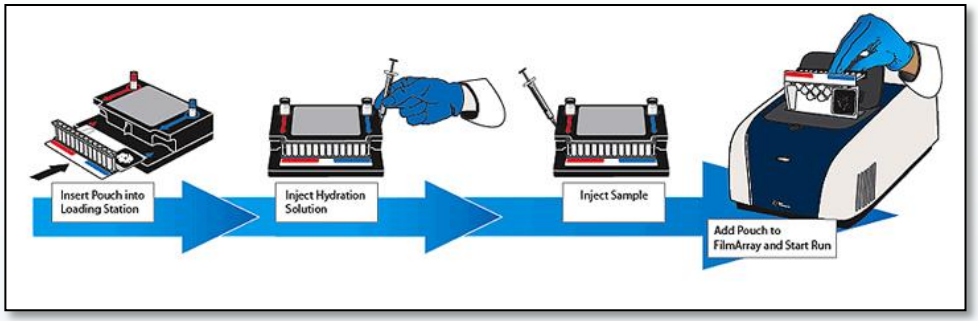




Cepheid Xpert Flu Assay

- Moderately complex
- Detects Flu A and B; discriminates 2009 H1N1.
- Flu + RSV cartridge available
- Sample to answer ~1h
- GeneXpert Xpress waived in 12/2015

FilmArray Respiratory Panel



- Moderately complex
  - Working toward waived
  - From: Biofire (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*
- Sample to answer ~1h

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

- Highly complex (Direct version is Moderately complex)
- From Focus Diagnostics / 3M
- Detects Influenza A&B and RSV; a separate test discriminates 2009 H1N1
- Sample to answer ~4h, ~2h for Direct

Verigene Respiratory Virus Nucleic Acid Test and RV+ Test

- Moderately complex
- From Nanosphere
- Detects Influenza A & B, RSV A&B, Plus version discriminates H1, H3, and 2009 H1N1
- Approved for NP swabs
- Sample to answer 3.5h

Are All Molecular Tests The Same?

- Of course not. That would be too simple.
- Numerous, rather confusing studies.
  - There are few comparisons of multiple methods. Sorry.
  - Don't take this as a comprehensive assessment of both assays; neither performed as well as the authors' homebrew RT-PCR.
- Performance DOES vary within the molecular tests.
- Pay attention not only to sensitivity / specificity numbers, but also to comparator method.
  - Comparisons with culture make a method look better; comparisons with a highly optimized molecular method or with a panel of different methods is a more stringent comparison.

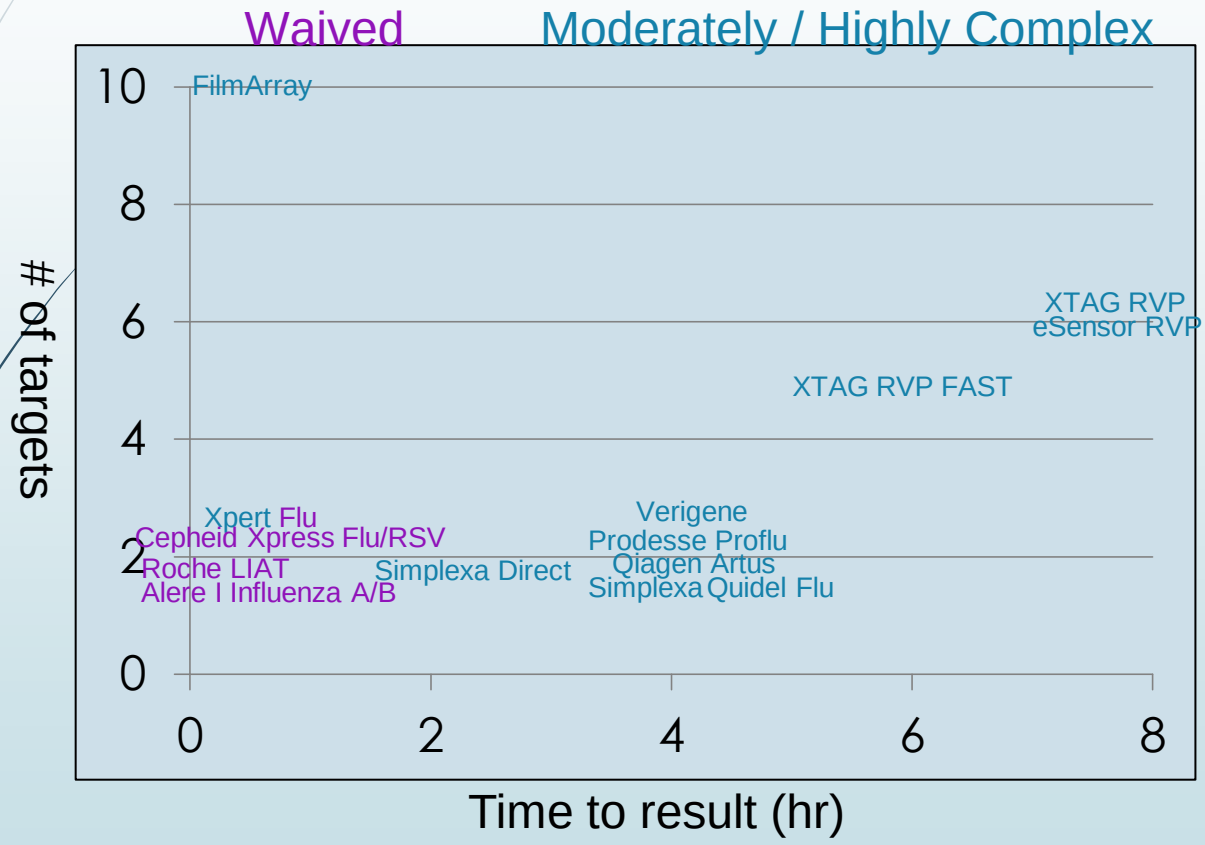
TABLE 1

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



Does it Make Sense to Test?

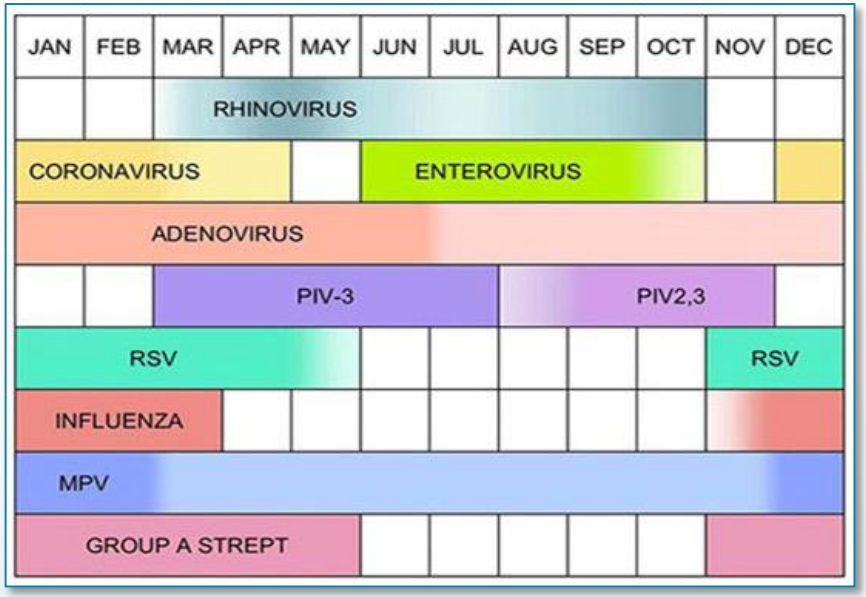
INFECTIOUS DISEASE/ORIGINAL RESEARCH

Cost-Utility of Rapid Polymerase Chain Reaction-Based Influenza Testing for High-Risk Emergency Department Patients

Andrea Freyer Dugas, MD; Sara Coleman, MPH, MBA; Charlotte A. Gaydos, DrPH, MPH; Richard E. Rothman, MD, PhD; Kevin D. Frick, PhD, MA

- Cost-effectiveness studies are tricky.
- Assuming a $50,000 per quality-adjusted life-year willingness-to-pay threshold, the most cost-effective treatment option is treatment according to provider judgment from 0% to 3% prevalence, treatment according to a PCR-based rapid influenza test from 3% to 7% prevalence, and treating all at greater than 7% prevalence.
  - …but this ignored induction of antiviral resistance, transmission of flu, and cost avoidance in tested patients; only treatment cost and effect was counted.
  - "Patients who did not have influenza were not evaluated further because influenza testing or treatment would have no further effect on their care or outcomes."
  - Ann Emerg Med. 2013;62:80-88

When to test?



- Remember – false-positives have potentially severe consequences, e.g. non-treatment of a serious bacterial infection.
- Test during the flu season.
  - This is the conventional wisdom, to be modified in travelers and people with contacts who are travelers. Note that other viruses don't have influenza's striking seasonality.
  - Molecular tests may have higher specificity than the old antigen tests, but still; question off-season positives.
- Potential strategies:
  - Seasonal: test Oct-Dec→March or so.
    - Early season – retain specimen for confirmatory testing!
  - Incidence-based testing – monitor regional influenza per CDC and State systems, begin testing only when influenza reported in the area.
- Remind providers to test *early in illness*; the best therapeutic results are when drugs are started within 48h of onset.

Who to Test?

- Expensive molecular flu tests may be best deployed selectively.
- Consider testing:
  - Patients destined for hospital admission.
  - Compromised patients at high risk likely to benefit from treatment.
- Consider not testing:
  - Otherwise healthy people who probably don't need anything but reassurance and good hydration.
- Remember that influenza and bacteria can and often do co-infect.
  - Really sick patients may have a bacterial superinfection facilitated by the virus.

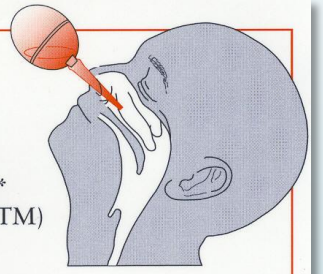
(Potential) Benefits of Flu Testing

- For positives…
  - Rapid treatment.
  - Avoidance of antibiotics and costs and complications thereof.
    - We all know what a large fraction of antibiotics are used for viral infections.
  - Avoidance of further workup / admission in some cases.
    - How much will test impact this versus clinical condition of the patient?
  - Infection control – inpatient and outpatient.
  - Patient flow in outpatient settings:
    - diagnosis – disposition/treatment – onward.
  - **All these depend on a result provided within the encounter time or shortly thereafter.**
- For negatives…
  - Save cost of antiviral therapy.
  - Save isolation cost / inconvenience
  - Continue diagnostic workup if patient's condition warrants it.

Influenza Specimen Collection

- Specimen collection is probably ***the*** critical step in influenza testing
  - **Good test on a bad specimen = bad test**

Nasopharyngeal Wash: Bulb Method

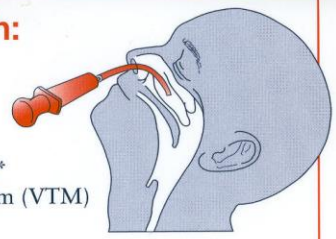


Materials: Saline
1-2 oz. tapered rubber bulb*
Viral Transport Medium (VTM)
Specimen container

1. Suction 3-5 ml saline into a new sterile bulb.
2. Insert bulb into one nostril until nostril is occluded.
3. Instill saline into nostril with one squeeze of the bulb and immediately release bulb to collect recoverable nasal specimen.
4. Empty bulb into suitable dry, sterile specimen container or one containing VTM, according to virology laboratory requirements.

* Length and diameter of bulb as appropriate for infant, child or adult.

Nasopharyngeal Wash: Syringe Method



Materials: Saline
3-5 ml syringe*
2" 18-20 gauge tubing*
Viral Transport Medium (VTM)
Specimen container

1. Fill syringe with saline; attach tubing to syringe tip.
2. Quickly instill saline into nostril.

3a. Aspirate the recoverable nasal specimen. Recovery must occur immediately, as the instilled fluid will rapidly drain.

3b. *(Alternate)* In appropriate cases, patients may tilt head forward to allow specimen to drain into suitable sterile container.

4. *(If aspirated)* Inject aspirated specimen from syringe into suitable dry, sterile specimen container or one containing VTM, according to virology laboratory requirements.

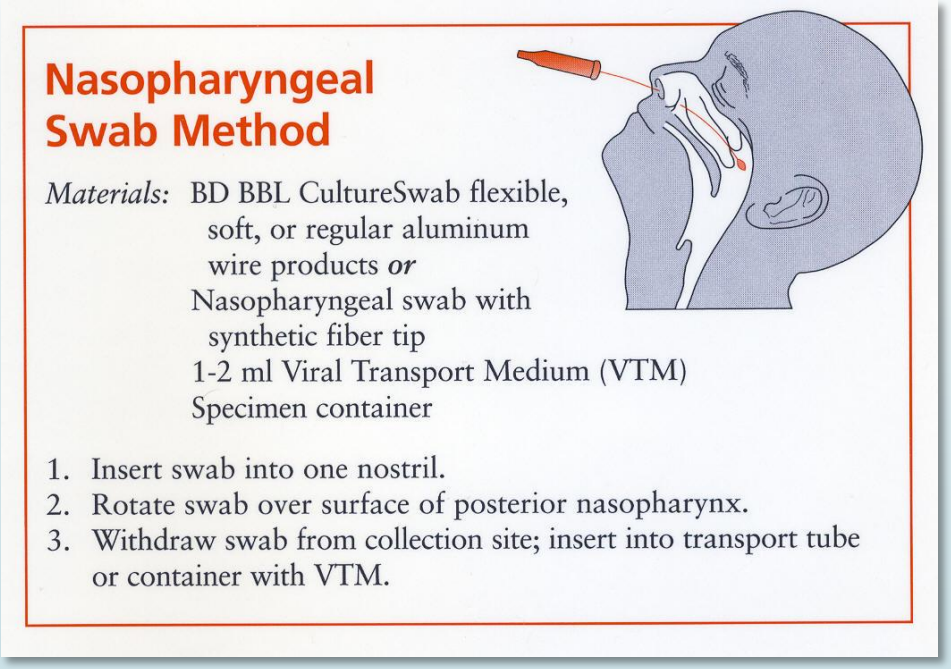
* Length and diameter of syringe and tubing as appropriate for infant, child or adult.

Washes are somewhat better than swabs*

*A general but not-quite universal rule of microbiology: swabs are evil

Specimen Collection – The NP Swab

- NOT A THROAT SWAB. NOT A NASAL SWAB. A NASOPHARYNGEAL SWAB.
- Important to get ciliated epithelial cells – this is a cell-associated virus
- Test early; more virus is shed early than later in disease.
  - A test a week after onset of symptoms is useless.
- Children shed more virus than adults
  - Tests tend to be more sensitive in kids



Polling question 4

Managing POC Molecular

- 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

- 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

Polling question