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Adoption of
Massively Parallel Real-time PCR
on a Nanofluidic Biochip
for Clinical Microbiology

Nick Antonishyn PhD, RMCCM
Saskatchewan Disease Control Laboratory

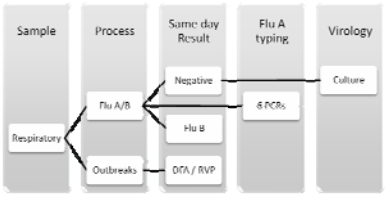
Outline

Why
Issues with algorithm-driven and multiplex testing.

What
Describe advances to massively parallel PCR approach.

How
Share results of SDCL's evaluations.

Rationalized State – Respiratory Screen



231 Consecutive Respiratory

- 85 concordant negatives
- 83 concordant positives
- 'Rationalized approach' missed 62 calls
  - missed 46 positives
  - missed 16 mixed infections

Nanofluidic Biochip 27% more calls

341 consecutive Cary-Blair stool samples

- 250 concordant negatives
- 25 concordant bacterial positives
- 'Rationalized approach' missed 59 calls
  - 62 enteric viruses

Nanofluidic Biochip 17% more calls

Pertussis/RSV example - 3 dept, 4 swabs, 14 days

Collected 2 swabs each - Jan 22 15:59					Final Report
	Jan 25 16:11	Jan 25 16:59	Jan 26 16:11	Feb 7 10:39	
10 year old brother	Neg Flu A	Indet Pert	Pos RSV	Pos Pert	**Pertussis/RSV**
	Neg Flu B	dropped swab	Day 2 - final	Day 14 - amend	
	Jan 25 16:11	Jan 25 17:00	Jan 26 12:09	Feb 4 12:03	
5 year old sister	Neg Flu A	Pos Pert	Neg Virology	Closed file	**Pertussis**
	Neg Flu B		Day 2 - final		

Issues with a stepwise approach

"Confirmation of another respiratory pathogen should not necessarily preclude testing for **MERS-CoV** in patients who develop fever and pneumonia or acute respiratory distress syndrome."

- September 26 issue of the Morbidity and Mortality Weekly Report

Issues with a stepwise approach

"The finding of human co-infection with **H7N9 and H3N2** viruses shows that human beings could act as mixing vessels for virus reassortment, which might facilitate human-to-human transmission. The public health and scientific communities **should enhance** surveillance for virus evolution."

- Lancet Vol 381 June 15, 2013

Issues with a Multiplexed approach

Detection of Influenza H7N9 Virus: All Molecular Tests Are Not Equal

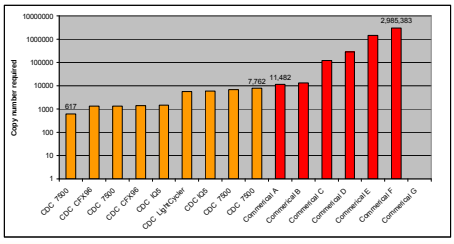
Todd F. Hatchette,[a,b] Steven J. Drews,[c,d] Nathalie Bastien,[e] Yan Li,[e] Gregory German,[f] Nick Antonishyn,[g] Hugues Charest,[h,i] Tony Mazzulli,[j,k] Kevin Fonseca,[l,d] Mel Krajden,[j,m] Martin Petric,[j,m] Kerry Dust,[n] Jason J. LeBlanc[a,b]

Division of Microbiology, Department of Pathology and Laboratory Medicine, Capital District Health Authority (CDHA), Halifax, Nova Scotia, Canada[a]; Department of Pathology, Dalhousie University, Halifax, Nova Scotia, Canada[b]; Provincial Laboratory for Public Health (ProvLab), Calgary, Alberta, Canada[c]; University of Calgary, Calgary, Alberta, Canada[d]; National Microbiology Laboratory (NML), Winnipeg, Manitoba, Canada[e]; Queen Elizabeth Hospital (QEH), Charlottetown, Prince Edward Island, Canada[f]; Saskatchewan Disease Control Lab (SDCL), Ministry of Health, Regina, Saskatchewan, Canada[g]; Laboratoire de Santé Publique du Québec (LSPQ)/Institut National de Santé Publique du Québec (INSPQ), Montréal, Québec, Canada[h]; Université de Montréal, Montréal, Québec, Canada[i]; Department of Microbiology, Mount Sinai Hospital and University Health Network, Toronto, Ontario, Canada[j]; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada[k]; British Columbia Centre for Disease Control (BCCDC), Vancouver, British Columbia, Canada[l]; University of British Columbia, Vancouver, British Columbia, Canada[m]; Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada[n]

The recent emergence of influenza A virus (H7N9) emphasizes the need for its rapid detection. While commercial nucleic acid amplification tests (NAATs) are commonly used to detect seasonal influenza virus, this study demonstrated that the analytical sensitivity of commercial assays is highly variable compared to that of CDC-based in-house NAATs for the detection of H7N9.

JCM 51(11):3835-3838 November 2013

Lab-developed Singleton Real-time PCR is better - H7N9 example



Issues with Molecular Diagnostics today

- Targeted testing = Opportunity for missed identification
- Expanded detection is expensive
- Movement toward 'Molecular Panels'
  - Lack sensitivity - still need singleton PCR for influenza A
  - Expensive - need to maintain multiple approaches
  - Slow to adopt
  - Typically slow to result – need same day

Issues with current options

"At this time, even with the most sophisticated devices available, physicians can only detect about **16** different viruses of approximately 30 or 40 that might be causing an illness."

- New Test Rapidly Distinguishes Viral, Bacterial Infections. Medscape. Sep 18, 2013.

Moving Molecular Diagnostics Forward

- Syndrome based instead of selective testing (40+ targets)
- Maintain sensitivity of current Flu & Norovirus screen
- Enable for rapid change (i.e. adapt to strains)
- Same day for outbreak investigations
- Scalable for surge capacity
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Fluidigm's NanoFlex Valves : Nanofluidic technology

"tens of thousands can be integrated into a dense network of channels for regulating solutions on a nanolitre scale"

- Gene Expression
- Single-Cell Genomics
- SNP Genotyping
- Targeted Resequencing
- Sample Quantitation
- Copy Number Variation

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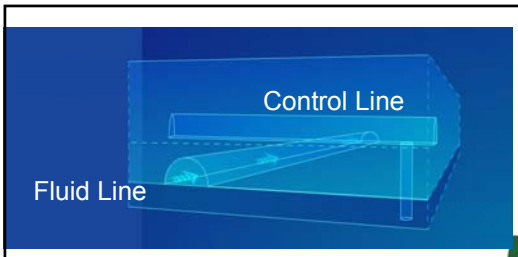
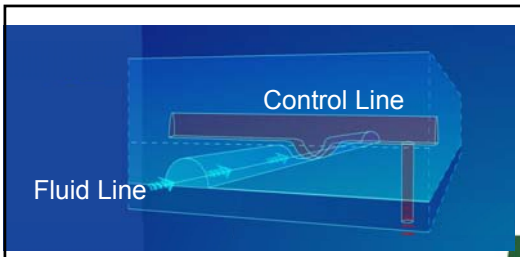
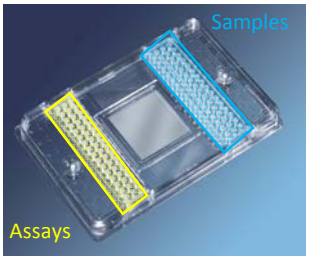
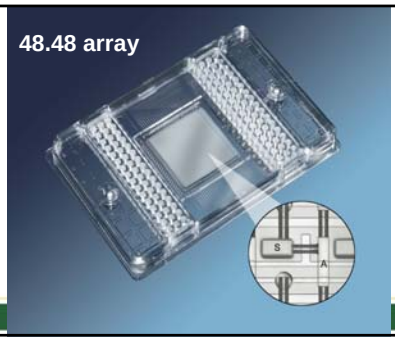
- **Clinical Microbiology**

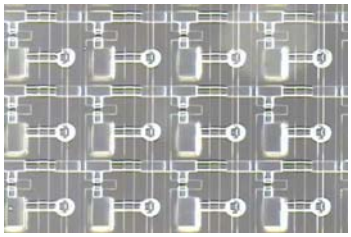




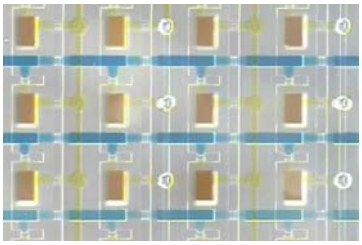


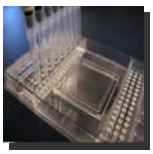


Fill



Mix



Pipette	Load	PCR
		
20 mins	60/90 mins	60/90 min

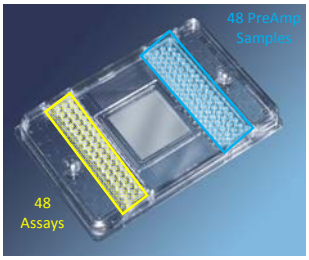
Pre-amplification Workflow

All primer mix (1:100 of assay strength) + Total NA + One-step RT-PCR Master mix

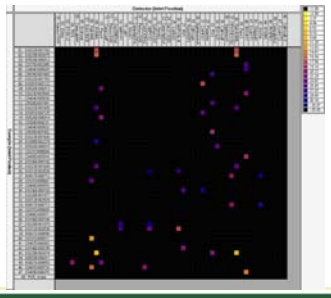
14 cycles

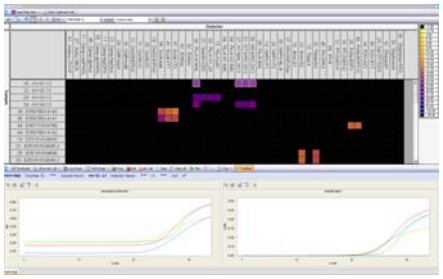
1:5 dilution

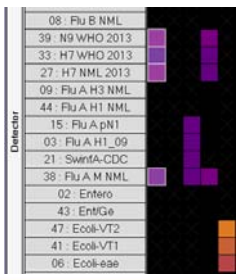
Pre-amplified template



Heat Map





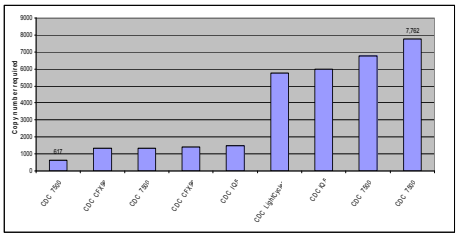


} **H7N9 data**

Easy Analysis

- 2,314 results to 'final call' with macros in excel
  - SDCL's results calculator
- Entire manual + automated calls into LIMS and certified within 15 minutes

Lab-developed Singleton Real-time PCR is better - H7N9 example



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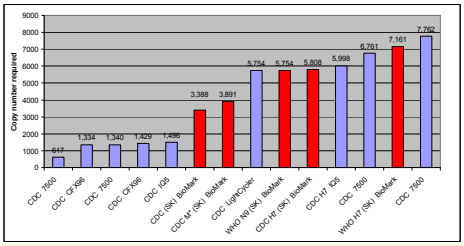
Nanofluidic Biochip in line with singleton real-time PCR



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Moving Molecular Diagnostics Forward

- ✓ **Syndrome based instead of selective testing (48+ targets)**
- Maintain sensitivity of current Flu & Norovirus screen
- Enable for rapid change (i.e. adapt to strains)
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Pertussis/RSV example Potential: **1 sample, 1 dept, 1 test, 1 report**

Collected 2 swabs each - Jan 22 15:59				Final Report	**BioMark SDCL**
10 year old brother	Jan 25 16:11 Neg Flu A Neg Flu B	Jan 25 16:59 Indet Pert dropped swab	Jan 26 16:11 RSV	Feb 7 10:39 Pertussis	**Day 1** **Pertussis/RSV**
5 year old sister	Jan 25 16:11 Neg Flu A Neg Flu B	Jan 25 17:00 Pertussis	Jan 26 12:09 Neg Virology	Feb 4 12:03 Closed file	**Day 1** **Pertussis/RSV**

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Study design

- Respiratory & Enteric targets
- LODs compared to singleton real-time PCR
- 159 respiratory samples for 28 defined outbreaks
  - 2 commercial panel kits, real-time PCR
- 231 consecutive respiratory samples with 12 defined outbreaks
  - positives confirmed by commercial kit and PCR
- 341 consecutive Cary-Blair stool samples
  - 2 commercial panel kits, real-time PCR, Culture
- 30 consecutive unpreserved stool samples
  - positives confirmed by PCR and Culture

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Results – Unpreserved stool

Norovirus real-time PCR

- 16 positives

BioMark SDCL

- 16 positives for Norovirus
- 2 positives for bacteria
  - 1 *Shigella*
  - 1 *Campylobacter*

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Results – 341 consecutive Cary-Blair stools

2 Commercial kits, Fluidigm & 7500 real-time PCR

Norovirus	Sensitivity	Specificity
BioMark SDCL	**95.7%**	**100%**
GPP	**95.8%**	**95.9%**
SeeGene	**95.8%**	**99.1%**

BioMark SDCL Performance Verification

Target	Sensitivity	Specificity	LR+	LR-
All Respiratory	96%	98%	50	0.038
Flu A	100%	100%	Perfect	Perfect
Norovirus	100%	100%	Perfect	Perfect

LR+ >10 means a positive result has a significant contribution to the diagnosis

LR- <0.1 means a negative result has a significant contribution of ruling out the target(s) as causing disease

LOD - Weakest dilution detected

	Singleton 7500	**BioMark**
44 assays		
Average of exponents*	**3.93**	**3.70**
40 assays		
Average of exponents*	**3.87**	**3.95**

*higher is better

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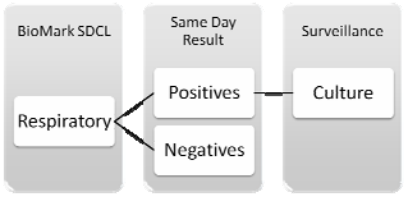
Results – 159 Respiratory Outbreak Samples

Targeted Organism	% Sensitivity			% Specificity			Likelihood ratio (LR+,LR-)		
	BioMark SDCL	RVP	RV16	BioMark SDCL	RVP	RV16	BioMark SDCL	RVP	RV16
All organisms	96	94	100	98	100	85	50, 0.038	(Perfect, 0.056	7, Perfect)
Influenza A	100	96	100	100	100	100	(Perfect, Perfect)	(Perfect, 0.040	(Perfect, Perfect)
Influenza B	100	80	100	100	100	100	(Perfect, Perfect)	(Perfect, 0.200	(Perfect, Perfect)
Metapneumovirus	100	100	100	100	100	100	(Perfect, Perfect)	(Perfect, 0.000	(Perfect, Perfect)
Parainfluenza	92	77	100	100	100	98	(Perfect, 0.077	(Perfect, 0.231	49, Perfect)
RSV	100	83	100	100	100	99	(Perfect, Perfect)	(Perfect, 0.167	153, Perfect)
Enteroviruses/Rhinovirus	91	100	100	99	100	99	135, 0.092	(Perfect, 0.000	148, Perfect)
Coronavirus	95	98	100	100	100	97	(Perfect, 0.050	(Perfect, 0.025	40, Perfect)

LR+ <10 means a positive has NO significant contribution to the diagnosis

LR- >0.1 means a negative result has NO significant contribution of ruling out the target(s) as causing disease

SDCL Current State – Molecular Diagnostics



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  - ✓ **Treat all samples like outbreak samples**
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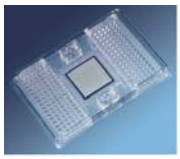
48+ targets with less reagent than 1 singleton real-time PCR

48.48

2,304

96.96



9,216

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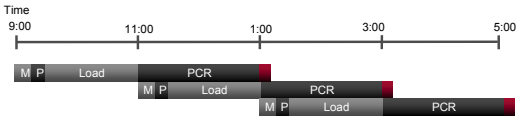
Throughput 96.96 array



288 samples/day or 27,648 PCRs/day

M: prime, P: pipette

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Stability of Assay Sensitivity

Average Ct of 26 assays using same controls for each test

Ver 1 Lot A	Ver 1 Lot B	Ver 2* Lot A	Difference V2-V1	Difference btw lots
21.7	**22.6**	**20.6**	**1.1**	**2.1**

***46% percent assay change between lots (22 assays replaced)**

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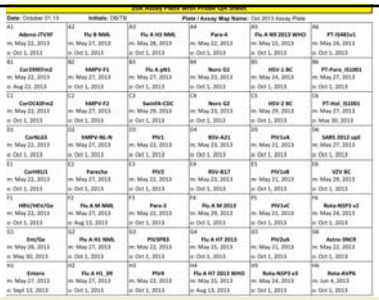

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Where are the savings?

9,216 PCR results

Master mix	240 µl
Primer/probe	240 µl
96.96 chips	1

PCR Cost Analysis

Cost	Influenza A/B PCR		BioMark SDCL	Commercial
	Negative	Positive		
TAT	2.5 h	Next day	4 h	5 h
HOT	20 min	45 min	30 min	90 min
Consumables	$5	$20	$10	$50

PCR Cost Analysis – with real numbers

Method	Slow / Busy		Slow Total	Busy Total
	PCR	RVP		
Old way	$1,420/$2,590	$1,590/$1,870	$3,010	$4,459
BioMark SDCL	$2,120/$2,944		$2,120	$2,944
Savings per week*			**$890**	**$1,515**

Using 2010 & 2011 specimen numbers

Save $274,000* using BioMark SDCL

Spend $658,000* more using commercial kit to get less results

*** Molecular consumables alone**

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Tipping point developments

- Consolidated process
  - Independent of sample type
- Unified assay conditions
- One-step RT and pre-amplification process
  - Independent of RNA / DNA target

Key QC points: 20X Assay stocks, Plate replication, 7500 backup

Why does this work?

➔**Laboratory developed real-time PCR** ←

- Simple
- Robust
- Flexible

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Nanofluidic technology and real-time PCR provides a **Paradigm Shift** for high-throughput infectious disease testing with no compromises and at a ***LOWER*** operating cost.

- ✓ Better for the patient
- ✓ Better for the clinician
- ✓ Better for the laboratory
- ✓ Better for the budget

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