

Analysis of 250 Pediatric NPA Samples for 21 Respiratory Pathogens Using an Automated, Nested Multiplex PCR Platform

Mark Poritz¹, Lindsay Meyers¹, Amber Lewis¹, Kody Nilsson¹, Paul Murphy¹, Meghan Hamilton¹, Mike Vaughn¹, Stephanie Thatcher¹, Jamey Hulsberg¹, Ron Mudrow¹, Ben Smith¹, Joanne Fisher¹, David Jones¹, Cory Estes¹, David Teng¹, Rob Crisp¹, Rich Abbott¹, Steven Dobrowolski¹, Anne Blaschke², Kent Korgenski², Judy Daly², Carrie Byington³, Kirk Ririe¹.
¹Idaho Technology Inc. (ITI), Salt Lake City, UT | ²Primary Children's Medical Center (PCMC), Salt Lake City, UT | ³School of Medicine, University of Utah, Salt Lake City, UT

CONTACT INFORMATION

Mark Poritz
 Mark_poritz@idahotech.com
 801-736-6354
 Lindsay Meyers
 Lindsay_meyers@idahotech.com
 801-736-6354

ABSTRACT

Background: Idaho Technology (ITI) has developed a lab-in-a-pouch system called "FilmArray™". It performs mesoscale (0.001 to 0.2 mL working volume) fluid manipulation in a self-contained, disposable, thin-film plastic pouch. The FilmArray pouch contains areas for nucleic acid purification, reverse transcription, a first-stage multiplex PCR, and multiple second-stage nested real time PCRs. A double-stranded DNA binding dye (LCGreen™) is used in the second-stage PCR to generate real time amplification curves. Additional high-resolution DNA melting curve analysis confirms the identity of the product. Reagents are stored in the pouch in a room-temperature-stabilized form so that only sample and water need be injected into the pouch. In the current pouch configuration the second-stage PCRs are performed in a 20 mm² hexagonal array of 120 wells (1 µL volume each). This number is sufficient to perform multiple tests for a large panel of pathogens and still include controls for process validation. The FilmArray instrument weighs 15lbs, measures 9" W x 15" L by 6" H, and is controlled from a laptop computer. The sample is processed and results reported in 56 min.

Methods: We have created nested PCR assays for: Adenovirus, HRV, PIV1, -2, -3, -4, RSV, Influenza B, BOCAvirus, Metapneumovirus, Influenza A (MA, H1, H3, H5, N1, N2), Polyomavirus WU, Coronaviruses 229E, NL63, OC43, HKU1, and SARS as well as the bacteria *Chlamydomphila pneumoniae* and *Bordetella pertussis*. A mixture of 77 primers is used to perform the reverse transcription and first PCR. Some viruses have multiple gene targets and some have single, degenerate primers to capture the diversity in a given target. The array second-stage PCR contains duplicate or triplicate replicates for each assay.

Results: Two hundred and fifty nasopharyngeal aspirate (NPA) samples from PCMC have been tested, ~150 at ITI and the rest in the microbiology laboratory at PCMC by the laboratory personnel (following the IRB protocol, data display is turned off on this instrument). With the exception of SARS and Avian Influenza (H5N1) we have identified all of the organisms listed above in patient samples from PCMC. The number of organisms detected per sample among the 250 NPAs is: zero: 44 (17.6%), one: 130 (52%), two: 59 (24%), three: 13 (5.0%), and four: 4 (1.6%). We are sequencing the triple and quadruple infections to confirm their identity. In general we find that most assays have consistency in CPs and LODs between the singleplex and multiplex formats.

Conclusions: Nested multiplex PCR chemistries have the potential for great sensitivity and specificity as well as the ability for moderate to high multiplexing. They have not been widely adopted in molecular diagnostics because of contamination concerns. The system described here substantially reduces this risk and enables the multiplex testing of pathogens.

The development of the FilmArray system has been funded in part by three National Institutes of Health grants to ITI: NIAID SBIR, 1R43 AI063695-01, PI Dobrowolski, "FilmArray – A closed system for Multi-Pathogen Screening". NIAID U01 AI061611, PI Poritz, "SARS Single Sample FilmArray". NIAID U01 AI074419, PI Dobrowolski, "Avian Influenza Panel and Multi-Sample Instrument".

MOLECULAR DETECTION OF RESPIRATORY PATHOGENS

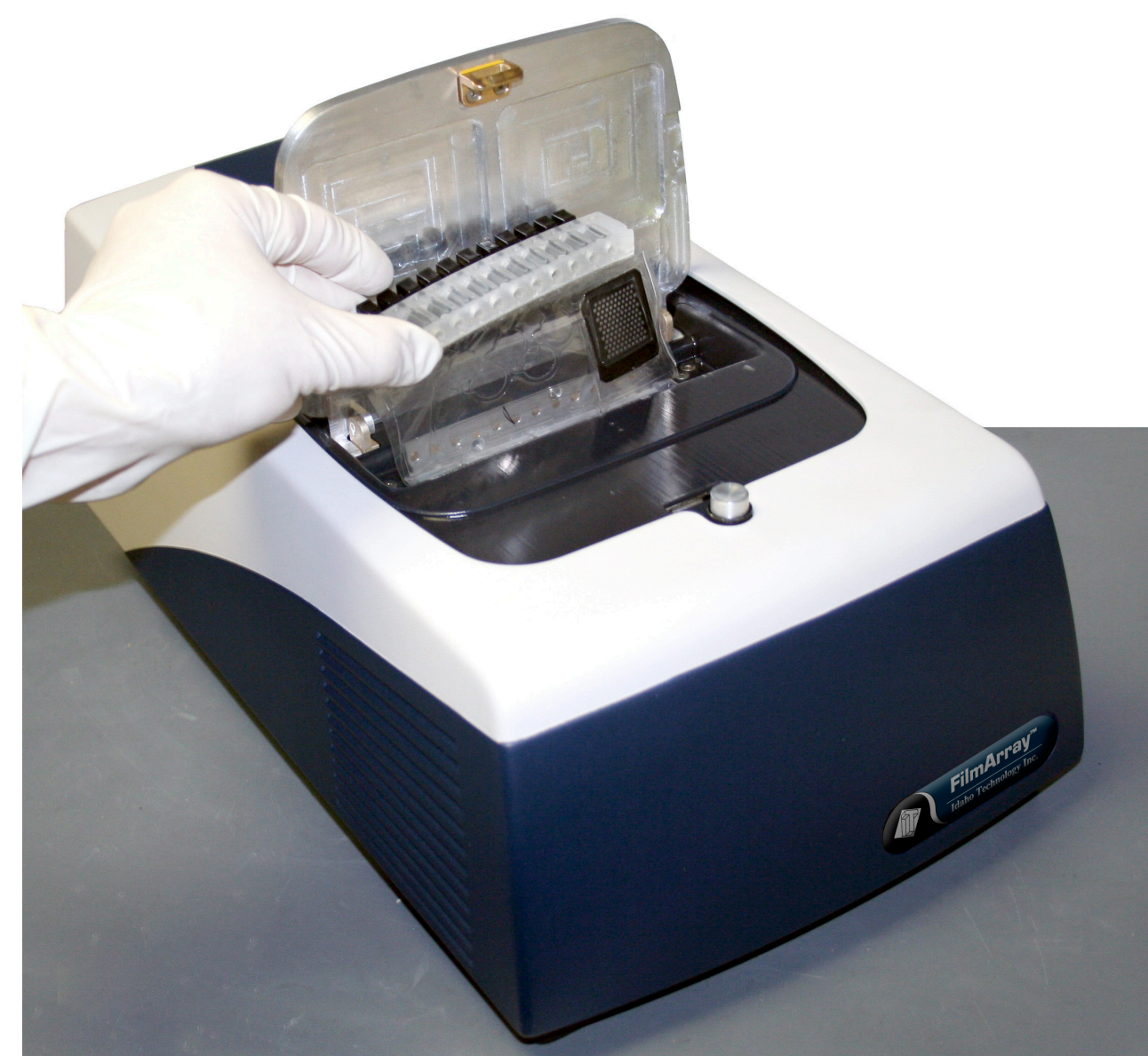
A comprehensive panel of assays for common and emerging respiratory pathogens would reduce the number of unidentified infections during seasonal and pandemic outbreaks. Direct Immunofluorescence Assay (DFA) is widely used for viral detection but only tests for a limited number of viruses. PCR assays are being used for the detection of respiratory pathogens but most real time assays test for one or a few organisms. Several multiplex PCR formats have been developed but since almost all require opening the reaction at the end of PCR they are subject to the standard issues of PCR contamination.

As new technologies continue to appear, the problem of identifying a viral or bacterial pathogen responsible for an infection has largely become an issue of engineering and costs. An engineering solution will attempt to optimize a specified combination of price, automation, speed, sensitivity, and comprehensiveness.

ITI has developed a lab-in-a-pouch system called "FilmArray" (Figures 1 and 3). It is a medium-scale fluid manipulation system performed in a self-contained, disposable, thin-film plastic pouch.

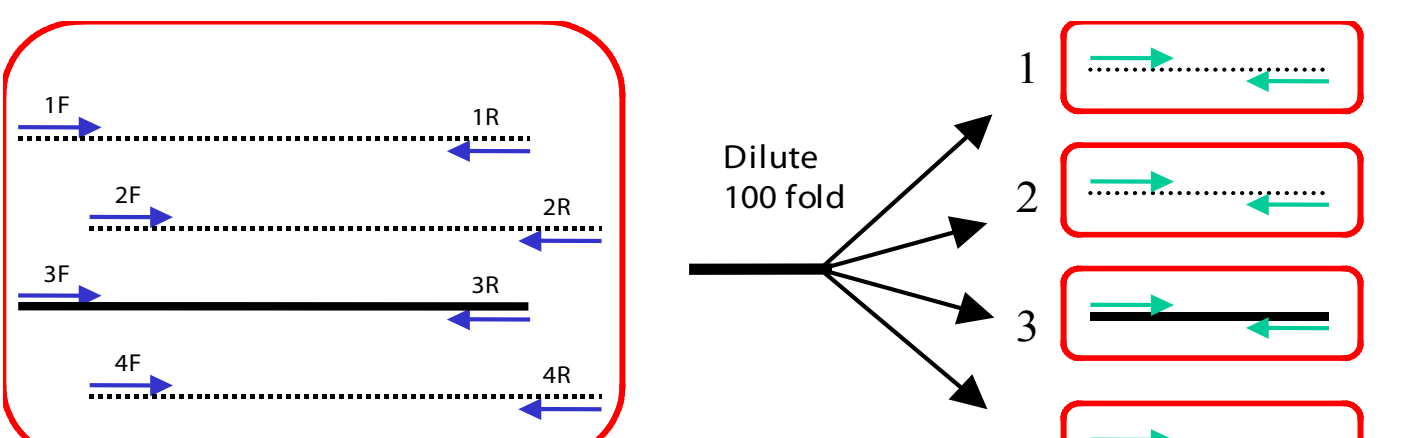
The FilmArray platform processes a single sample, from NPA to result, in a fully automated fashion. These system characteristics are ideal for the multiplex testing of pathogens in standard diagnostic sample matrices.

Figure 1: The FilmArray Instrument



The FilmArray pouch is being inserted into the instrument. Weight: 15 pounds. Size: 9" W x 15" L by 6" H.

Figure 2: Schematic of Nested Multiplex PCR



A large volume multiplex PCR (shown here as 4-plex on the left side of figure) is run for a limited number of cycles (20). The reaction is diluted and distributed to individual small real time PCR reactions that contain primers (green) nested inside the primers (blue) of the first PCR reaction. A template amplified in the first reaction (by the 43 primers) is further amplified in only one of the second reactions.

Table 1. Steps in Running a FilmArray pouch

Steps	Time
Mix sample with lysis buffer	5 min.
Inject mix and water into pouch	
Read pouch and sample barcodes	
Load pouch, press start	
Instrument run-time	
Total time	60 min.

The FilmArray pouch has a fitment (Figure 3, see label A) containing all needed freeze-dried reagents. The FilmArray instrument depresses plungers (B) in the fitment to move reagents through channels and into blisters in the pouch (C – H). The film portion of the pouch has stations for:

1. Cell lysis (Blister C)
2. Magnetic-bead based nucleic acid purification (D & E)
3. Reverse transcription (RT) to detect RNA viruses (F & G)
4. First-stage multiplex PCR (F & G)
5. Array of 120, second-stage nested PCRs (I).

PCR primers are dried into the wells of the array and each primer set amplifies a unique product of the first-stage multiplex PCR. A fluorescent-double-stranded DNA binding dye, LCGreen Plus, developed by ITI, is used to detect amplification. A CCD camera collects and software processes fluorescent images to generate real time PCR amplification curves for each individual PCR reaction (shown in Figure 5). Post-PCR product identity is confirmed by high-resolution melt profiling (data not shown).

Figure 3: The FilmArray Pouch

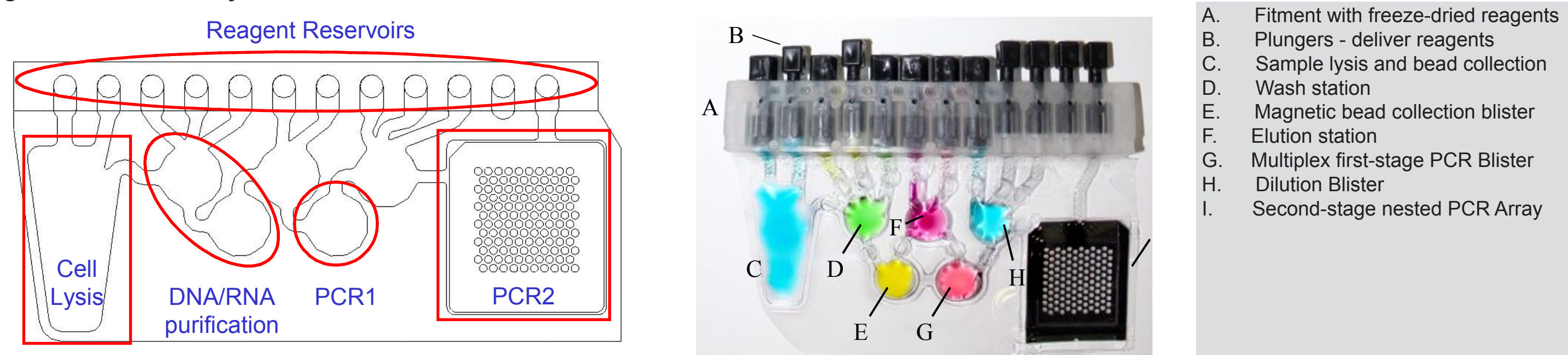


Table 2: FilmArray Target Pathogens

Organism	Target	First-Stage Primers	Second-Stage Wells
Complex, Multiprimer Targets			
Adenovirus	Hexon	5	4x2
hRV	5'UTR	5	4x2
FluA H1	HA	4	4x2
FluA N1	NA	2	3
FluA N2	NA	2	3
FluA pan	Matrix	2	3
Less Diverse Targets			
Bocavirus	NP101	2	2
	NS102	2	2
<i>B. pertussis</i>	Toxin	2	2
CoV 229E	Pol	2	2
	N	2	2
CoV HKU1	N	2	3
CoV NL63	N	2	2
	Pol	2	2
CoV OC43	N	2	2
	Pol	2	2
<i>C. pneumoniae</i>	gyrB	2	3
FluA H3	HA	2	3
FluB	HA	2	3
<i>M. pneumoniae</i>	gyrB	2	3
Legionella sp.	16S	2	2
hMPV	N	3	3
	Pol	3	3
PIV1	HN	2	3
PIV2	FG	2	3
PIV3	FG	2	3
PIV4	FG	2	3
	FG	2	3
RSV*	Matrix	2	3

Process Controls			
First-stage PCR	Synthetic 1	2	2
First-stage PCR	Synthetic 2	0	2
Dilution	Synthetic 2	0	2
DNA extraction	<i>S. pombe</i> gDNA	2	2
RNA extraction	<i>S. pombe</i> mRNA	2	2
Total Primer number		60	79

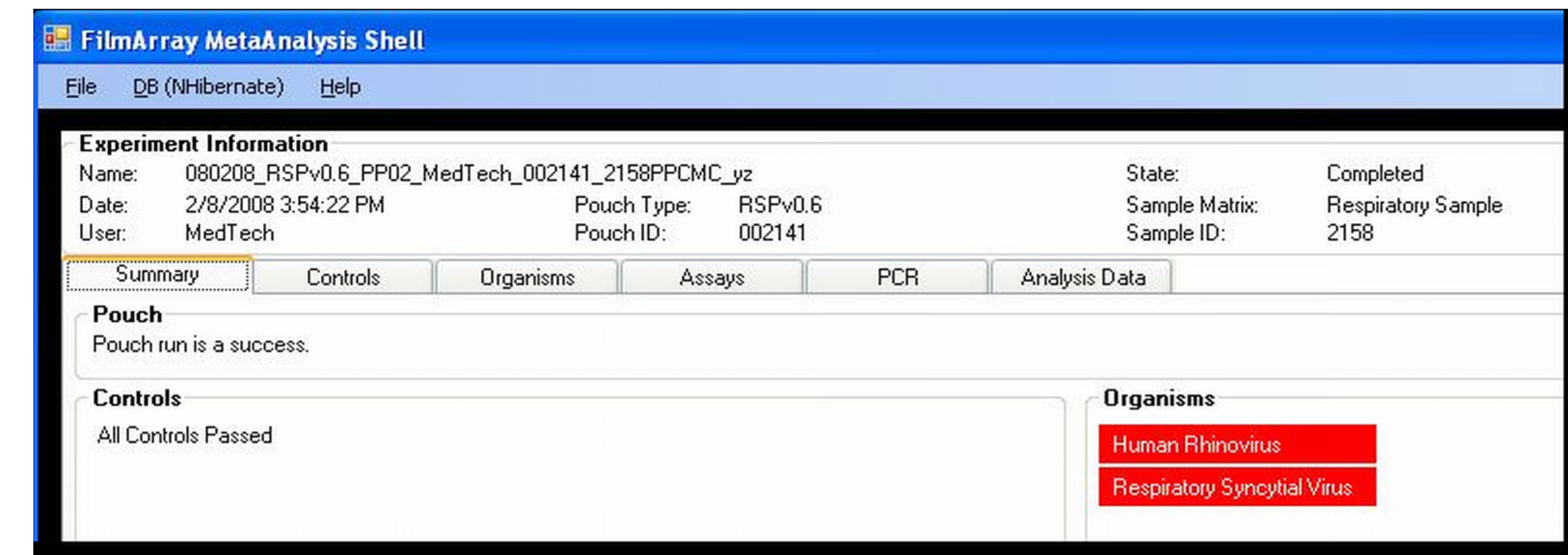
At the conclusion of a run, the FilmArray software automatically analyzes fluorescence data, generating positive, negative, or equivocal calls for each assay and for process validation controls. While most users will only see the calls made by the automated software (Figure 4), advanced operators can view detailed information such as the amplification curves (Figure 5).

Respiratory Virus Assay Design

The development of nmPCR assays for each of the common respiratory viruses follows standard protocols. Whole and partial genome alignments were used to identify conserved regions. Inner and outer primer sets targeting these regions were designed to a target Tm of 60C so that primer sets can be mixed and matched in future pouch layouts.

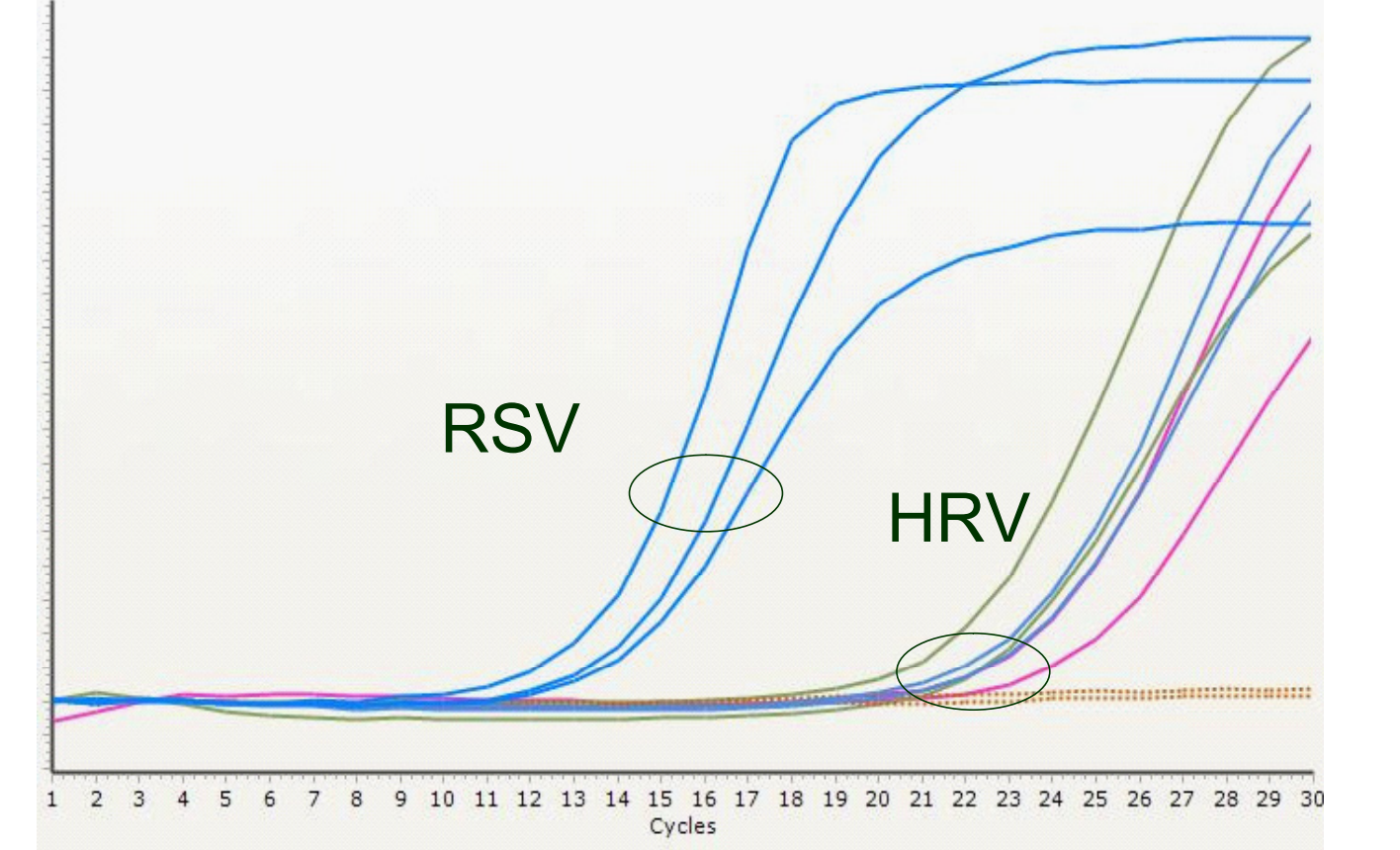
In some cases (e.g. Adenovirus and human Rhinovirus) a combination of degenerate primers and primers targeting specific branches of the phylogenetic tree were designed. The viruses, gene targets, strain subtypes and number of outer and inner primer sets are shown in Table 2. In total 60 primers are used in the first-stage PCR and 43 primer sets are used in the second-stage PCRs (in duplicate or triplicate). This combination of outer and inner primers is termed the FilmArray RP (respiratory panel) pouch.

Figure 4. FilmArray RP Software Display at the end of a Run



This report indicates that the controls passed and hRV and RSV were detected.

Figure 5. FilmArray RP analysis of an NPA sample



A PCMC NPA sample was injected into a FilmArray RP pouch. The resulting real time amplification curves are shown. DFA called this sample positive for RSV; hRV is not tested for by DFA. The RSV assays are spotted in triplicate in the array. The hRV assays consist of 4 inner primer sets spotted in duplicate. The inner primer sets anneal to the same target region but differ in sequence to capture different members of the phylogenetic tree. This data is from the same FilmArray run shown in Figure 4.

RESULTS

Testing Clinical Samples on the FilmArray Instrument

We have tested several hundred pediatric NPA samples isolated at PCMC. The samples were tested both at ITI and by onsite instrument placement in PCMC's microbiology laboratory for two months during the last respiratory season.

Figure 6 compares the distribution of pathogens detected by DFA at PCMC (Adenovirus, hMPV, PIV 1, 2, 3, Flu A, Flu B, and RSV) to those detected by the FilmArray RP in 387 samples.

1. More organisms were detected by the FilmArray RP than by DFA
2. The FilmArray RP has significantly fewer negative test results (18% than DFA (53%).
3. The distribution of pathogens includes a few major contributors (hRV, Adenovirus, RSV, FluA) and a long tail of lower prevalence pathogens (PIV1, PIV2, CoV OC43, CoV 229E, etc)

The larger number of viruses and bacteria that can be tested for in the FilmArray RP pouch has also resulted in the observation of more multiply infected samples (Figure 7), with 32% of samples having two or more pathogens identified. We are exploring the significance of multiple infections on patient outcomes.

FilmArray results are 72% concordant with the DFA results (if all organisms detected by DFA must match to show concordance). The concordance rises to 80% if at least one DFA result matches a FilmArray result for a given sample.

Sensitivity and Robustness of FilmArray Detection

A demonstration of the robustness of nmPCR is shown in Figure 8. In this experiment, nmPCR amplified both a high-concentration as well as several low-concentration species in a FilmArray BioThreat pouch. Traditional multiplex PCR using the same 40-primer mixture was unable to detect any of the species using Taqman probes for specific targets within the multiplex assay (data not shown). The LODs for these assays are all less than 10,000 pfu per mL for viruses and 1000 cfu per mL for bacteria.

Controls in the FilmArray

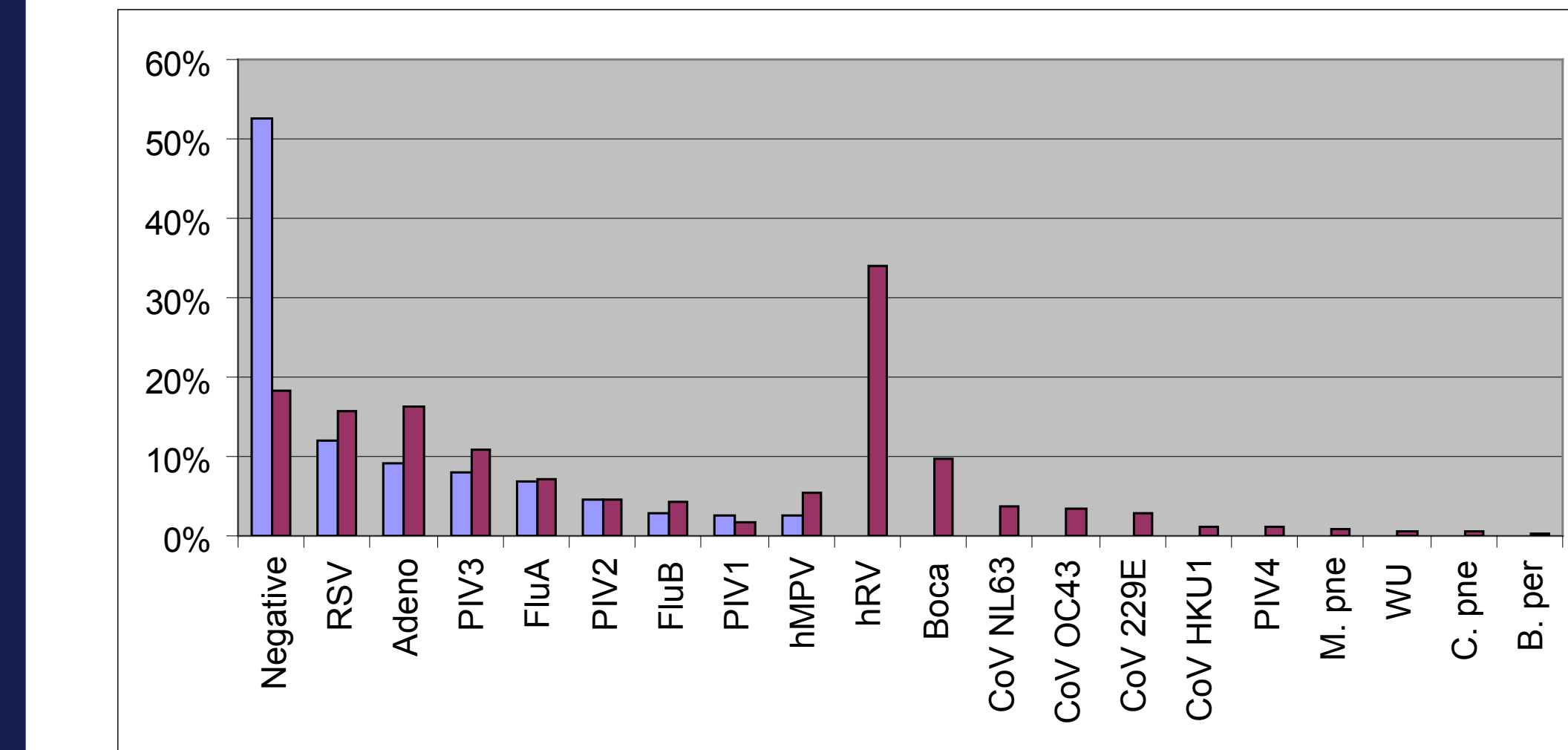
The ability to perform 120 independent second-stage PCRs allows for extensive full process as well as process specific controls. An outline of these controls is shown in Table 3. The full process control is based on PCR of specific genomic DNA and mRNA targets in the yeast, *Schizosaccharomyces pombe*. This yeast is freeze-dried in the pouch.

Defects in any of the steps of the FilmArray RP process (cell lysis, nucleic acid purification, RT, etc.) will result in either no amplification or a delay in the crossing point (Cp) value. The FilmArray RP system full process controls contain both DNA and RNA targets to control for extraction and amplification of both forms of nucleic acid.

Additional amplification reactions that control for individual steps of the process are incorporated in the FilmArray RP pouch. For example, the second-stage PCR control consists of a short synthetic sequence spiked into a well of the PCR array along with the primers necessary to amplify this sequence.

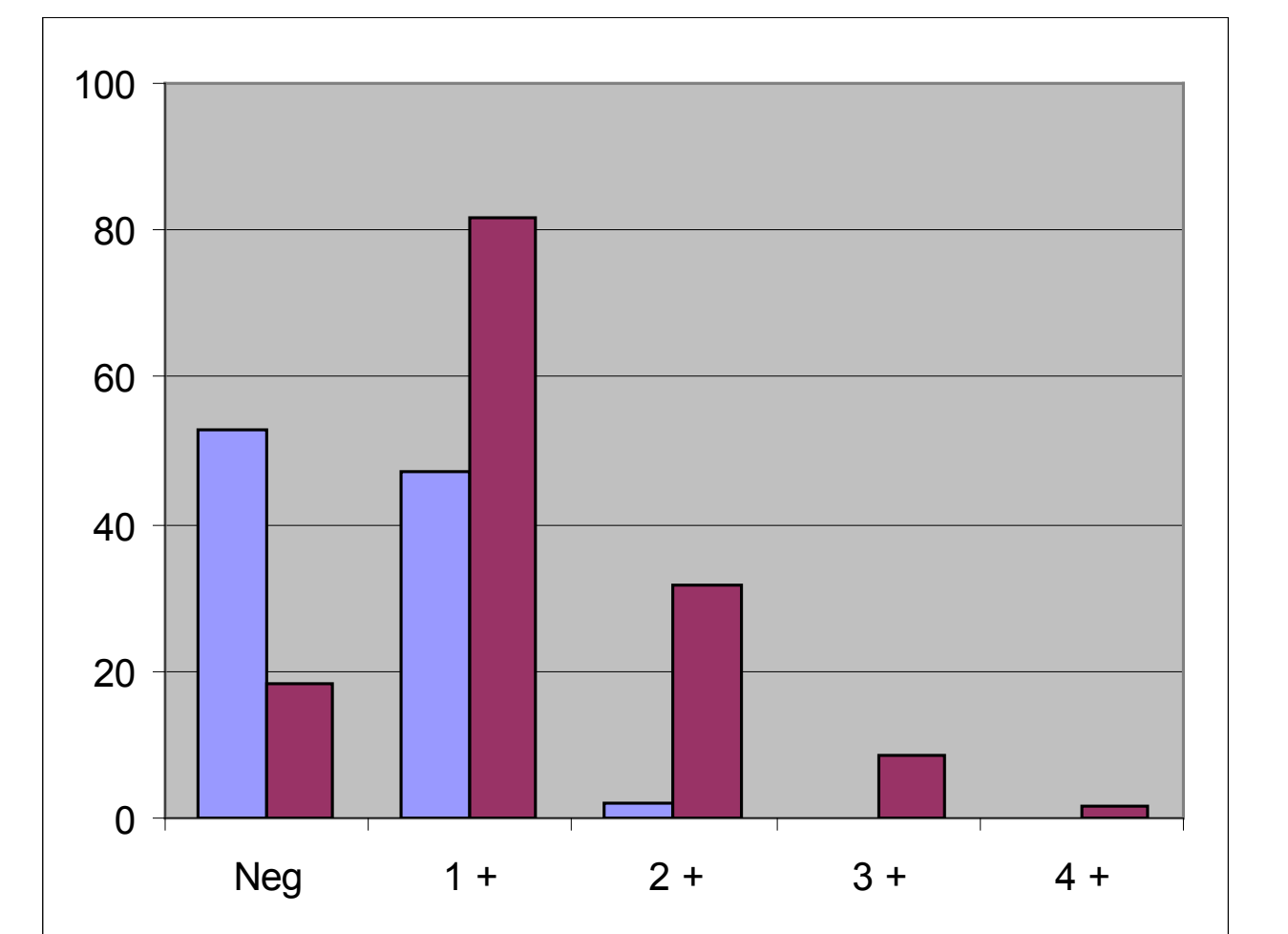
In addition to process controls we routinely run analyte specific controls on every batch of pouches. This consists of a mixture of the target nucleic acids at a predetermined, normalized concentration. The nmPCR protocol is robust in the presence of up to 20 different targets at one time (data not shown).

Figure 6. Distribution of Viruses and Bacteria Detected among 387 Pediatric NPAs



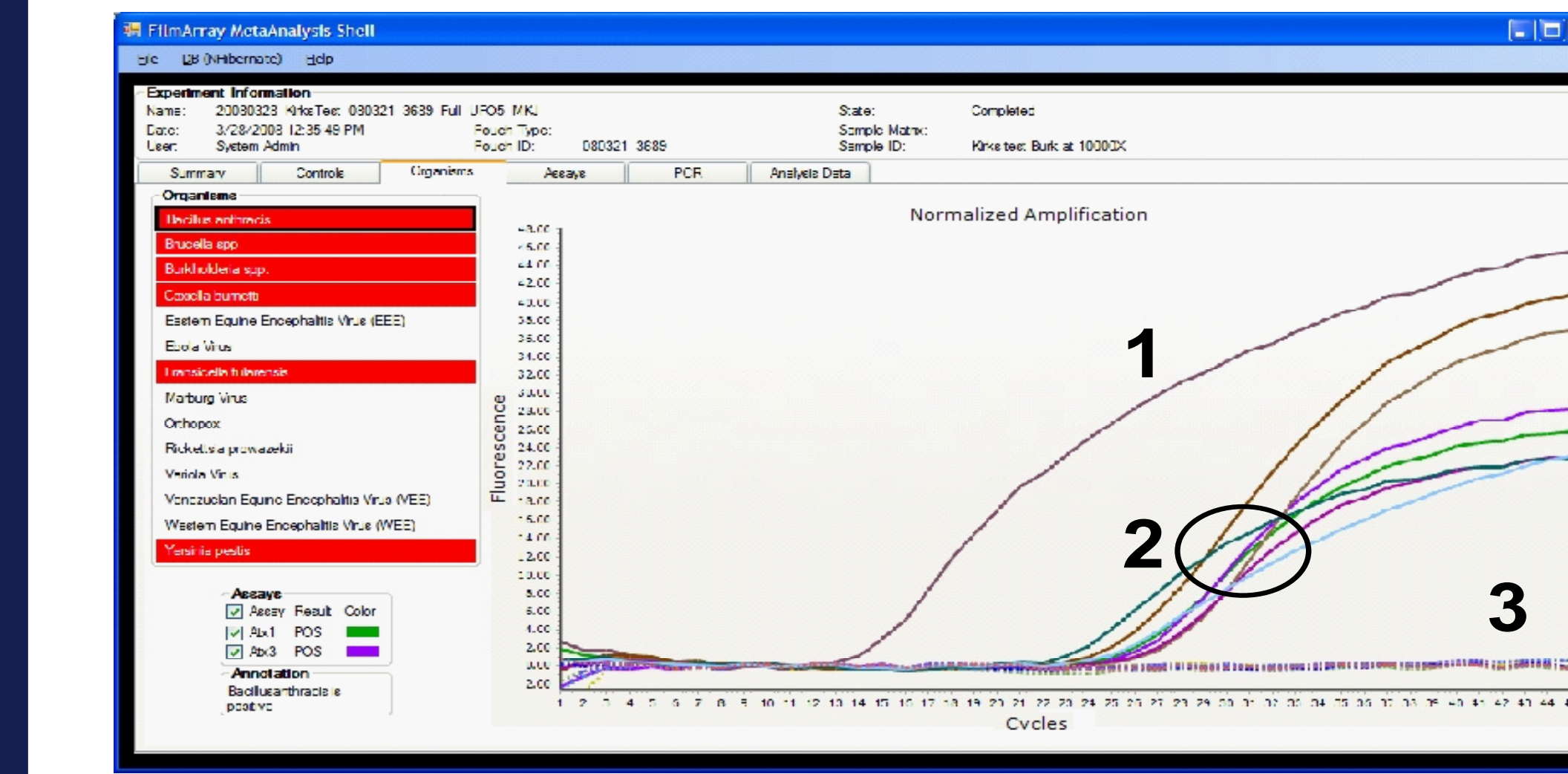
Pediatric NPA samples were tested by DFA (blue) and by FilmArray (maroon). Results are expressed as the percent of the 387 samples testing positive for a given pathogen. (PIV4 and WU assays were added later so the true number of PIV4 and WU is under-represented)

Figure 7. Number of Pathogens Detected per sample: DFA vs FilmArray RP



NPA samples (Figure 2) were tested by DFA (blue) and by FilmArray (maroon). The percentage of samples in which 0 (negative), 1 or more, 2 or more, 3 or more, 4 or more pathogens is indicated.

Figure 8 Sensitivity and Robustness of FilmArray



A FilmArray pouch containing 20 assays covering all of the JBAIDS BioThreat agents (and controls), was injected with a sample containing *Burkholderia malieri* nucleic acid at 10,000 times LOD and 5 other organisms at LOD. (1) indicates the *B. malieri* amplification curve, (2) indicates the curves for the 5 organisms spiked at LOD and (3) shows the curves for all other organisms.

Table 3. Process and Internal Controls in the Pouch.

Control Name	Template		Primers	
	Type	Location	Outer	Inner
Full Process Controls				
DNA Process	<i>S. pombe</i> cells: gDNA	with sample	yes	yes
RNA Process	<i>S. pombe</i> cells: mRNA	with sample	yes	yes
Process Specific Controls				
Second-stage PCR	Target 2	PCR 2	no	yes
Dilution: First to Second-stage	Target 2	PCR 1	no	yes
First-stage PCR	Target 1	PCR 1	yes	yes

CONCLUSION

The data presented here support the idea that the FilmArray RP system will provide valuable diagnostic information and that the system is ready for the formal analytic and clinical testing required for FDA clearance. ITI will begin clinical trials of the FilmArray RP system in the fall of 2008.

FilmArray as an Enabling Technology

In other work we are developing a FilmArray BioThreat pouch that detects biothreat agents such as *B. anthracis*, *F. tularensis* and *Y. pestis*. The ultimate goal is a "dual use" system that will have the ability to identify common respiratory pathogens as well as biothreat agents. An advantage of such a system is that the platform is used efficiently in a clinical laboratory and in the event of a biothreat attack, the platform and the personnel with expertise are already available. The preliminary data described above suggest that widespread deployment of FilmArray RP could facilitate two novel forms of disease tracking and pandemic early warning.

FilmArray + Network Software = Real Time Respiratory "Weather Maps"

There is recent and rapidly growing medical literature on the idea of "syndromic surveillance" since the anthrax attacks and SARS outbreak. Tracking outbreaks of disease symptoms via the internet will speed up the detection of novel pathogens, natural or man-made. It is hypothesized that syndromic surveillance will precede diagnosis by a few critical days and will allow for an earlier signal of pandemics.

FilmArray RP has the potential to truly be a point-of-care diagnostic – usable in schools, walk-in clinics and nursing homes. ITI has been developing software that will make the FilmArray "network aware". The goal is to have the locally generated (de-identified) FilmArray RP results reported back to a central database in real time. As the number of instrument placements grows, epidemiologists will be able to observe respiratory pathogen flow through a community, a state or the nation, in real time. Syndromic surveillance will turn into "diagnostic surveillance".

New Pandemics as an "Outbreak of FilmArray Negatives"

The lower "negative" rate and the long tail of low frequency infections detected by the FilmArray RP system (Figures 6 and 7) has important implications for the early detection of pandemics using a deep multiplex platform.

An emerging pathogen may signal its appearance by a sudden spike in FilmArray RP negative results from symptomatic patients. With an overall negative rate below 20% it should be possible to observe an unusual increase that is temporally or spatially concentrated. This concept applies even within a single virus type such as Flu A. An outbreak of Flu A detection events that are positive for the pan Flu A target (using a nested assay for the highly conserved Matrix gene) but negative for H1 or H3 subtypes suggest the emergence of either a novel H1 or H3 sequence variant or a switch to a different HA subtype.

A highly pathogenic variant of one of the low prevalence viruses will be evident by a new correlation between FilmArray RP detection of that virus and severe illness. Finally, for some viruses (Adenovirus, Flu A and hRV) the FilmArray RP pouch has multiple outer and inner primer sets that collectively capture the phylogenetic diversity of these organisms (Table 2). In this case the combination of Cps and melting profiles for each assay in the array becomes a fingerprint for the particular virus.