Abstract #2602

Adaptation of Immunoassays for Multiplexed Diagnosis of a Diverse Panel of Pathogens Associated With Acute Febrile Illness

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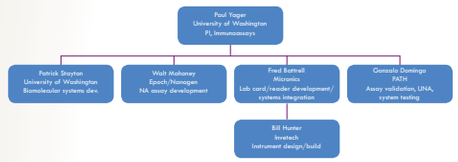
Project overview

PATH is part of a consortium of public and private enterprises working to develop an affordable microfluidics-based diagnostic tool capable of accurately diagnosing rapid-onset fever in the developing world. The system (Dx Box) is expected to be composed of two components: (1) a rugged, portable, user-friendly instrument and (2) low-cost disposable cards that will contain all reagents for the diagnostic assays. The prototype is being developed to diagnose typhoid fever, dengue fever, rickettsial diseases, malaria, measles, and influenza from a single 50 µL specimen in under 20 minutes. This panel of pathogens represents a diverse array of organisms requiring different diagnostic approaches. For each pathogen, the platform will perform enzyme immunoassays for antigen detection, serology, or PCR to detect the presence of pathogen genomic material. PATH's role in the project is composed of three activities:

Activity 1: Immunoassay Development
Activity 2: Field Studies
Activity 3: User Needs Assessment

Recent work on each of these activities is outlined below.

Consortium participants and structure



Activity 1. Immunoassay Development

Aim

Adapt immunoassays for use in multiplexed microfluidic immunoassay format.

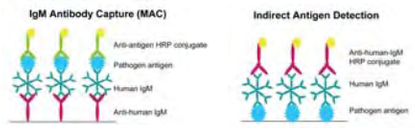
Introduction

Prior to integration on the microfluidic card, assays are developed and optimized in a traditional ELISA format. There are two common formats used for IgM ELISA: IgM antibody capture (MAC) and indirect detection (Figures 1 and 2). We have chosen to utilize the indirect detection format because this format relies on a single detector for all assays. This allows for easier multiplexing of assays on a single surface and eliminates the costs associated with having multiple detection reagents (Figure 3). One common problem with the indirect format is interference of IgG that can lead to false-positive results in the presence of rheumatoid factor (RF) IgM and false-negative results in the absence of RF (Figure 4). This problem can be mitigated by the addition of goat α-human IgG to the serum diluent (Martins T; Clin & Diag Lab Immuno; 1995). Understanding the behavior of goat α-human IgG complexed with human IgG is critical for successful adaptation of assays to a microfluidic format. We have explored this issue and adapted MAC assays for two pathogens (measles and dengue) to indirect detection format.

Figure 1: PCR, serology, and antigen detection will be performed simultaneously on the microfluidic card.

Pathogen	Nucleic Acid Detection	Immunoassay Type	Target Antigen
Dengue virus	rt-PCR	Serology (IgM)	Envelope protein
Influenza A & B virus	rt-PCR	Serology (IgM)	Virus
Measles virus	rt-PCR	Serology (IgM)	Virus
Rickettsia	PCR	Serology (IgM)	Cell lysate
Salmonella typhi / paratyphi	PCR	Antigen detection	LPS
		Serology (IgM)	LPS
			OMP50
			Cell lysate
Plasmodium spp	PCR	Antigen detection	*pan-Plasmodium*: Aldolase
			P. falciparum: HRP2

Figure 2: Indirect detection is more amenable to multiplexed immunoassay arrays than MAC ELISA.



Pros:
High IgM specificity.
Does not require additional reagents to remove interfering IgG.

Cons:
Difficult to multiplex.
Requires pathogen-specific detection antibody.

Pros:
Easier to multiplex.
Lower reagent costs (no pathogen-specific antibodies).

Cons:
Requires removal of interfering IgG.
Higher risk of false-positive or negative result.

Figure 3: Multiplexed immunoassay arrays can be integrated into microfluidic circuits and allow a high degree of scalability.

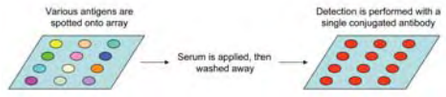
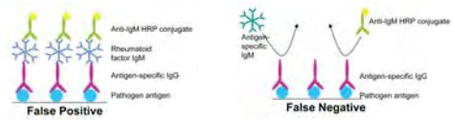


Figure 4: Interfering, antigen-specific IgG can lead to false-positive or false-negative IgM results depending on the presence of rheumatoid factor IgM.



- MAC ELISA captures IgM prior to the addition of pathogen antigen.
  - Potentially confounding IgG is washed away prior to exposure to antigen.
- Indirect ELISA relies on binding of IgM directly to pathogen antigen.
  - IgG is exposed to antigen.
  - IgG interference has been shown to be mitigated by adding goat α-human IgG to serum diluent.

Methods

We have tested and optimized various combinations of monoclonal antibody- and antigen-coated plates for IgM serology of measles and dengue fever. Once optimal antigen coating, blocking, and washing conditions were identified, we proceeded to investigate the role of goat α-human IgG in eliminating potentially confounding IgG.

Results

Initial studies showed that adding goat α-human IgG dramatically reduced IgG signal without diminishing IgM signal (Figure 5). Experiments were then performed to identify the cause of this signal reduction (Figures 6–8). These experiments revealed that the addition of goat α-human IgG effectively removes IgG from the reaction well. We then compared our indirect ELISA to commercially available MAC ELISAs and determined that they have equivalent sensitivity (Figure 9).

Figure 5: Adding goat α-human IgG to serum diluent dramatically reduces IgG signal without diminishing IgM signal.

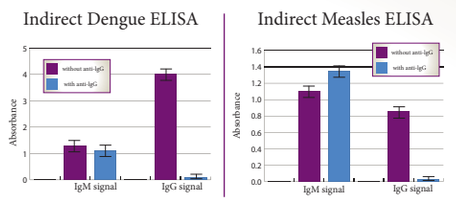
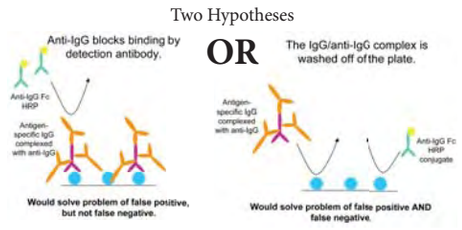
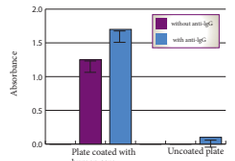


Figure 6: Why does IgG signal drop when anti-IgG is added to serum diluent?



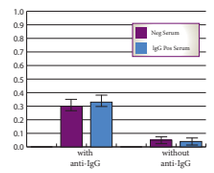
A reduction in IgG signal is not sufficient evidence to prove that we are preventing IgG from binding with antigen.

Figure 7: Detection of human IgG coated with goat α-human IgG.



- ELISA plate was coated overnight with human IgG, then with goat α-human IgG for 1 hr.
- Coating with goat α-human IgG does not inhibit detection of IgG.
- This supports the hypothesis that IgG complexed with goat α-human IgG washed out of the reaction well.

Figure 8: Elevated levels of goat α-human IgG are not detected when pathogen specific IgG is present.



- Measles ELISA was performed, but rather than probing for human IgG we probed for goat α-human IgG.
- There is no difference in signal when pathogen-specific IgG is present.
- This supports the hypothesis that IgG complexed with goat α-human IgG washed out of the reaction well.

Figure 9: Sensitivity of indirect ELISA compared with MAC ELISA.

		Serum Titers	
		MAC ELISA	Indirect ELISA
Measles	Positive Sera 1	1:4000	1:4000
	Positive Sera 2	1:256	1:128
Dengue Fever	Positive Sera 1	1:800	1:800
	Positive Sera 2	1:800	1:800

Activity 2. Field Studies

Malaria diagnosis study in collaboration with Walter Reed Army Institute of Research (WRAIR), Kisumu, Kenya.

Aim

To determine the sensitivity and specificity of combined nucleic acid and antigen detection for diagnosis of malaria infection or clinical disease in a holoendemic region.

Objectives of study

- Determine sensitivity and specificity data for the malaria assays intended for the DxBox platform.
- Collect data that simulates the diagnostic output of a multiplexed immuno and nucleic acid diagnostic platform such as the DxBox. Published or commercial diagnostic assays will be performed for serology and nucleic acid detection of each of the target pathogens (Figure 10).
- Determine the etiology both for respiratory infection and bloodborne pathogens in febrile patients in this region.
- Collect and archive samples for microfluidic assay validation.

Next steps

Typhoid diagnosis study in collaboration with Oxford University Clinical Research Unit (OUCRU), Ho Chi Minh City, Vietnam.

Figure 10: Panel of diagnostic assays for simulation of lab-on-a-card data output.

	Immunoassay	Real-Time PCR
Malaria	HRP2 and Aldolase ELISA	Rougemont M et al; J Clin Micro (2004), Vol 42 (12)
Typhoid	Typhidot	Song J et al; J Clin Micro (1993), Vol 31 (6)
Rickettsia	PanBio IgM ELISA	Jiang J et al; Am J Trop Med Hyg (2004), Vol 70 (4)
Dengue Fever	PanBio MAC ELISA	McAvin J et al; Mil Med (2005), Vol 170 (12)
Influenza	None	van Elden L et al; J Clin Micro (2001), Vol 39 (1)
Measles	Light Diagnostics MAC ELISA (Chemicon)	Hummel K et al; J Vir Met (2006), Vol 132

Activity 3. User Needs Assessment

User Needs Assessment, India.

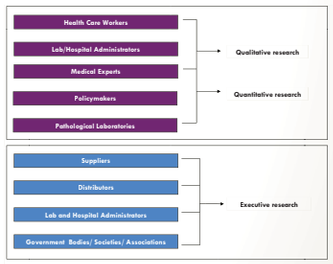
Aim

Inform product development and commercialization efforts by understanding the needs of stakeholders.

Study details

Interviews were performed in two regions of India:
- Delhi - (urban)
- Tamil Nadu - (rural)

Stakeholders and study approach



Objectives of study

To obtain an overview of the diagnostic industry, to understand the particulars of the market environment and health care infrastructure, and to solicit feedback on key product attributes for acceptance by stakeholders.

Next steps

User needs assessments will be performed in Brazil and sub-Saharan Africa.

We thank PanBio for providing access to their dengue antigen for research purposes.

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