Immobilized CRISPR Chemistry for Massively Multiplexed Target Detection

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Abstract

Current biosurveillance and diagnostic systems aim to detect a broad range of biothreats and pathogens of interest in a timely and user-friendly manner. Traditional PCR-based detection devices can be used to detect relatively small panels of assays. Typically, it takes weeks to months to develop and transition new assays onto closed platform devices. However, as Ebolavirus, SARS-CoV-2 and Monkeypox virus outbreaks have shown, novel, emerging, and re-emerging pathogens present an ever evolving challenge. We are developing a Massively Multiplexed Detection (MMD) device to detect >500 pathogenic targets in a microfluidic platform. By using CRISPR technologies, we aim to be able to quickly reconfigure the open platform to accommodate new pathogens and/or new sequence variations within 24 hours of discovery.

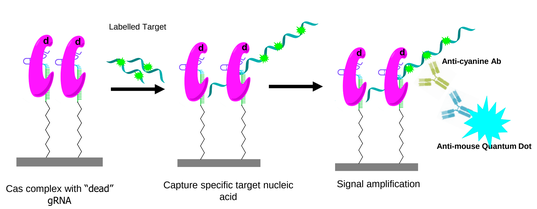
The current panel for the MMD device includes upper and lower respiratory bacteria and viruses, bacterial anti-microbial resistance genes, fungal pathogens, select agents and their near neighbors, vector-borne viruses, food-borne and water-borne pathogens, zoonotic pathogens, biomarkers for disease severity, and endogenous controls. In addition, several variant assays are included, since alterations in CRISPR guide RNA can distinguish single-nucleotide polymorphisms (SNPs) with specificity. The MMD platform requires advances in automated sample preparation, microfluidics, optics, and immobilized CRISPR-based detection chemistry. In this work we will describe strategies and progress on developing optimized CRISPR-based detection chemistry.

Overview of Chemistry

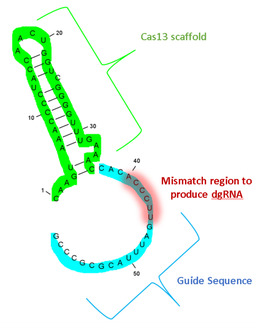
This strategy relies on the use of "dead gRNA" (d-gRNA) sequences, which are modified to include mismatches at defined sites within the guide region.

- gRNA is altered so that the Cas complex captures target specifically (and stays bound), but does not cleave the target.
- Captured nucleic acid is detected directly by pre-labelling with dye.
- Cyanine label is amplified through quantum dot-labeled anti-cyanine antibodies

Schematic of Immobilized Assay



Cas13 "dead" gRNA



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500-plex Panel

Targets for the MMD 500-plex panel are shown below. The number in each box is the estimated number of spots on the array for each target.

Target			Target			Target			Target		
Bordetella bronchoseptica	1	D	Influenza A-H1	1	D	Influenza A-H8	1	D	RSV-A	1	D
Bordetella holmesii	1	D	Influenza A-H10	1	D	Influenza A-H9	1	D	RSV-B	1	D
Bordetella pertussis & Bordetella parapertussis	1	D	Influenza A-H11	1	D	Influenza A	1	D	*Legionella pneumophila*	1	D
Chlamydophila pneumoniae	1	D	Influenza A-H12	1	D	Influenza B	1	D	*Mycoplasma pneumoniae*	1	D
Exogeneous control MS2	1	C	Influenza A-H13	1	D	Influenza A-N1	1	D	MERS-CoV	1	D
hCoV-229E	1	D	Influenza A-H14	1	D	Influenza A-N2	1	D	Sarbecovirus subgenus	1	D
hCoV-HKU1	1	D	Influenza A-H15	1	D	Influenza A-N3	1	D	SARS-CoV-1	1	D
hCoV-NL63	1	D	Influenza A-H16	1	D	Influenza A-N4	1	D	SARS-CoV-2	1	D
hCoV-OC43	1	D	Influenza A-H2	1	D	Influenza A-N5	1	D	SARS-related viruses	1	D
Human adenovirus Type A	1	D	Influenza A-H3	1	D	Influenza A-N6	1	D	Human parainfluenza 1	1	D
Human adenovirus Type B	1	D	Influenza A-H4	1	D	Influenza A-N7	1	D	Human parainfluenza 2	1	D
Human adenovirus Type C	1	D	Influenza A-H5	1	D	Influenza A-N8	1	D	Human parainfluenza 3	1	D
Human adenovirus Type D	1	D	Influenza A-H6	1	D	Influenza A-N9	1	D	Human parainfluenza 4	1	D
Human adenovirus Type E	1	D	Influenza A-H7	1	D	Human bocavirus	1	D	Human metapneumovirus	1	D
Human adenovirus Type F	1	D	Argentinian mammarenavirus (Junin)	1	D	AlphaCoV genus	1	D	*Enterobacter aerogenes*	1	D
Acinetobacter baumannii	1	D	Lassa mammarenavirus	1	D	BetaCoV genus	1	D	*Enterobacter cloacae susp. cloacae*	1	D
Acinetobacter calcoaceticus-baumannii complex	1	D	Lujo mammarenavirus	1	D	DeltaCoV genus	1	D	*Enterobacter cloacae subsp. hormaechei*	1	D
Acinetobacter spp.	1	D	Machupo mammarenavirus	1	D	GammaCoV genus	1	D	*Enterobacter cloacae subsp. kobei*	1	D
Actinomyces spp.	1	D	*Francisella tularensis - pan*	1	D	Andes Orthohantavirus	3	D	*Enterobacter cloacae subsp. roggenkampii*	1	D
Aspergillus flavus	1	D	*F. tularensis holartica*	1	D	Dobrava-Begrade orthohantavirus	1	D	*Enterobacter cloacae complex*	2	D
Aspergillus fumigatus	1	D	*F. tularensis novicida*	1	D	Puumala orthohantavirus	3	D	*Escherichia coli - pan*	2	D
Aspergillus spp.	3	D	*F. tularensis tularensis*	1	D	Seoul orthohantavirus	3	D	*Escherichia coli O157:H1*	2	D
Bacillus anthracis	4	D	Flaviviridae DENV	4	D	Sin Nombre orthohantavirus	3	D	*Pneumocystis jerovecci*	1	D
Bacillus atrophaeus	1	D	Flaviviridae JEEV	1	D	Hantaan orthohantavirus	3	D	Chikungunya virus	1	D
Bacillus thuringiensis	2	D	Flaviviridae SLEV	1	D	Influenza A oseltamivir resistance	2	D	Rabies virus	1	D
Brucella spp.	1	D	Flaviviridae WNV	2	D	Influenza A/H1-2009	1	D	African Swine Fever Virus	1	D
Burkholderia cepacia	2	D	*Giardia spp.*	1	D	Influenza B-Victoria	1	D	Yellow Fever Virus	1	D
Burkholderia mallei	1	D	*Haemophilus influenzae*	1	D	Influenza B-Yamagata	1	D	Tick-borne encephalitis virus	1	D
Burkholderia pseudomallei	1	D	Human Rhinovirus/Enterovirus	6	D	Goat pox virus	1	D	*Listeria monocytogenes*	1	D
Campylobacteraceae spp.	3	D	Extended spectrum b-lactamase (CTX-M, TEM)	7	D	Lumpy Skin Disease Virus	1	D	Vaccinia virus	1	D
Candida albicans	1	D	Human Endogenous controls	35	C	Sheep pox virus	1	D	Nipah virus	1	D
Candida lusitaniae	1	D	*Klebsiella pneumoniae complex*	4	D	Monkeypox virus	1	D	Hendravirus	1	D
Candida spp.	4	D	*Chronobacter spp.*	1	D	Omsk hemorrhagic fever virus	1	D	Foot and Mouth Disease virus	3	D
Carbapenemase (KPC, NDM,Oxa-23, Oxa 24/40, Oxa-48, Oxa48-like, Oxa-58, VIM, IMP)	18	D	Porcine Reproductive and Respriatory Syndrome (PRRS) - Eastern Europe	1	D	Porcine Reproductive and Respiratory Syndrome (PRRS) - Europe	1	D	Porcine Reproductive and Respiratory Syndrome (PRRS) - North America	1	D
Citrobacter freundii	1	D	Ravn Marburgvirus	1	D	Host Biomarker - ARL17B	1	D	*Chlamydophila psittaci*	1	D
Clostridium botulinum	1	D	Marburg Marburgvirus	2	D	Host Biomarker - CCL20	1	D	*Vibrio cholerae*	2	D
Clostridium botulinum toxin A	5	D	Measles morbillivirus	3	D	Host Biomarker - CLEC5A	1	D	*Vibrio parahaemolyticus*	2	D
Clostridium botulinum toxin B	1	D	Methicillin resistance (mecA, mecC)	6	D	Host Biomarker - CNTN1	1	D	*Vibrio vulnificus*	2	D
Clostridium botulinum toxin C/D	1	D	*Moraxella catarrhalis*	1	D	Host Biomarker - CREB3L3	1	D	Camelpox virus	1	D
Clostridium botulinum toxin E	1	D	*Morganella morganii*	1	D	Host Biomarker - IGF1	1	D	Powassan virus	1	D
Clostridium botulinum toxin F	1	D	Mumps virus	1	D	Host Biomarker - IL1R2	1	D	Rinderpest virus	1	D
Clostridium botulinum toxin G	1	D	Murray Valley Virus	1	D	Host Biomarker - KLK5	1	D	*Borrelia burgdorferi*	1	D
Clostridium difficile	1	D	*Mycobacterium bovis*	1	D	Host Biomarker - MMP19	1	D	*Shigella spp.*	2	D
Clostridium perfringens	1	D	*Mycobacterium tuberculosis*	2	D	Host Biomarker - MUC3A	1	D	Shigella Virulence Factor	2	D
Clostridium tetani	1	D	TB - fluoroquinolones resistance	11	V	Host Biomarker - PLAU	1	D	*Staphylococcus aureus*	2	D
Corynebacterium diphtheriae	1	D	TB - isoniazid resistance	6	V	Host Biomarker - TNFRSF12A	1	D	*Stenotrophomonas maltophilia*	2	D
Corynebacterium striatum	1	D	TB - rifampicin resistance	6	V	*Rickettsia spp.*	2	D	*Streptococcus agalactiae*	2	D
Corynebacterium ulcerans	1	D	TB- SLID resistance	6	V	*Salmonella enterica Choloreaesuis*	2	D	*Streptococcus pneumoniae*	2	D
Corynebacterium pseudotuberculosis	2	D	*Mycoplasma capricolum capripneumoniae*	1	D	*Salmonella enterica Paratyphi*	2	D	*Streptococcus pyogenes*	1	D
Corynebacterium spp.	3	D	*Mycoplasma mycoides mycoides*	1	D	*Salmonella enterica Enteritides*	4	D	*Streptococcus spp.*	4	D
Coxiella burnetii	2	D	*Neisseria meningitides*	1	D	*Salmonella enterica Typhi*	3	D	Togaviridae EEEV	1	D
Cryptococcus spp.	5	D	Newcastle disease virus	1	D	*Salmonella enterica Typhimurium*	5	D	Togaviridae VEEV	3	D
Cytomegalovirus	1	D	*Nocardia spp.*	1	D	SARS2 - Alpha variant	18	V	Togaviridae WEEV	1	D
Ebolavirus - Bombali	1	D	Orthopox virus	2	D	SARS2 - Beta variant	18	V	Varicella Zoster virus	1	D
Ebolavirus - Bundibugyo	1	D	Peste des petits ruminants virus	2	D	SARS2 - Delta variant	18	V	*Yersinia enterocolitica*	2	D
Ebolavirus - Reston	1	D	Poliovirus	1	D	SARS2 - Gamma variant	17	V	*Yersinia pseudotuberculosis*	2	D
Ebolavirus - Sudan	1	D	*Proteus spp.*	4	D	SARS2 - Omnicron variant	18	V	*Yersinia pestis*	6	D
Ebolavirus - Tai Forest	1	D	*Pseudomonas aeruginosa*	1	D	*Serratia marcescens*	2	D			
Ebolavirus - Zaire	1	D	Pseudorabies Virus	1	D	Shiga Toxin	1	D			

Target Type	RNA	DNA	Both	Assay Type	D	V	C
					Detection	Variant	Control

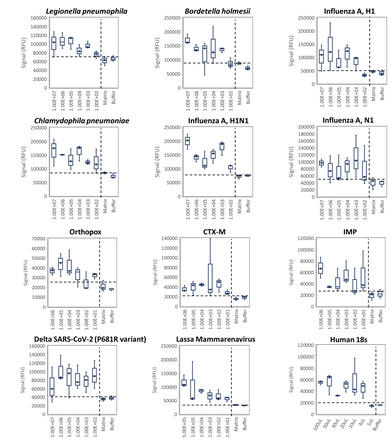
Results

Results for a subset of 12 assays are shown. Testing was done with a background of artificial nasal matrix. Target material was quantified by droplet digital PCR prior to testing. All plate-based assay experiments were in triplicate.

Assay Sensitivity

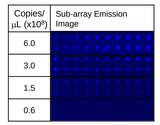
Assay	Target Type	Limit of Detection
Bordetella holmesii	Bacteria	1000 copies
Legionella pneumophila	Bacteria	1000 copies
Chlamydophila pneumoniae	Bacteria	100 copies
Human 18S	Host internal control	3mL of nasal matrix
Orthopox	DNA virus	10,000 copies
FluA	RNA virus	100 copies
Influenza A-H1	RNA virus	1000 copies
Influenza A-N1	RNA virus	1000 copies
Lassa mammarenavirus	RNA virus	10 copies
IMP	AMR marker	1000 copies
CTX-M-2	AMR marker	10 copies
P681R SARS-CoV-2 Delta	Variant assay	100 copies

Dynamic Range

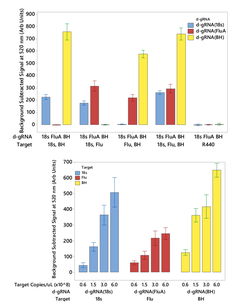


Inclusivity and Exclusivity

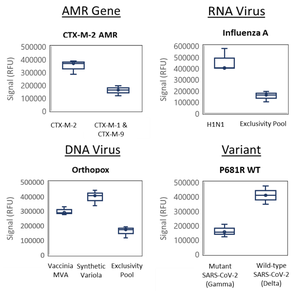
A **Example Subarray Images for 18s**



B



Inclusivity and Exclusivity Testing using printed microarray assay. Microarray results only used cyanine dye without signal amplification. **A.** Example Subarray image **B.** Specificity test (top); Sensitivity test (Bottom)



Inclusivity and Exclusivity in plate-based assay. Inclusivity organisms: CTX-M-2 - 1000 copies *Klebsiella pneumoniae* with CTX-M-2 gene; Influenza A – 100 copies H1N; Orthopox - 1000 copies Vaccinia MVA, 1E8 copies synthetic Variola sequence (equivalent in ng to 5E4 copies); P681R WT - 500 copies Gamma SARS-CoV-2 genome (does not include P681R mutation). Exclusivity Organisms: CTX-M-2 - Pooled 1000 copies of each: *Klebsiella pneumoniae* with CTX-M-15 gene (CTX-M-1 group), *Escherichia coli* with CTX-M-14 gene (CTX-M-9 group); Influenza A - Pooled 5000 copies each: SARS-CoV-2, SARS-CoV, Influenza B, RSV-A, RSV-B, Cov229E; Orthopox - Pooled 5000 copies each: SARS-CoV-2, SARS-CoV, Influenza B, RSV-A, RSV-B, Cov229E; P681R WT - 500 copies Delta SARS-CoV-2 genome (does include P681R mutation). N=3 replicates

Summary

- Dead-gRNA capture assay with signal amplification has shown highly promising results
- Current LoDs indicate 1000 copies and fewer can be detected
- Dynamic range for detection covers over 4 logs
- Specific detection show for DNA virus, RNA, viruses, AMR genes, and sequence variants.

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