Novel Assays to Support Human Respiratory Syncytial Virus Drug and Vaccine Development

Justin De La Cruz, Terri Wrin, Yolanda Lie, Andrew Gale, Christos J. Petropoulos

Monogram Biosciences, Inc., Laboratory Corporation of America® Holdings
South San Francisco, CA, USA

JUSTIN DE LA CRUZ, Ph.D
Monogram Biosciences
345 Oyster Point Blvd
South San Francisco, CA 94080
Telephone: (650) 624-4179
Email: *delacj7@labcorp.com*

Background

Respiratory syncytial virus (RSV) is a negative-sense, single-stranded RNA virus of the paramyxovirus family and can be separated into two main groups, genotypes A and B. Human RSV (hRSV) is the major cause of severe respiratory infections such as bronchiolitis and lower tract illness, affecting mostly newborns and young children, as well as the elderly, and immune-compromised.

Over 200,000 deaths per year can be attributed to hRSV and there are no effective therapies to counteract the disease. Therefore, efforts are focused on generating a vaccine to prevent hRSV infection as well as developing new therapeutic drugs to treat RSV infection and reduce the potential long-term effects caused by RSV infection.

Objectives

Although disease severity for hRSV remains high for at-risk populations, current treatment options are limited. Furthermore, assays to support the development of effective drug and vaccine efforts are cumbersome and/or inadequate.

Consequently, we have developed capabilities to (a) rapidly quantify hRSV viral load, (b) simultaneously assign A or B subtype, (c) derive F (fusion) gene sequences, and (d) phenotypically assess the susceptibility of clinical hRSV isolates to F protein-mediated neutralization.

Methods

Nucleic Acid Extraction

Primary hRSV samples (cultured isolate, nasal swab) were spiked with MS2 (recovery control) and incubated in lysis buffer. Nucleic acids were extracted using the bioMérieux NucliSENS easyMAG instrument. Following the addition of magnetic silica beads, the easyMAG automatically performed the incubation, washing, and elution steps.

Quantitative Real-Time PCR

We developed a multiplex, quantitative real-time PCR (qRT-PCR) assay that simultaneously quantifies viral load (copies/mL) and distinguishes A versus B subtype. Universal primers were used to reverse transcribe and amplify a segment of the hRSV N (nucleocapsid) gene. Differentially-labeled probes were used to distinguish A and B strains; MS2 control detected in triplex (MGB 3plex). C_t values were standardized to *in vitro* transcription products representing RSV A and RSV B.

Sequencing Fusion Glycoprotein

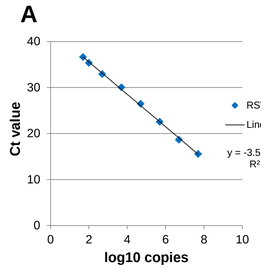
Fusion (F) glycoprotein sequences of cultured hRSV isolates were amplified by RT-PCR. Samples were then subjected to AxyPrep magnetic PCR purification, Nextera DNA library preparation, and MiSeq paired-end sequencing. Analysis of reads was performed using Monogram Biosciences' deep sequencing pipeline.

Neutralization Susceptibility

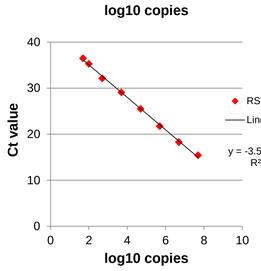
Codon-optimized hRSV F protein expression vectors were constructed and used to generate luciferase reporter pseudovirus preparations to assess susceptibility to polyclonal and monoclonal anti-RSV antibodies. Target cells were infected with pseudovirus preparations +/- antibodies, and relative light units (RLU) were measured at day 3 post-infection.

Results

A



Input		Measured		
copies	log10 copies	RSVA Ct	log10 copies	copies
5.00E+07	7.70	15.54	7.69	48,769,747
5.00E+06	6.70	18.64	6.81	6,421,794
5.00E+05	5.70	22.53	5.70	503,450
5.00E+04	4.70	26.44	4.59	39,169
5.00E+03	3.70	30.03	3.57	3,748
5.00E+02	2.70	32.89	2.76	575
1.00E+02	2.00	35.33	2.07	117
5.00E+01	1.70	36.62	1.70	50



Input		Measured		
copies	log10 copies	RSVB Ct	log10 copies	copies
5.00E+07	7.70	15.39	7.57	37,163,632
5.00E+06	6.70	18.24	6.76	5,782,907
5.00E+05	5.70	21.72	5.78	595,884
5.00E+04	4.70	25.48	4.71	51,425
5.00E+03	3.70	29.05	3.70	5,000
5.00E+02	2.70	32.10	2.83	681
1.00E+02	2.00	35.27	1.94	87
5.00E+01	1.70	36.44	1.60	40

RSV A	Known VL	MGB 3plex
1A.1	1,100,000	2,249,378
1A.2	1,300,000	2,172,043
1A.3	1,200,000	2,227,539
1B.1	893,000	256,557
1B.2	136,000	265,169
1B.3	120,000	210,356
1C.1	12,600	27,987
1C.2	14,200	36,763
1C.3	13,100	27,952
1D.1	1,960	3,437
1D.2	2,350	3,240
1D.3	1,650	4,506
1E.1	140	445
1E.2	ND	2,196
1E.3	1,530	998

RSV B	Known VL	MGB 3plex
2A.1	44,000,000	30,513,179
2A.2	24,000,000	21,187,163
2A.3	26,000,000	25,762,193
2B.1	2,100,000	3,414,058
2B.2	1,400,000	2,360,924
2B.3	2,900,000	2,426,159
2C.1	134,000	120,437
2C.2	67,600	100,514
2C.3	229,000	176,436
2D.1	12,200	4,886
2D.2	45,900	9,821
2D.3	5,800	10,809
2E.1	500	116
2E.2	800	814
2E.3	700	206

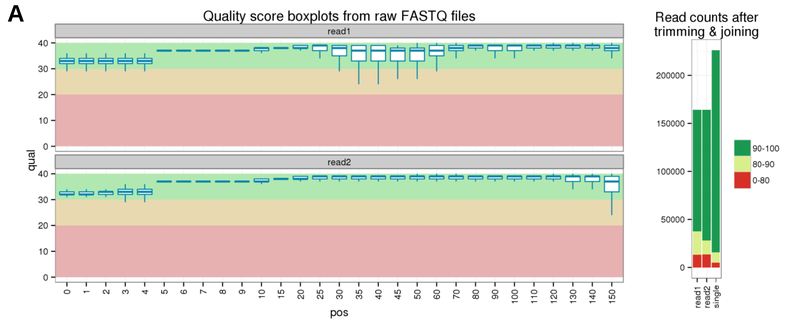
B

Sample	MGB 3plex RSVA	MGB 3plex RSVB	GenMark RVP RSVA	GenMark RVP RSVB
2014-1	UND	25.59		X
2014-2	UND	27.35		X
2014-3	UND	27.28		X
2014-4	UND	28.17		X
2014-5	UND	37.70		X
2014-6	27.48	UND	X	
2014-7	UND	31.76		X
2014-8	UND	29.35		X
2014-9	25.65	UND	X	
2014-10	31.93	UND	X	

Sample	MGB 3plex RSVA	MGB 3plex RSVB	GenMark RVP RSVA	GenMark RVP RSVB
2015-1	26.12	UND	X	
2015-2	27.03	UND	X	
2015-3	UND	28.23		X
2015-4	UND	28.10		X
2015-5	29.17	UND	X	
2015-6	UND	31.11		X
2015-7	UND	29.76		X
2015-8	UND	28.94		X
2015-9	UND	29.13		X
2015-10	UND	26.19		X

Figure 1. Quantifying and subtyping hRSV using a multiplex qRT-PCR assay. **A**) Linearity, sensitivity, and accuracy defined with *in vitro* transcripts and a proficiency panel of samples with known viral load. **B**) Comparative study of our qRT-PCR assay (C_t values shown, UND=undetermined) with the qualitative GenMark eSensor RVP assay utilizing previously uncharacterized hRSV isolates.

A



B

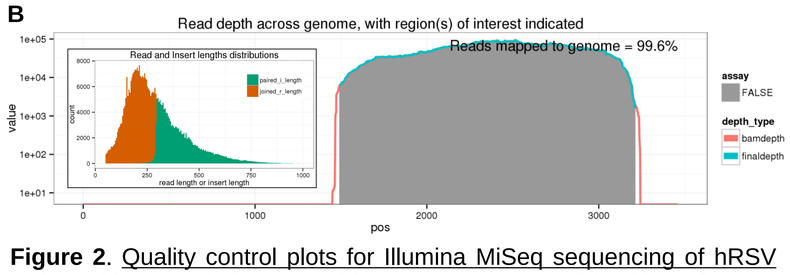


Figure 2. Quality control plots for Illumina MiSeq sequencing of hRSV isolate, 2015-1. **A**) Q-score >30 across reads and **B**) >99% of reads mapped to the reference genome with evenly distributed read and insert lengths. Minor variants detected using 3-10% thresholds, with accompanying data on each variant codon, amino acid and frequency.

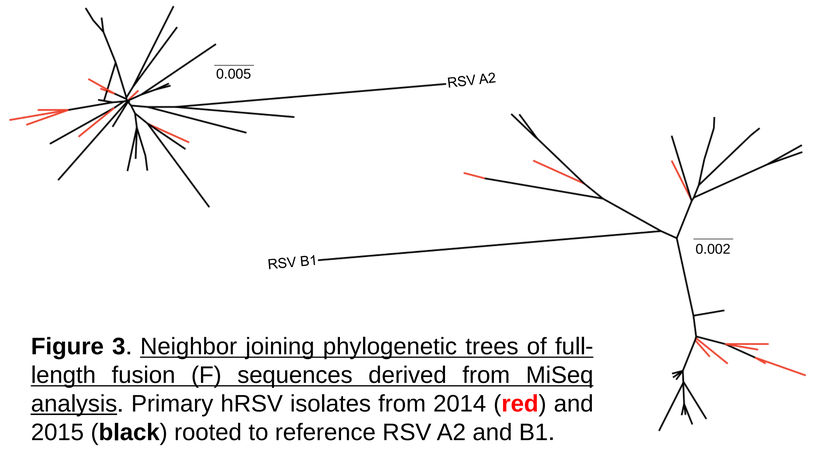
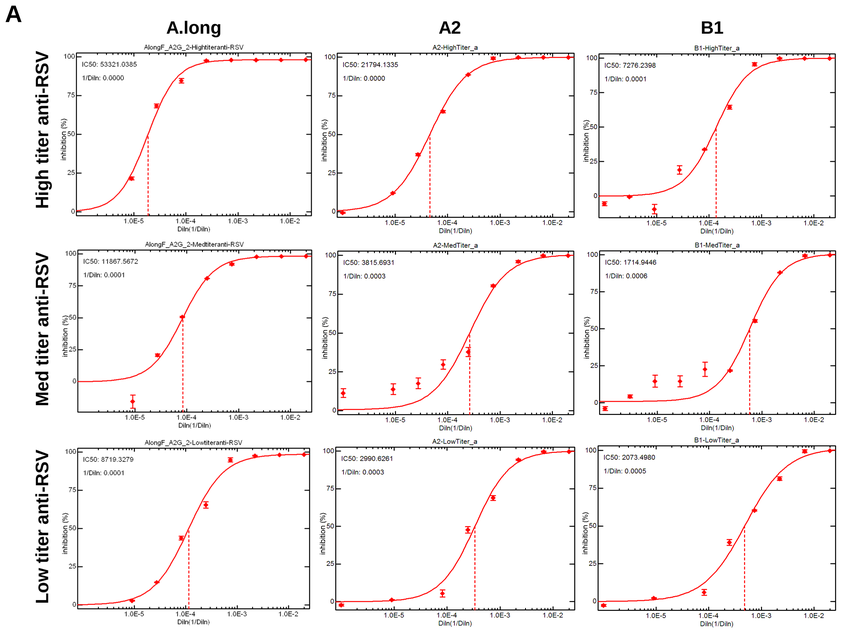


Figure 3. Neighbor joining phylogenetic trees of full-length fusion (F) sequences derived from MiSeq analysis. Primary hRSV isolates from 2014 (**red**) and 2015 (**black**) rooted to reference RSV A2 and B1.

A



B

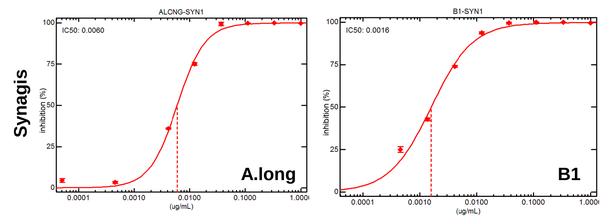


Figure 4. Assessing susceptibility of RSV subtypes (A.long, A2, and B1) to polyclonal and monoclonal antibodies. **A**) polyclonal antisera (high, medium and low) and **B**) a monoclonal antibody fusion inhibitor, Synagis®. IC50 values reported as 1/*n* dilution or *n* ug/ml, respectively.

Conclusions

This new suite of assays enables the simultaneous quantification and subtyping of hRSV and the genotypic and phenotypic characterization of hRSV.

1. The qRT-PCR assay provides accurate, sensitive, and linear assessments of hRSV viral load
2. Subtype specificity is 100% concordant with an FDA-approved qualitative assay (GenMark eSensor)
3. Amplification and sequencing of the F ORF is achieved at an LLOQ of ~500 copies; minor variants are detected at a 3-10% threshold
4. Antibody neutralization is measured using a panel of viruses pseudotyped with reference or patient-derived F protein, demonstrating the ability of the assay to assess the efficacy of fusion inhibitors and neutralizing antibodies to treat or prevent hRSV infection