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Short Report: Comprehensive Panel of Real-Time TaqMan™ Polymerase Chain Reaction Assays for Detection and Absolute Quantification of Filoviruses, Arenaviruses, and New World Hantaviruses

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Abstract. Viral hemorrhagic fever is caused by a diverse group of single-stranded, negative-sense or positive-sense RNA viruses belonging to the families *Filoviridae* (Ebola and Marburg), *Arenaviridae* (Lassa, Junin, Machupo, Sabia, and Guanarito), and *Bunyaviridae* (hantavirus). Disease characteristics in these families mark each with the potential to be used as a biological threat agent. Because other diseases have similar clinical symptoms, specific laboratory diagnostic tests are necessary to provide the differential diagnosis during outbreaks and for instituting acceptable quarantine procedures. We designed 48 TaqMan™-based polymerase chain reaction (PCR) assays for specific and absolute quantitative detection of multiple hemorrhagic fever viruses. Forty-six assays were determined to be virus-specific, and two were designated as pan assays for Marburg virus. The limit of detection for the assays ranged from 10 to 0.001 plaque-forming units (PFU)/PCR. Although these real-time hemorrhagic fever virus assays are qualitative (presence of target), they are also quantitative (measure a single DNA/RNA target sequence in an unknown sample and express the final results as an absolute value (e.g., viral load, PFUs, or copies/mL) on the basis of concentration of standard samples and can be used in viral load, vaccine, and antiviral drug studies.

The disease-causing hemorrhagic fever RNA viruses have the potential to be used as aerobiological weapons, indicating that accurate and timely identification of these agents is necessary.[1,2] The implicated hemorrhagic fever viruses include Ebola, Marburg, Lassa, Junin, Machupo, Sabia, Guanarito, and hantavirus. TaqMan™-based, real-time reverse transcription–polymerase chain reaction (RT-PCR) can rapidly detect and identify the selected gene targets found within the genomes of these RNA viruses. Although many research groups have developed and tested standard and real-time RT-PCR assays for detection of various species and corresponding strains of Ebola,[3–8] Marburg,[4,8,9] Lassa[8,10–12] Junin,[13] and hantavirus,[14–18] a comprehensive panel of TaqMan™-based real-time PCR assays using identical cycling conditions is not currently available.

We designed 48 TaqMan™-based PCR assays for specific and absolute quantitative detection of multiple hemorrhagic fever viruses. A select few of these assays were used with unidentified human isolates drawn from suspected Lassa fever cases at the Kenema Government Hospital in Sierra Leone. The approved human use protocols for this study are United States Army Medical Research Institute of Infectious Diseases FY09-32 and Tulane University Institutional Review Board Study #09-00332.

Primer pairs and TaqMan™/TaqMan™-minor groove binder (MGB) probes were designed with either Primer Express version 2.0 (PE2) (Applied Biosystems, Foster City, CA; www.appliedbiosystems.com) or AlleleID 4/5/6 (PREMIER BioSoft, Palo Alto, CA; www.premierbiosoft.com) using gene targets and genomes identified by accession numbers in Table 1. Hemorrhagic fever virus family genomes were initially aligned using ClustalW2 (http://www.ebi.ac.uk/clustalw/index.html) with nonhomologous regions identified visually. The PE2 software was then implemented to design virus-specific TaqMan™ and TaqMan™-MGB assays. Viral genomes aligned with AlleleID used the integrated ClustalW2 algorithms with the Species-Specific Design option selected in the software. After primer/probe targets were chosen by the software, corresponding viral genes were identified. Development of pan assays, defined as a single assay capable of detecting multiple viral sequences, were also attempted using the Taxa-Specific/Cross-Species Design option of AlleleID. AlleleID was able to design pan assays for detection of Marburg virus only (Table 1). The design of virus-specific assays by both software programs resulted in 48 primer/probe pairs (27 TaqMan™-MGB and 21 TaqMan™) using several gene targets: nucleoprotein, glycoprotein, virus protein 40, polymerase, and zinc-binding protein.

Virus preparations were produced in continuous cell lines appropriate for each virus. Virus stocks were harvested prior to peak cytopathic effects to minimize contaminating cellular components such as RNA or DNA. The cell culture supernatant containing intact virions was clarified by high-speed centrifugation. For extraction of viral RNA, virus stock was added to a virus-inactivating volume (3:1) of TRIzol reagent (Invitrogen, Grand Island, NY; www.invitrogen.com). Four-hundred microliters of TRIzol-inactivated virus was either extracted manually using the Ambion MagMAX™ Viral RNA Isolation Kit (Ambion, Austin, TX; www.ambion.com) or automated using the BioRobot EZ1 Virus Mini Kit V 2.0 (Qiagen, Valencia, CA; www.qiagen.com). All viral genomic RNAs were eluted in 60 μL of elution buffer (Ambion) or AVE buffer (Qiagen) and stored at –80°C. In a related study, viral RNA extracted by the Qiagen and Ambion methods was directly compared using identical assay conditions and viral genomic RNA concentrations and resulted in uniform cycle threshold (C_t) regardless of extraction method.[19]

All real-time RT-PCR assays were performed with specific primers and probes using the Invitrogen SuperScript™

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TABLE 1

Real-time PCR assay primers, probes, reaction concentrations and sensitivities*

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Ebola Zaire-MGB	NP	AF086833	76	F565	5′ - TCT GAC ATG GAT TAC CAC AAG ATC - 3′	0.9	0.001
				R640	5′ - GGA TGA CTC TTT GCC GAA CAA TC - 3′	0.9	
				p597S	6FAM - AGG TCT GTC CGT TCA A - MGBNFQ	0.2	
Ebola Sudan-MGB	NP	AY729654	80	F1051	5′ - CAT GCA GAA CAA GGG CTC ATT C - 3′	1.0	0.1
				R1130	5′ - CTC ATC AAA CGG AAG ATC ACC ATC - 3′	1.0	
				p1079S	6FAM - CAA CTT CCT GGC AAT - MGBNFQ	0.2	
Ebola Reston-MGB	GP	AB050936	55	F1129	5′ - TCA CCG CGA ACC CAA TG - 3′	0.8	1.0 (34 copies)‡
				R1183	5′ - TCG CTT GTC ATG GTT GGA CTT - 3′	0.8	
				p1149S	6FAM - ACC ACC ATT GCC C- MGBNFQ	0.2	
Ebola Ivory Coast-MGB	GP	U28006	64	F1123	5′ - CCC ATC TCC GCC CAC AA - 3′	0.7	1.0 (586 copies)‡
				R1186	5′ - GAG TGG AAT CCT CTG AAA CCA ATT - 3′	0.7	
				p1143S	6FAM - CGC AGG CGA AGA C - MGBNFQ	0.2	
Ebola Zaire-TM	GP	AF086833	80	F2000	5′ - TTT TCA ATC CTC AAC CGT AAG GC - 3′	1.0	0.0001 (584 copies)‡
				R2079	5′ - CAG TCC GGT CCC AGA ATG TG - 3′	1.0	
				p2058A	6FAM - CAT GTG CCG CCC CAT CGC TGC - TAMRA - 3′	0.1	
Ebola Sudan-TM	GP	AY729654	77	F583	5′ - AGG ATG GAG CTT TCT TCC TCT ATG - 3′	0.8	0.1
				R659	5′ - TAC CCC CTC AGC AAA ATT GAC T - 3′	0.8	
				p608SB	6FAM - CAG GCT GGC TTC AAC TGT AAT TTA CAG AGG - TAMRA -3′	0.1	
Ebola Reston-TM	VP40	AF522874	80	F5645	5′ - CTA TGG TTA TCA CCC AGG ATT GTG - 3′	0.9	1.0
				R5724	5′ - GTA ACT ATC CTG CTT GTC CAT GTG - 3′	0.9	
				p5674S	6FAM - TGC CAC TCT CCA GCC AGC CAT CCG - TAMRA - 3′	0.1	
Ebola Ivory Coast-TM	GP	U28006	79	F564	5′ - TGT ACA CAA AGT CTC AGG AAC TGG - 3′	1.0	0.1
				R642	5′ - GTC ATA CAG GAA GAA GGC TCC TTC - 3′	1.0	
				p589S	6FAM - CCA TGC CCA GGA GGA CTC GCC TTT - TAMRA -3′	0.1	
Ebola Bundibugyo (MGB and TM)	NP	FJ217161	74	F2016	5′ - ATG GAA ACC AAG GCG AAA CTG - 3′	0.9	10^{-6} (RNA dilution)§ (MGB and TM)
				R2089	5′ - TAC TTG TGG CAT TGG CTT GTC T - 3′	0.9	
				p2045S	6FAM - CGG GTA GCC CCC AAC - MGBNFQ	0.2	
					or		
				p2038S	6FAM - ATC CCC ACG GGT AGC CCC CA - TAMRA	0.1	
***pan*-Marburg-MGB assay:** (Ravn, Ci67, Musoke, and Angola-MGB)	GP	EF446131 (Ravn) EF446132 (Ci67) DQ217792 (Musoke) DQ447660 (Angola)	64	F6121	5′ - GAT TCC CCT TTG GAA GCA TCT - 3′	1.0	0.1 (Ravn) 1.0 (Ci67) 10 (Musoke) 1.0 (Angola)
				F6121-1	5′ - GAT TCC CCT TTA GAG GCA TCC - 3′	1.5	
				R6184	5′ - CAA CGT TCT TGG GAG GAA CAC - 3′	1.0	
				p6144	6′FAM - ACG ATG GGC TTT CAG - MGBNFQ - 3′	0.2	
***pan*-Marburg-TM assay:** (Ravn, Ci67, Musoke, and Angola-TM)	GP	EF446131 (Ravn) EF446132 (Ci67) DQ217792 (Musoke) DQ447660 (Angola)	64	F6121	5′ - GAT TCC CCT TTG GAA GCA TCT - 3′	0.5	0.1 (Ravn) 10 (Ci67) 1.0 Musoke) 10 (Angola)
				F6121-1	5′ - GAT TCC CCT TTA GAG GCA TCC - 3′	1.0	
				R6184	5′ - CAA CGT TCT TGG GAG GAA CAC - 3′	1.0	
				p6145A-5	6FAM - AAA CGA TGG GCC TTC AGG GCA GG-TAMRA-3′	0.1	
				p6145A-7	6FAM - AAG CGA TGG GCT TTC AGG ACA GG-TAMRA-3′	0.1	

(*continued*)

TABLE 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Marburg Musoke-MGB	NP	DQ217792	65	F391	5′ - CAA CCC GCT TTC TGG ATG TG - 3′	1.0	10
				R455-3	5′ - CTT AAG GGC TAG AAT TAA AGG GCT - 3′	1.0	
				p429A	6FAM - TAA TGA GGT TCG TTA GGA A - MGBNFQ	0.2	
Marburg Ci67-MGB	NP	EF446132	65	F391	5′ - CAA CCC GCT TTC TGG ATG TG - 3′	0.9	0.01 (120 copies)‡
				R455	5′ - CTT AAG GGC CAA AAT TAA AGG ACT G -3′	0.9	
				p413S	5′ - 6FAM - TCC TAA CGA ACC TCA - MGBNFQ	0.2	
Marburg Ravn-MGB	NP	EF446131	66	F350	5′ - GGA CGC GGG CTA TGA GTT TG - 3′	0.9	0.1
				R415	5′ - GGA ATA ACC TCT AGA AAG CGA GTT G -3′	0.9	
				p371S	6FAM - TGT CAT CAA GAA TCC TG - MGBNFQ	0.2	
Marburg Angola-MGB	VP40	DQ447660	66	F4573T	5′ - CCA GTT CCA GCA ATT ACA ATA CAT ACA - 3′	0.6	0.1
				R4638G	5′ - GCA CCG TGG TCA GCA TAA GGA - 3′	0.6	
				p4601CT	6FAM - CAA TAC CTT AAC CCC C - MGBNFQ	0.2	
Marburg Ravn-TM	NP	DQ447649	77	F1788	5′ - TTA TAT GCT CAG GAA AAG AGA CAG G - 3′	0.9	0.1
				R1864	5′ - CCA ATA CTG CCA AAG GGA TCT TG - 3′	0.9	
				p1815S	6FAM - CCC ATA CAG CAT CCA GCC GTG AGC - TAMRA - 3′	0.1	
Marburg Angola-TM	NP	DQ447660	80	F985	5′ - TCT ATC CTC AGC TCT CAG CAA TTG - 3′	1.0	0.1
				R1064	5′ - TTC GCC GAC ATT GAC ACC AG - 3′	1.0	
				p1035A	5′ - 6FAM - TGC CAT GTG CTG TCG CTA CAC CCA - TAMRA - 3′	0.1	
Lassa Josiah-MGB	GPC	AY628203	80	F3569	5′ - TGC TAG TAC AGA CAG TGC AAT GAG - 3′	0.9	0.1 (268 copies)‡
				R3648	5′ - TAG TGA CAT TCT TCC AGG AAG TGC - 3′	0.9	
				p3598S	6FAM - TGT TCA TCA CCT CTT C - MGBNFQ	0.2	
Lassa Macenta-MGB	NP	AY628201	73	F1079	5′ - CAG GAA GGG CAT GGG AAA - 3′	0.8	10^{-6} (RNA dilution)§ (257 copies)‡
				R1151	5′ - TTG TTG CTC CCA ATT TTT TGT G - 3′	0.8	
				p1106S	6FAM - TTG ATT TGG AAT CAG GCG AG - MGBNFQ	0.2	
Lassa Weller-MGB	NP	AY628206	72	F1695	5′ - GCA TTG ATG GAC TGC ATT ATG TTT - 3′	0.9	0.1 (583 copies)‡
				R1766	5′ - CAC AGC TCT TAG GAC CTT TGC AT - 3′	0.9	
				p1720S	6FAM - ATG CAG CAG TCT CGG GA - MGBNFQ	0.2	
Lassa Pinneo-MGB	GPC	AY628207	78	F3525	5′ - CCA ATA ATC CCA CAT GTA GCG ATG - 3′	0.9	0.001
				R3602	5′ - GAA CAT TGT GCT AAT TGC GCT TTC - 3′	0.9	
				p3559S	6FAM - CCT TCA AGA TTG CCA - MGBNFQ	0.2	
Lassa Mozambique (Mopeia)-MGB	NP	DQ328874	78	F1788	5′ - TCT GGG GAC CGG CAA TTG TG - 3′	0.9	1.0
				R1865	5′ - ACA CCA CAT TGT GCC TTA CTA GAC - 3′	0.9	
				p1815S	6FAM - TAT GAC TGC TGC TTC - MGBNFQ	0.2	
Lassa Mobala (Acar)-MGB	GPC	AY342390	80	F1858	5′ - TAC AGA CCA CAG CTA CAC ACA CC - 3′	1.0	0.001
				R1937	5′ - ACT CAC CGT CAC CTG GTT GG - 3′	1.0	
				p1915A	6FAM - AGC CGT GCC CAA AG - MGBNFQ	0.2	
Lassa Josiah-TM	NP	AY628203	70	F548	5′ - GGA ATG AGT GGT GGT AAT CAA GG - 3′	0.6	0.1 (11,711 copies)‡
				R617	5′ - TTT TCA CAT CCC AAA CTC TCA CC - 3′	0.6	
				p594A	6FAM - ACT CCA TCT CTC CCA GCC CGA GC-TAMRA - 3′	0.1	

(*continued*)

TABLE 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Lassa Macenta-TM	NP	AY628201	73	F1079	5′ - CAG GAA GGG CAT GGG AAA - 3′	0.8	10^{-5} (RNA dilution)§ (257 copies)‡
				R1151	5′ - TTG TTG CTC CCA ATT TTT TGT G - 3′	0.8	
				p1098S	6FAM – CAC TGT TGT TGA TTT GGA ATC AGG CGA GAA G - TAMRA - 3′	0.1	
Lassa Weller-TM	NP	AY628206	72	F1695	5′ - GCA TTG ATG GAC TGC ATT ATG TTT - 3′	0.9	0.01 (583 copies)‡
				R1766	5′ - CAC AGC TCT TAG GAC CTT TGC AT - 3′	0.9	
				p1720S	6FAM - ATG CAG CAG TCT CGG GAG GGC TC-TAMRA - 3′	0.1	
Lassa Pinneo-TM	GP	AY628207	81	F2730	5′ - CCC AGT TTC CCT TTC CTG AGT - 3′	0.8	0.1 (234 copies)‡
				R2810	5′ - CCA ACG GAG TGT TGC AAA CA - 3′	0.8	
				p2757S	6FAM - CAA TGT ATC TTC CAC CCC AGG CCA TTC - TAMRA - 3′	0.1	
Lassa Mozambique (Mopeia)-TM	NP	DQ328874	81	F2687	5′ - CCT GAT GGT CTC CAG CAT ATT TC - 3′	0.9	0.1
				R2768	5′ - GCT ACA ATT TCA GCT TGT CTG C - 3′	0.9	
				p2712S	6FAM - CCC GTC TAT GAG GCA AGC CCC AGC - TAMRA - 3′	0.1	
Lassa Mobala (Acar)-TM	GP	AY342390	85	F1860	5′ - CAG ACC ACA GCT ACA CAC ACC - 3′	1.0	0.0001
				R1944	5′ - AAT TCC AAC TCA CCG TCA CCT G - 3′	1.0	
				p1915A	6FAM - AGC CGT GCC CAA AGC CTC ATC GTC TC - TAMARA - 3′	0.1	
Machupo Carvallo-MGB	GPC	AY619643	80	F699	5′ - ATG ACC CGT GTG AGG AAG GG - 3′	0.7	0.001
				R778	5′ - GCC ACA GTA GTC AAA GGA ACT GG - 3′	0.7	
				p721S	6FAM - AGT GTG CTA CCT GAC CAT - MGBNFQ - 3′	0.2	
Machupo Mallele-MGB	GPC	AY619645	80	F406	5′ - CAC CTT CCT GAT TCG GGT CTC - 3′	0.8	0.001
				R485	5′ - CAA GGT CTT CTG GTT CAT AGA CTG - 3′	0.8	
				p428S	6FAM - AGT GTA TCT GTC CTC A - MGBNFQ - 3′	0.2	
Machupo Carvallo-TM	GPC	AY619643	78	F681	5′ - ATC TCT TCA GGG GCT TCC ATG - 3′	0.7	0.001
				R758	5′ - TGG GGT CAC CAC ACT GAT TG - 3′	0.7	
				p728A	6FAM - AGC ACA CTT TCC CTT CCT CAC ACG G - TAMRA - 3′	0.1	
Machupo Mallele-TM¶	Polymerase	AY619642	78	F1009	5′ - TTC GAT CAA CAT TGC AGC TAA ATC - 3′	0.5	0.001
				R1086	5′ - GAT GGT GTA TCG GTT GTT GCA G- 3′	0.5	
				p1059A	6FAM - CCA TTG TTC ACC GGG CAG GCC AGT - TAMRA - 3′	0.1	
Junin-MGB	NP	AY619641	65	F1933	5′ - CAT GGA GGT CAA ACA GCT TCC T - 3′	0.8	0.001 (234 copies)‡
				R1997	5′ - GCC TCC AGA CAT GGT TGT GA - 3′	0.8	
				p1956S	6FAM - ATG TCA TCG GAT CCT T - MGBNFQ	0.2	
Junin-MGB	Polymerase	AY619640	68	F2427	5′ - TTG CCA AAT TGA CCC ATC TGT A - 3′	0.8	0.001 (234 copies)‡
				R2494	5′ - GCA ATA TGC CGG GTG TAG TGA - 3′	0.8	
				p2452S	6FAM - TAG AGT TCG TCT GAT TTC A - MGBNFQ	0.2	
Junin-TM	NP	AY619641	79	F3032	5′ - CAG TTC ATC CCT CCC CAG ATC - 3′	1.0	0.0001
				R3110	5′ - GGT TGA CAG ACT TAT GTC CAT GAA GA - 3′	1.0	
				p3083A	6FAM - TGT TCA ACG AAA CAC AGT TTT CAA GGT GGG - TAMRA - 3′	0.1	

(*continued*)

TABLE 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Junin-TM	Zinc Binding Protein (Z)	AY619640	66	F282	5′ - AGG AAT TCG GAC CTC TGC AA - 3′	1.0	0.001
				R347	5′ - CTC CAC CGG CAC TGT GAT T - 3′	1.0	
				p303Sa	6FAM - ATC TGT TGG AAG CCC CTA CCT ACC A - TAMRA -3′	0.1	
Sabia-MGB	GP	U41071	65	F903	5′ - CGT CAG ATC TTA AGT GCT TTG GAA - 3′	0.8	0.001
				R967	5′ - TTC CGA ATC GTG GTC AAG GT - 3′	0.8	
				p928S	6FAM - CAC AGC ACT AGC AAA A - MGBNFQ	0.2	
Sabia-MGB	Polymerase	AY358026	73	F948	5′ - TGT GAT CAT TGG TCG CAG CTA - 3′	0.7	0.01
				R1020	5′ - CTG AGA GGG AAG AAG GCA GTG A - 3′	0.7	
				p970S	6FAM - ATC GTT ATC CAT TAG AAT CT - MGBNFQ	0.2	
Sabia-TM	GP	U41071	83	F903	5′ - CGT CAG ATC TTA AGT GCT TTG GAA - 3′	0.8	0.001
				R985	5′ - TTT CAA CAT GTC ACA GAA TTC CG - 3′	0.8	
				p959A	6FAM - CGT GGT CAA GGT TAC ATT TTG CTA GTG CTG TGT - TAMRA - 3′	0.1	
Sabia-TM	Hairpin Intergenic Region and RNA Polymerase	AY358026	79	F433	5′ - CCG GGG TGG TGT GGT TTA - 3′	0.7	0.01
				R511A	5′ - AAG GCT CAA GGG TGT TAC CTG - 3′	0.7	
				p460S	6FAM - TTC AAC GGA CTG CTG CTG TCT AAA TAG TCT - TAMRA - 3′	0.1	
Guanarito-MGB	GP1	AY129247	73	F485	5′ - TGG ATT CTT GGG TGG ACA ATT AA - 3′	0.6	0.01
				R557	5′ - TAG GCT CAC AGC AGA TTC TTG GA - 3′	0.6	
				p513S	6FAM - TGG GAC ATG ACT TTT T - MGBNFQ - 3′	0.2	
Guanarito-MGB	Polymerase	AY358024	80	F521	5′ - GCT GCC GGA GCT GTC TGA - 3′	0.7	0.1
				R599	5′ - ATG GTG CGA GTT TGT GGA CTT - 3′	0.7	
				p542S	6FAM - CAC CAA GTC CCT TAA AG - MGBNFQ - 3′	0.2	
Guanarito-TM	GP1	AY129247	73	F485	5′ - TGG ATT CTT GGG TGG ACA ATT AA - 3′	0.8	0.001
				R557	5′ - TAG GCT CAC AGC AGA TTC TTG GA - 3′	0.8	
				p532A1	6FAM - CCT CAA AAA GTC ATG TCC CAA TCC C - TAMRA - 3′	0.1	
Guanarito-TM	Polymerase	AY358024	80	F521	5′ - GCT GCC GGA GCT GTC TGA - 3′	0.7	0.01
				R599	5′ - ATG GTG CGA GTT TGT GGA CTT - 3′	0.7	
				p542S	6FAM - CAC CAA GTC CCT TAA AGA TTT GCT ATA GCA GAC AC - TAMRA -3′	0.1	
Hantavirus-MGB (Andes)	NP	AF291702	67	F41	5′ - GAA TGA GCA CCC TCC AAG AAT TG - 3′	0.9	10^{-5} (RNA dilution)§
				R107	5′ - CGA GCA GTC ACG AGC TGT TG - 3′	0.9	
				p71S	6FAM - ACA TCA CAG CAC ACG A - MGBNFQ	0.2	
Hantavirus-MGB (Sin Nombre and NY)	NP	L37904	71	F26	5′ - CTA CGA CTA AAG CTG GAA TGA GC - 3′	0.8	10^{-6} (SN) 10^{-2} (NY) (RNA dilution)§
				R96	5′ - GAG TTG TTG TTC GTG GAG AGT G - 3′	0.8	
				p59S	6FAM - AAG TGC AAG ACA ACA - MGBNFQ	0.2	

* PCR = polymerase chain reaction; PFU = plaque-forming units; MGB = minor groove binder; NP = nucleoprotein; GP = glycoprotein; VP = virus protein; GPC = glycoprotein precursor.
† Limit of Detection (LOD) for all assays were measured in PFU/PCR unless otherwise stated; all sensitivities were done in triplicate and were positive in three of three replicates.
‡ Synthetic RNA was used to determine LODs in genome copy number (see Materials and Methods).
§ LODs measured as dilution of stock RNA because PFU information was not available.
¶ Machupo Mallele-TM assay run at 62°C.

One-Step RT-PCR Kit plus bovine serum albumin and ran on the LightCycler 2.0 (Roche Applied Science, Indianapolis, ID; www.roche.com). The amplicon size, primer/probe sequence, optimum assay concentration, and sensitivity (limit of detection [LOD]) are shown in Table 1. All assays were conducted at a final concentration of 3 mM $MgSO_4$ and the following cycling conditions: 50°C for 15 minutes (1 cycle); 95°C for 5 minute (1 cycle); 95°C for 1 second and 60°C for 20 seconds (45 cycles); and 40°C for 30 seconds (1 cycle). A single fluorescence read was taken at the end of each 60°C step. The Machupo Mallele TaqMan™ assay was conducted at 62°C for 45 amplification cycles.

Initial primer/probe down selection was accomplished by testing for cross-reactivity to individual members of the applicable virus family. Each pair that passed this test was then evaluated for optimum primer concentration by using an internal protocol. The final concentration selected resulted in the earliest C_t, lowest LOD, and highest end point fluorescence. Further exclusivity analysis involved a screening against two RNA panels: a United States Army Medical Research Institute of Infectious Diseases viral hemorrhagic fever panel containing infected cell lysate genomic RNAs of Ebola (Zaire, Sudan Gulu and Boniface, Reston, Ivory Coast, Bundibugyo), Marburg (Musoke, Angola, Ravn, Ci67), Lassa (Josiah, Weller, Macenta, Pinneo, Mobala, Mozambique), Machupo (Carvallo, Mallele), Junin (Romero), Sabia, and Guanarito viruses. The second arthropod-borne virus panel contained purified viral genomic RNAs from Venezuelan equine encephalitis viruses (VEE) IA/B (strain Trinidad donkey), VEE IC (CO951006), VEE ID (1D V209-A-TVP1163),VEE IE (68U201),VEE IF (78V3531), VEE II (Everglades Fe3-7c), VEE IIIA (Mucambo),VEE IV (BeAR40403),VEE V (Cabassou Be508), VEE VI (AG80-663), eastern equine encephalitis viruses (Georgia 97, ARG-LL and 76V-25343), western equine encephalitis virus (CBA 87/4), Barmah Forest (Aus BH2 2193), Nduma, Sindbis (UgMP6440), Highlands J, Mayaro (BEH256), Middleburg, Semliki Forest, yellow fever, Japanese encephalitis (B-0005/85), chikungunya (vaccine strain 15561), and Getah (Amm2021) viruses.

Sensitivity testing using cell culture supernatant viral genomic RNA with a known plaque-forming units (PFU) concentration determined the LOD of each assay. The LOD is defined as the lowest concentration of target RNA detected three of three times. The Lassa Macenta RNA PFU concentration was unknown. Therefore, the RNA was serially diluted 10-fold and the LOD was determined to be the lowest dilution detected three of three times. Additionally, for several assays, single-stranded full-length target RNA sequences synthesized by Dharmacon (Lafayette, CO; www.dharmacon.com), were used to determine the target copy number. A 10-fold serial dilution (1 pg–1 ag/PCR) was used to determine the LOD. The equivalent target copy number was calculated based on the molecular weight and length of the target sequence. Moreover, once the LOD was established, a standard curve could easily be used for an absolute quantitative real-time RT-PCR (qRT-PCR) analysis of unknown samples.

Although the viral hemorrhagic fever virus assays were designed using the Roche LightCycler 2.0, all assays should be easily transferable with minimal master mixture adjustments to most TaqMan™-compatible machines, including the Fast Real-Time PCR System (Applied Biosystems), the SmartCycler (Cepheid, Sunnyvale, CA; www.cepheid.com) the R.A.P.I.D and JBAIDS (Idaho Technology, Salt Lake City, UT; www.idahotech.com), the Mx QPCR (Stratagene, La Jolla, CA; www.stratagene.com) and the iCycler (Bio-Rad, Hercules, CA; www.bio-rad.com). Recent data indicated an easy transfer of both probe chemistries from one platform to another through similar assay performance, sensitivity, and specificity on three of these instruments.[20]

In a pilot evaluation of the TaqMan™-MGB assay designed to detect Lassa Josiah genomic RNA, 39 blood samples from patients infected with Lassa virus in Sierra Leone[21,22] were tested as part of the Lassa surveillance and treatment program based at Kenema Government Hospital.[23,24] Five of these samples were serially collected from one patient (#G-104) on days 1, 2, 3, 8, and 15 after admission to the Lassa Fever Ward. Viral genomic RNA was extracted from TRIzol-treated samples as described above and tested with the six Lassa TaqMan™-MGB assays (Josiah, Weller, Macenta, Pinneo, Mobala, and Mozambique). The resulting data from the LightCycler 2.0 software determined that blood of the patient was positive for Lassa Josiah on days 1, 2, 3, and 8 but was not detectable on day 15. An absolute quantification analysis of viral load indicates that 714, 633, 447 and 555 PFU/mL of patient blood were detected in samples on days 1, 2, 3 and 8, respectively. Of the remaining patient samples, 23 were positive for Lassa Josiah. Every Lassa Josiah–negative patient sample was also negative in the other five Lassa assays. All assays exhibited robust positive controls, and all negative controls were as expected. These results indicate that the six Lassa virus-specific TaqMan™-MGB assays were highly specific with the capability to detect potential clinically relevant levels of Lassa virus.

In conclusion, the assays presented in this report are highly specific and sensitive for detection of multiple hemorrhagic fever viruses. Rigorous design of primers and probes (length, melting point, amplicon size), stringent down selection, a strict optimization regiment, and common rapid-cycling parameters demonstrate that precise detection of multiple species/strains of filoviruses, arenaviruses, and New World hantaviruses (Sin Nombre and Andes viruses) is possible. Unknown samples can be tested simultaneously with multiple assays on TaqMan™-compatible instruments because these assays were designed, optimized, and validated by using identical cycling parameters. An additional benefit is the ability for researchers to multiplex the assays in any preferred combination.

Most importantly, because these are absolute qRT-PCR assays, researchers will be able to detect and identify unknown organisms in a sample and use the assays for viral load, vaccine, and antiviral drug studies. Additional validation of the assays with in-field samples will increase data on assay specificity and indicate their utility for clinical and environmental matrices. This collection of TaqMan™-based viral RNA real-time qRT-PCR assays provide a repertoire of diagnostic tools that can serve as a foundation for the simultaneous identification and analysis of these potential biothreat agents when multiple, rapid cycling, real-time PCR platforms are used.

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