

1 **Sequence optimized real-time RT-PCR assay for detection of Crimean-Congo hemorrhagic fever**  
2 **virus**

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12  
13 **Abstract**

14  
15 Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* within  
16 the family *Bunyaviridae*. Infection can result in general myalgia, fever, and headache with some patients  
17 developing hemorrhagic fever with mortality rates ranging from 5-30%. CCHFV has a wide geographic  
18 range that includes Africa, Asia, the Middle East, and Europe with nucleotide sequence variation  
19 approaching 20% across the three negative-sense RNA genome segments. While phylogenetic clustering  
20 generally aligns with geographic origin of individual isolates, distribution can be wide due to  
21 tick/CCHFV dispersion via migrating birds. This sequence diversity negatively impacts existing  
22 molecular diagnostic assays, leading to false negative diagnostic results. Here, we updated our previously  
23 developed CCHFV real-time RT-PCR assay to include CCHFV strains not detected using that original  
24 assay. Deep sequencing of eight different CCHFV strains, including three that were not detectable using  
25 the original assay, identified sequence variants within this assay target region. New primers and probe  
26 based on the sequencing results and newly deposited sequences in GenBank greatly improved assay  
27 sensitivity and inclusivity. Subsequent comparison of this assay to another commonly used CCHFV real-  
28 time RT-PCR assay targeting a different region of the viral genome showed improved detection, and both  
29 assays could be used to mitigate CCHFV diversity with diagnostics. Overall, this work demonstrated the  
30 importance of viral sequencing efforts for robust diagnostic assay development with specific  
31 improvement in our currently fielded CCHFV assay.

## Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV; family *Bunyaviridae*, genus *Nairovirus*) infection of humans can result in a disease spectrum ranging from a nonspecific febrile illness to hemorrhagic fever manifestations with a mortality rate of 5-30% [1]. The relatively low rate of disease in seropositive populations has spurred research into potential host susceptibility factors [2-5], although the availability of appropriate supportive care may provide a more direct correlation with clinical outcomes. CCHFV is predominantly transmitted by ixodid ticks of the genus *Hyalomma*, and CCHFV has a wide geographic distribution with endemic foci in eastern Europe, sub-Saharan and southern Africa, the Middle East, and Asia [6, 7]. Handling of tick-infested livestock and proximity to vegetated areas with high tick burdens are significant risk factors for CCHFV infection. In addition, nosocomial exposure to CCHFV-infected individuals in low resource facilities can result in severe disease among healthcare workers [1, 7].

CCHFV is an enveloped virus with a trisegmented, negative-sense RNA genome that encodes an RNA-dependent RNA polymerase (L), two major structural glycoproteins ( $G_N$  and  $G_C$ ), and a nucleoprotein (N) on the L, M, and S genome segments, respectively. CCHFV has the largest genome of any bunyavirus at 19.1 kb total with 12.1, 5.4, and 1.6 kb in the three genome segments, respectively. To date, the NIAID Virus Pathogen Database and Analysis Resource (ViPR) lists complete genome sequences available for 54 (L), 75 (M), and 102 (S) genome segments of CCHFV isolates [8]. Pairwise alignments of these sequences indicate that their mean sequence identities are 89.4 % (L), 80.0 % (M), and 88.1 % (S).

Currently, there are no CCHFV vaccines or therapeutics approved for human use by the United States Food and Drug Administration, although immunoglobulin therapy and ribavirin have been used abroad with mixed results [14]. In the absence of approved countermeasures, effective diagnostics remain an invaluable means to identify and control CCHFV outbreaks. A variety of assay platforms for CCHFV can detect viral nucleic acids to include low density microarrays [15], high density resequencing arrays [16], padlock probes with colorimetric readout [17], LAMP [18], and polymerase chain reaction [19-23]. Real-time reverse-transcription PCR remains the gold standard for quantitative, sensitive, and specific detection of CCHFV; however, these assays have sensitivity issues due to the genetic diversity of different CCHFV strains [24].

Previously, Garrison et al developed a TaqMan MGB real-time RT-PCR assay capable of detecting eighteen strains of CCHFV [25]. In subsequent testing of this assay identified several additional strains which were undetectable by this assay [20]. We suspected the inherent diversity of CCHFV genome contributed to inefficient primer/probe hybridization. To improve the assay performance, we sequenced these strains and designed a set of degenerate primers and probes to take into account CCHFV diversity in the assay target region. This optimization increased assay sensitivity compared to the original Garrison et al. assay and to a commonly used assay developed by Atkinson et al [20, 26-31].

## Materials and Methods

**Viruses.** Multiple CCHFV strains including IbAr10200 (UCC# R4401), DAK8194 (UCC# R4416), SPU 128/81 (UCC# R4417), SPU 115/87 (UCC# R4448), UG 3010 (UCC# R4432), JD-206 (UCC# R4413), HY-13 (UCC# R4459), and Drosdov (UCC# R4405) were acquired from the Unified Culture Collection (UCC) maintained at US Army Medical Research Institute of Infectious Diseases (USAMRIID). Total RNA was extracted from 200  $\mu$ l of cell culture supernatant using TRIzol LS (Thermo Fisher Scientific, Waltham, MA), the EZ1 Advanced XL (Qiagen, Valencia, CA), and the EZ1 Virus Mini Kit V 2.0 (Qiagen) according the manufacturers' recommendations. Total nucleic acid was eluted in 90  $\mu$ l elution buffer and stored at -80 °C until use. Previously extracted RNA from additional CCHFV strains maintained at the USAMRIID including I-40, 2219 KKK28, I-248, SPU 97/85, SPU 134/ 87, SPU 415/85, and SPU 41/84 were used for assay inclusivity testing.

*S segment sequencing and analysis.* The S segment of each CCHFV isolate was amplified from 15 µl of total nucleic acid eluate using previously described primers [32] that were modified for Nextera-based Illumina sequencing. The sequencing and assembly of these segments were previously described (reference Genome Announcement). Briefly, the S segment of each virus was amplified using the SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific) and gel-purified [QIAquick Gel Extraction Kit (Qiagen)]. Next-generation sequencing libraries were generated using the Nextera XT DNA Library Kit (Illumina) according to the manufacturer's instructions. Libraries were pooled and sequenced on the MiSeq Desktop Sequencer (Illumina). Consensus sequences were generated using CLC Genomics Workbench (Qiagen). The SRA files were deposited into Bioproject PRJNA360092, and the consensus sequences were deposited into GenBank [IbAr10200 (KY484036), DAK8194 (KY484027), SPU 128/81 (KY484044), SPU 115/87 (KY484040), UG3010 (KY484048), JD-206 (KY484037), HY-13 (KY484031), and Drosdov (KY484028)].

The S segments from these newly sequenced viruses were aligned, and the assay target region was isolated for variant analysis and assay redesign. Additionally, existing CCHFV S segment sequences from GenBank that covered the assay target region were aligned with CLC Genomics Workbench (Supplementary Figure 1).

*Real-time RT-PCR assays.* The existing CCHFV-S assay [25] was run as previously described with modifications described below. For the new assay described here (CCHFV-S2), primers and probe were designed within the same assay target region based on the data from the newly sequenced CCHFV isolates (see Table 1 for the primer and probe sequences and concentrations). Both assays (CCHFV-S and CCHFV-S2) were run on a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) using the SuperScript One-Step RT-PCR Kit (Thermo Fisher Scientific), 5 µl purified nucleic acid, and a final concentration of 3 mM MgSO<sub>4</sub>. Cycling conditions were 50 °C for 15 minutes, 95 °C for 5 min, and then 45 cycles of 94 °C for 1 s, 55 °C for 20 s, and 68 °C for 5 s. For the comparison with the Atkinson assay, primers, probe, and reaction conditions were the same as previously published [20]. The fluorescence was measured at the end of each 68 °C extension step, and a positive call required a quantification cycle (Cq) value of less than 40 cycles. All negative calls were given a Cq value of 40. The modified assay (CCHFV-S2) was optimized for primer and probe concentrations using CCHFV IbAr10200 RNA. This process involved testing multiple primer concentrations ranging from 0.5 to 1.0 mM with 0.2 mM probe. The optimal primer concentration was selected based on the lowest Cq value and the highest endpoint fluorescence (data not shown).

A preliminary limit of detection (LOD) determination was conducted for both assays by serially diluting viral RNA either ten-fold or five-fold in two different series, and samples were run by real-time RT-PCR in triplicate. The preliminary LOD was the lowest RNA dilution where all replicates were positive. The LOD was confirmed by running 60 replicates at the LOD, requiring at least 58 of 60 replicates to be positive. Exclusivity testing was conducted using a viral RNA reference panel maintained at USAMRIID and acquired from the UCC. These viruses included Rift Valley fever virus (MP12), Hantaan virus (76118), yellow fever virus (17D), dengue virus serotype 1 (WestPac, UCC# R4423), dengue virus serotype 2 (S16803, UCC# R4424), dengue virus serotype 3 (CH53489, UCC# R4425), dengue virus serotype 4 (341750, UCC# R4426), West Nile virus [EG101 (UCC# R4310T) and NY99 (UCC# R4272T)], Chikungunya virus [B8636 and 38635], Lassa fever virus Josiah (UCC# R4086T), and Ebola virus variant Mayinga (UCC# R3828S). Inclusivity for both assays was determined using the 15 different strains of CCHFV maintained at USAMRIID and the UCC described above.

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128 *Statistics.* Statistical analyses were performed using GraphPrism 6 (GraphPad Software, San Diego, CA).  
129 Assay linearity based on the preliminary LOD was determined based on the linear range of the curve  
130 using a nonlinear regression analysis. A two-way ANOVA with Sidak's multiple comparisons test was  
131 done to determine differences between the CCHF-S and CCHF-S-pan assay using multiple CCHFV strain  
132 RNAs.

## 133 134 **Results**

135  
136 *CCHFV strain sequencing and analysis.* Since the development of our original CCHFV-S assay [25], we  
137 (CCHF-S, Table 2) and others [20] identified decreased assay performance including nondetection of  
138 several CCHFV strains (JD-206, Drosdov, and DAK8194). To address this problem, we conducted deep  
139 sequencing of multiple CCHFV S segments, including the three nondetectable CCHFV strains ([reference](#)  
140 [Genome Announcement](#)). The S segment consensus sequences for these viruses were aligned to identify  
141 mismatches within the assay target region (Figure 1).

142  
143 Multiple nucleotide variants were identified in the primer and probe region for each strain sequenced  
144 (Figure 1), resulting in suboptimal primer/probe binding. Of note, a deletion in the 5' end of the published  
145 probe sequence, along with additional 3' probe variants for JD-206 and DAK 8194, likely resulted in  
146 nondetection of those two virus strains. Multiple variants in the reverse primer of Drosdov likely  
147 contributed to that strain's nondetection.

148  
149 *Assay evaluation.* New primers and probe (assay CCHF-S2, Table 1) were redesigned to incorporate as  
150 much sequence diversity at the assay target location as possible. Comparison of the CCHFV-S2 assay  
151 primer/probe sequence to all CCHFV S segment sequences available in GenBank at the time of the assay  
152 redesign (n = 138, Supplementary Figure 1) showed the forward, reverse, and probe sequences had no  
153 greater than 1 mismatch (and an exact match within the last 2 bases of the 3' end) for 93.5, 99.3, and  
154 98.6% of these S segment sequences, respectively.

155  
156 The analytical characteristics of both the CCHF-S and CCHF-S2 assays were determined using a well-  
157 characterized stock of IbAr10200 (Figure 2, Table 3). The preliminary limit of detection (LOD), the  
158 highest dilution of virus where 3 of 3 replicates were all positive, was 1.28 PFU/reaction or 256 PFU/ml  
159 for the CCHF-S2 assay (Figure 2). Considering the linear segment of the dilution series, the  $R^2$  value was  
160 0.980, and the y-intercept was 43.64. This LOD was confirmed by running 60 replicates at this LOD,  
161 resulting in 58 of 60 positive replicates (Figure 2). For the CCHF-S assay using the same IbAr10200  
162 RNA (Figure 2) identified the preliminary LOD, confirmed by 59 of 60 replicates being positive, to be  
163  $1.28 \times 10^4$  PFU/rxn or  $2.56 \times 10^6$  PFU/ml. The assay linearity over the linear part of the dilution series  
164 was 0.845, and the y-intercept was 53.41.

165  
166 The CCHF-S2 assay was then compared to another commonly used assay, the Atkinson assay [20], which  
167 targets a different region within the CCHFV S segment. Using the same RNA as a template and the  
168 reaction conditions described in [20], we identified a greater assay sensitivity compared to the CCHFV-S  
169 assay but decreased sensitivity compared to the CCHF-S2 assay (Figure 2, Tables 2 and 3). The assay  
170 LOD with IbAr10200 RNA was  $2.56 \times 10^4$  PFU/ml with 60/60 replicates being positive. While several  
171 nucleotide variants were identified for the Atkinson assay within the assay target region of the CCHFV  
172 isolates previously sequenced ([reference Genome Announcement](#)), incorporating sequence-optimized  
173 reverse primer and the probe into the Atkinson assay did not change assay sensitivity (data not shown).

174  
175 All three assays were then screened against an inclusivity panel of 15 different CCHFV isolates (Table 3).  
176 The CCHF-S2 assay and the Atkinson assay detected all of the CCHFV isolates including the three that  
177 the CCHF-S assay did not detect. Assay sensitivity, reflected in the Cq values, was generally better for the

CCHF-S2 assay (Table 3). In comparing the CCHF-S and the CCHF-S2 assays, almost all of these viruses had large improvements in the C<sub>q</sub> values. For example, SPU 115/87 had ~10 C<sub>q</sub> (~3 log) improvement in sensitivity, and IbAr10200 had ~15 C<sub>q</sub> (>4 log) improvement (Table 3). Exclusivity testing for multiple viruses (see Materials and Methods) with the CCHF-S2 assay resulted in negative detection for each virus tested.

## Discussion

Due to the low fidelity of the viral RNA-dependent RNA-polymerase, RNA viruses generally rapidly evolve under selective pressure, resulting in significant phylogenetic heterogeneity. This diversity can be problematic for diagnostic assays and therapeutics, requiring assay modification as additional sequence information becomes available. Indeed, two recently published studies investigating the genomic diversity of the Ebola virus circulating in West Africa [33, 34] identified multiple nucleotide variants among several commonly used Ebola virus real-time RT-PCR assays and therapeutics in development. These studies suggest such variants could negatively impact efficacy of diagnostic assays and therapeutics. To mitigate the diagnostic risk of CCHFV diversity, we re-designed our currently fielded CCHFV assay by incorporating sequencing data from several CCHFV isolates that were previously undetectable with the original assay.

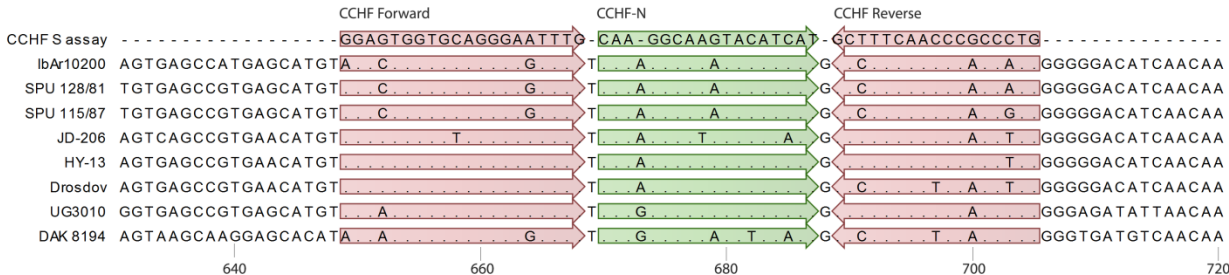
Deep sequencing of the CCHFV S segments of these and other CCHFV isolates identified multiple nucleotide variants within the CCHF-S assay target region. These variants likely led to the decreased assay performance we observed the CCHFV-S assay. Variant analysis within this assay region did not identify intra-viral nucleotide differences (data not shown), suggesting some signature stability within each isolate and supporting continued targeting of this genomic region as a diagnostic signature. Based on these sequencing data and the CCHFV genomic data deposited into GenBank since the original assay design, a new assay (CCHF-S2) incorporated degenerate primers and probe taking into account the assay target sequence diversity. These primers greatly improved CCHFV detection, reflected in lower C<sub>q</sub> values and detection of the three isolates not detected by the CCHF-S assay.

For highly diverse viruses like CCHFV, it is advantageous to have several diagnostic assays that target different regions of the viral genome in order to further minimize the diagnostic risk of a false negative call due to primer/probe mismatches. We conducted a comparison with another commonly used CCHFV assay developed by Atkinson and colleagues that targets the 5' untranslated region of the CCHFV S segment [20]. While both the CCHF-S2 assay and the Atkinson assay positively detected all of the CCHFV strains tested here, the CCHF-S2 assay had improved sensitivity for most of the tested strains. Since both of these assays target different regions of the CCHFV genome, both assays could be used for increased confidence in diagnostic and biosurveillance efforts in order to mitigate the risk of nondetection due to CCHFV's diversity.

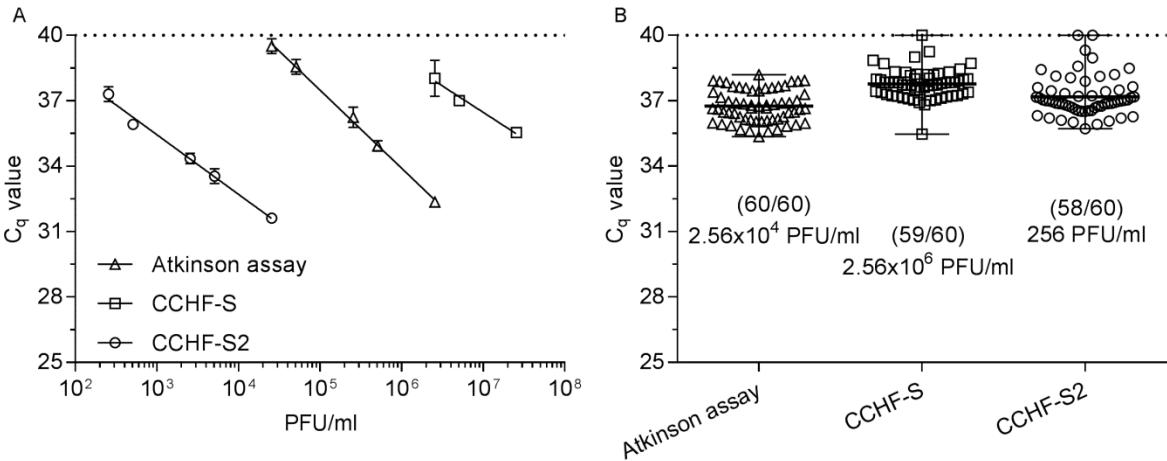
In summary, we redesigned a CCHFV real-time RT-PCR assay that was initially developed when limited sequence information was available and did not perform optimally with newly acquired CCHFV isolates. This new assay contains degenerate primers and probe that accounts for a significant amount of the diversity within CCHFV, resulting in dramatically improved isolate detection and assay sensitivity. These data increase the confidence in the new assay detecting true positives, and this approach can be used to improve assay sensitivity existing nucleotide-based assays.

**Acknowledgements**

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. This effort was funded by Defense Threat Reduction Agency (DTRA) through the JSTO-CBD project 1143798.



**Figure 1. CCHFV isolate sequence analysis.** Consensus sequences from eight newly sequenced CCHFV isolates (reference Genome Announcement) and the Garrison assay primer/probe sequences [25], indicated with red and green arrows, respectively, were aligned. Nucleotides identical to the primer and probe sequence are shown as dots, and nucleotide numbers are relative to IbAr10200. Degenerate primers and probe for the Garrison assay (see Table 1) were designed based off of these aligned sequences.



**Figure 2. CCHFV assay characterization.** (A) CCHFV IbAr10200 RNA was serially diluted in two series, 1:5 and 1:10, and assayed with the CCHFV assays. Shown is a nonlinear fit of the linear range where all three of the replicates were positive. (B) The preliminary LOD was confirmed by running 60 replicates at the preliminary LOD. The dashed line in each figure indicates the Cq positive/negative cutoff (40 cycles).

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**Table 1. CCHFV real-time primers and probe**

| Assay  | Primers/pro | Sequence (5'-3')         | Conc. | Amplicon | Referenc |
|--------|-------------|--------------------------|-------|----------|----------|
| CCHF-S | CCHF        | GGAGTGGTGCAGGGAATTTG     | 1.25  | 57       | [25]     |
|        | CCHF        | CAGGGCGGGTTGAAAGC        | 1.25  |          |          |
|        | CCHF-N      | 6FAM-CAAGGCAAGTACATCAT-  | 0.1   |          |          |
| CCHFV- | CCHF-SF2    | GGA VTGGTGVAGGGARTTTG    | 1.0   | 57       | here     |
|        | CCHF-SR2    | CADGGTGGRTTGAARGC        | 1.0   |          |          |
|        | CCHF-N2     | 6FAM-CAARGGCAARTACATMAT- | 0.2   |          |          |

**Table 2. CCHFV assay detection**

| virus      | detection (average Cq ± STDEV) |                     |                |
|------------|--------------------------------|---------------------|----------------|
|            | CCHFV-S2                       | CCHF-S <sup>2</sup> | Atkinson assay |
| I-40       | 20.66 ± 0.380                  | 17.13 ± 0.242       | 27.62 ± 0.3    |
| 2219 KKK28 | 25.12 ± 0.04                   | 22.34 ± 0.290       | 35.01 ± 0.16   |
| I-248      | 22.95 ± 0.04                   | 23.56 ± 0.174       | 31.23 ± 0.27   |
| JD-206     | 34.29 ± 0.511                  | nd                  | 31.69 ± 0.3    |
| Drosdov    | 29.00 ± 0.091                  | nd                  | 39.37 ± 0.64   |
| HY13       | 19.66 ± 0.07                   | 17.85 ± 0.11        | 24.94 ± 0.21   |
| SPU 97/85  | 21.69 ± 0.133                  | 34.10 ± 1.308       | 38.94 ± 0.51   |
| SPU 134/87 | 25.48 ± 0.182                  | 30.30 ± 2.081       | 31.61 ± 0.13   |
| SPU 115/87 | 24.28 ± 0.489                  | 34.35 ± 0.474       | 31.96 ± 0.26   |
| SPU 415/85 | 18.97 ± 0.025                  | 32.18 ± 0.219       | 26.81 ± 0.22   |
| SPU 41//84 | 25.68 ± 0.083                  | 32.86 ± 0.321       | 25.68 ± 0.2    |
| SPU 128/81 | 23.16 ± 0.216                  | 37.37 ± 0.525       | 28.97 ± 0.04   |
| UG3010     | 24.20 ± 0.059                  | 24.01 ± 0.050       | 29.84 ± 0.14   |
| IbAr10200  | 24.28 ± 0.201                  | 39.47 ± 0.924       | 30.55 ± 0.52   |
| DAK8194    | 28.89/29.28 <sup>1</sup>       | nd                  | 33.46 ± 0.54   |

<sup>1</sup>2 of 3 replicates were positive<sup>2</sup>nd is not detected**Table 3. Analytical assay characteristics with IbAr10200**

| assay          | linearity (R <sup>2</sup> ) | slope  | y-intercept | LOD, PFU/ml (positives/60 replicates) | Cq ± STDEV   | coefficient of variance |
|----------------|-----------------------------|--------|-------------|---------------------------------------|--------------|-------------------------|
| CCHF-S         | 0.845                       | -2.419 | 53.41       | 2.56 x 10 <sup>6</sup> (59/60)        | 37.77 ± 0.68 | 1.79%                   |
| CCHFV-S2       | 0.980                       | -2.730 | 43.64       | 256 (58/60)                           | 37.18 ± 0.91 | 2.95%                   |
| Atkinson assay | 0.987                       | -3.581 | 55.4        | 2.56 x 10 <sup>4</sup> (60/60)        | 36.75 ± 0.74 | 2.02%                   |

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381

**Supplementary**

**Figure S1. CCHFV sequence analysis.** CCHFV S segment sequences containing the assay target region were identified in GenBank and aligned to show the forward and reverse primers (red) and the probe (green) region of the CCHF-S assay. The dominant nucleotide variants within these sequences, with the exception of one, are covered in the newly designed primers and probe. The single variant, the G at the 5' forward primer end, should have little impact on primer binding and extension.

# CCHFV 10200 Standard Curve with Hewson assay

Run 10200 RNA in triplicate at 8 dilutions starting at 1:10  
with one NTC (H<sub>2</sub>O)

## Reaction Mix

(SuperScript III Platinum One-Step Quantitative RT-PCR System)

|                                        |       |         |      | # Rxns = |
|----------------------------------------|-------|---------|------|----------|
| Reagents                               | Stock | [Final] | 1rxn | 28       |
| 2X Reaction Mix                        | 2     | 1       | 10   | 280      |
| Nuclease-free water                    |       |         | 1.7  | 47.6     |
| 100 µM F. Primer                       | 18    | 0.9     | 1    | 28       |
| 100 µM R. Primer                       | 18    | 0.9     | 1    | 28       |
| 100 µM Probe                           | 25    | 0.625   | 0.5  | 14       |
| RT/Platinum Taq Mix                    |       |         | 0.8  | 22.4     |
| X pg/µL RNA-pos cntrl/H <sub>2</sub> O |       | X pg/µL | 5    |          |

20

## Cycling Conditions:

| Temp | Time   | Cycles |
|------|--------|--------|
| 50C  | 10 min | 1      |
| 95C  | 2 Min  | 1      |
| 95 C | 10 sec | 45     |
| 60 C | 40 sec |        |
| 40 C | 30 sec | 1      |

## Results:

|                            |  | Hewson |       |       |       |
|----------------------------|--|--------|-------|-------|-------|
| pfu/PCR rxn                |  | CP     |       |       |       |
| Standard Log Concentration |  | Rep1   | Rep2  | Rep3  | Avg   |
| 1.28E+04                   |  | 32.25  | 32.21 | 32.61 | 32.36 |
| 2.56E+03                   |  | 34.83  | 34.75 | 35.2  | 34.93 |
| 1.28E+03                   |  | 36.33  | 36.66 | 35.73 | 36.24 |
| 2.56E+02                   |  | 38.24  | 38.5  | 38.91 | 38.55 |
| 1.28E+02                   |  | 39.47  | 39.17 | 39.86 | 39.50 |
| 2.56E+01                   |  | 41.06  | ND    | 40.88 | 40.97 |
| 1.28E+01                   |  | 42.77  | 42.7  | ND    | 42.74 |
| 2.56E+00                   |  | ND     | ND    | ND    |       |

Error: 0.127  
Efficiency: 1.994  
Slope: -3.337  
Yintercept: 46.28

## CCHFV Standard Curve

Run 10200 RNA in triplicate at 7 dilutions starting with stock and one NTC (H2O)

### Reaction Mix

|                                 |       |               |      | # Rxns = |
|---------------------------------|-------|---------------|------|----------|
| Reagents                        | Stock | [Final]       | 1rxn | 27       |
| Mater Mix                       |       |               | 14.6 | 394.2    |
| RT/Platinum Taq Mix             |       |               | 0.4  | 10.8     |
| X pg/ $\mu$ L RNA-pos cntrl/H2O |       | X pg/ $\mu$ L | 5    |          |

20

### PCR Cycling Conditions

| Temp | Time   | Cycles |
|------|--------|--------|
| 50C  | 15 min | 1      |
| 95C  | 5 Min  | 1      |
| 94 C | 1 sec  | 45     |
| 55 C | 20 sec |        |
| 68 C | 5 sec  |        |
| 40 C | 30 sec | 1      |

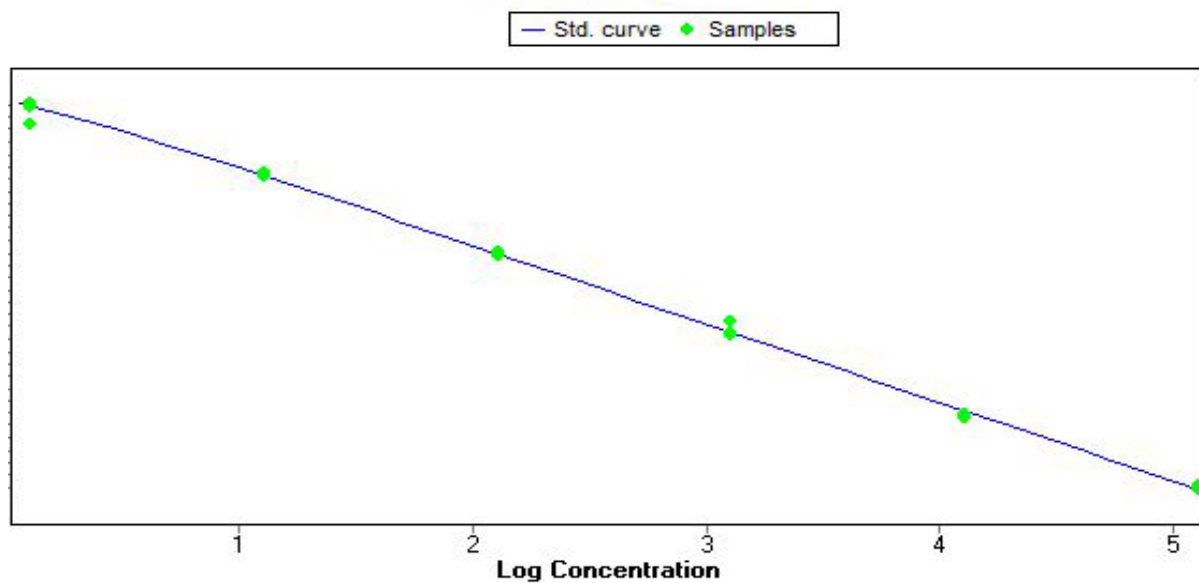
Crossing Point  
36  
34  
32  
30  
28  
26  
24  
22

### Results:

| Standard Log Concentration | CP    |       |       |           |
|----------------------------|-------|-------|-------|-----------|
|                            | Rep1  | Rep2  | Rep3  |           |
| 1.28E+05                   | 21.04 | 20.87 | 21.03 | 20.98     |
| 1.28E+04                   | 23.9  | 23.97 | 23.79 | 23.886667 |
| 1.28E+03                   | 27.14 | 27.69 | 27.21 | 27.346667 |
| 1.28E+02                   | 30.49 | 30.37 | 30.37 | 30.41     |
| 1.28E+01                   | 33.55 | 33.77 | 33.71 | 33.676667 |
| 1.28E+00                   | 36.44 | 36.57 | 35.74 | 36.25     |
| 1.28E-01                   | ND    | ND    | 37.07 |           |
| 1.28E-02                   | ND    | ND    | ND    |           |

21.01 20.91 20.89 20.936667  
24.1 23.99 24.2 24.096667  
27.31 27.39 27.26 27.32  
30.17 30.24 30.22 30.21  
33.66 33.62 33.74 33.673333  
35.74 35.84 35.79

Standard Curve



| x        | y        |
|----------|----------|
| 1.28E+05 | 20.98    |
| 1.28E+04 | 23.88667 |
| 1.28E+03 | 27.34667 |
| 1.28E+02 | 30.41    |
| 1.28E+01 | 33.67667 |
| 1.28E+00 | 36.25    |

$R^2$   
0.503227

| X        | Y     |
|----------|-------|
| 1.28E+05 | 21.04 |
| 1.28E+05 | 20.87 |
| 1.28E+05 | 21.03 |
| 1.28E+04 | 23.9  |
| 1.28E+04 | 23.97 |
| 1.28E+04 | 23.79 |
| 1.28E+03 | 27.14 |
| 1.28E+03 | 27.69 |
| 1.28E+03 | 27.21 |
| 1.28E+02 | 30.49 |
| 1.28E+02 | 30.37 |
| 1.28E+02 | 30.37 |
| 1.28E+01 | 33.55 |
| 1.28E+01 | 33.77 |
| 1.28E+01 | 33.71 |
| 1.28E+00 | 36.44 |
| 1.28E+00 | 36.57 |
| 1.28E+00 | 35.74 |

$R^2$   
0.502586

Error: 0.023  
Efficiency: 2.06  
Slope: -3.187  
Yintercept: 36.95

|          |          |
|----------|----------|
| 1.28E+05 | 20.93667 |
| 1.28E+04 | 24.09667 |
| 1.28E+03 | 27.32    |
| 1.28E+02 | 30.21    |
| 1.28E+01 | 33.67333 |
| 1.28E+00 | 35.79    |

0.520809