PCRassays.com

ASSAY NAME: Hepatitis A virus (hepatovirus A)

Quantity: 100 x 20µL PCR reactions

3-plex assay: Hepatitis A virus site-1
Hepatitis A virus site-2
and human RPP30 DNA

SKU: BUN-HAV-D-QS

(RUO). Research Use Only. Not for use in Diagnostic Procedures.

SCOPE OF THIS DOCUMENT

The recipes are optimized for each instrument. For QuantStudio instruments use SKUs: BUN-HAV-D-QS-100 (3-plex assay including the human RPP30-DNA control assay). The verification data for BUN-HAV-D-QS-100 on a QuantStudio 7 Flex are presented in this product information sheet (PIS). The performance of the other SKUs should be similar. Contact PCRassays.com if you need to use a different instrument.

CONTENTS

The primers and probes in the 3-plex Hepatitis A virus assay (also known as hepatovirus A) are provided in Tube 1 as a 20X concentrated working solution. The 3-plex assay is designed as a "double positive" assay that targets 2 independent sites in the HAV virus so that even if one site mutates in the future, the assay would still detect the positive infection. Bioinformatic analysis (using ThermoSleuth from DNA Software, Inc. (not shown)) of the 3-plex against 287 full-length HAV genomes (all known publicly available in January 2024) suggests that the assay will detect all of them. The primers and probe for site 1 (**TEX615** channel) bind to domain IV of the 5'-UTR of the virus. The HAV site-1 assay amplifies all 6 types of hepatitis A viruses (namely types 1A, 1B, 2A, 2B, 3A, 3B). The primers and probes for site 2 (**FAM** channel) bind to domain III of the 5'-UTR of the virus. The HAV site-2 assay amplifies HAV types 1A, 1B, 2A, and 2B efficiently, but detects types 3A and 3B less efficiently (later Cq and less sloped PCR curve). The same mix also contains primers/probe targeting the human RPP30DNA Intron I as an endogenous qPCR positive control assay for human samples. The current assay does not detect the human mRNA because typical blood samples contain little mRNA due to abundant nucleases. If you are interested in a version of the assay using an RNA spike-in control, please contact PCRassays.com for a custom shipment. The probes are designed as TaqMan[6] cleavage mechanism and thus the reaction requires a DNA polymerase with 5'-exonuclease activity.

Assay contents:

Tube 1: 20X Primer/Probe mix for Hepatitis A virus and hRPP30DNA.

Tube 2: (**Do NOT add to specimen unknowns**) Positive control: Synthetic ~500 bp DNA for Hepatitis A virus and hRPP30DNA.

Tube 3: RT-qPCR enzyme Mastermix (enough for 100 rxns. with 20 µL total volume). This is a custom formulation from Fortis Life Sciences to the specifications of PCRassays.com.

Table of Dyes used in this assay:

Pathogen/Target	Dyes	Quencher	Refs.
Hepatitis A site-1	TEX615	BHQ-2	3,4
Hepatitis A site-2	FAM	BHQ-1	1,2
RPP30-DNA control	HEX	BHQ-1	5

ASSAY HANDLING

The Hepatitis A virus assay is shipped at ambient temperature, but should be stored at -20 °C. The tubes should be kept on ice once thawed. Do not subject the enzyme to multiple freeze-thaw cycles. Contamination should be avoided by using appropriate personal protective equipment (PPE), powder free gloves, aerosol barrier pipette tips, and a clean hood.

EXPERIMENTAL

Perform nucleic acid extraction/purification (recommended). Set up your reaction (20 µL) as follows on ice:

Component	Volume (µL)
RT-qPCR enzyme mastermix (2X)	10
Primer/Probe mix (20X)	1
Sample	2
Water	7

Notes:

1. To improve assay sensitivity, up to 9 µL of sample can be added (water volume adjusted accordingly) for a total reaction volume of 20 µL.

2. For positive control rxns., add 2 µL solution from Tube 2.

3. Molecular biology grade water (NOT included) should be used to prepare the PCR reactions.

An RT-qPCR protocol was used for verification on a QuantStudio 7 Real-Time System, with the following program:

Step	Thermocycling Protocol:
1	Incubate @ 50 °C for 10 minutes
2	Incubate @ 95 °C for 3 minutes
3	Incubate @ 95 °C for 5 seconds
4	Incubate @ 55 °C for 22 seconds
5	Plate Read
6	Go to Step 3, repeat 44x more

RESULT INTERPRETATION

After running the qPCR reaction, perform a regression analysis on the data to determine the quantification cycle, Cq. (Cq is preferred over Ct). Each fluorescence channel with a Cq < 38 cycles and final RFU >200,000 is considered "positive" or "+" in the Table below.

HAV site-1 TEX615™	HAV site-2 FAM™	hRPP30 HEX™	Recommended Interpretation
–	–	–	The PCR reaction failed. Please repeat the experiment
–	–	+	The sample does not contain HAV viral RNA. The sample contains human RPP30 DNA.
+	-	–	The sample contains HAV RNA. The sample may not contain human RPP30 DNA. Site 2 of HAV may contain a mutation.
+	-	+	The sample contains HAV RNA and human RPP30 DNA. Site 2 of HAV may contain a mutation.
-	+	–	The sample contains HAV RNA. The sample may not contain human RPP30 DNA. Site 1 of HAV may contain a mutation.
-	+	+	The sample contains HAV RNA and human RPP30 DNA. Site 1 of HAV may contain a mutation.
+	+	–	The sample contains HAV RNA double positive. The sample may not contain human RPP30 DNA.
+	+	+	The sample contains HAV RNA double positive and human RPP30 DNA.

VERIFICATION EXPERIMENTS

Experiments were performed in triplicate on a QuantStudio 7 using the experimental procedure given above, but with different samples added to each reaction. RNA extracts for HAV types 1A and 1B, Human serum control, and Hepatitis C virus extract (HCV) were generously provided by the Michigan DHHS shown in **Figure 1**. Figure 2 shows experiments for all 6 HAV types (1A, 1B, 2A, 2B, 3A, 3B) were performed using 9 synthetic DNA constructs (from Twist Biosciences) harboring ~500 bp regions of the 5'-UTR from the target genomes. The limit of detection (LOD) was estimated by performing serial dilution experiments (**Figure 3)** used samples with 1×10^4 copies/reaction of transcribed 500 nt. RNA for HAV type 1A (results shown for the fluorescence from site-2 assay).

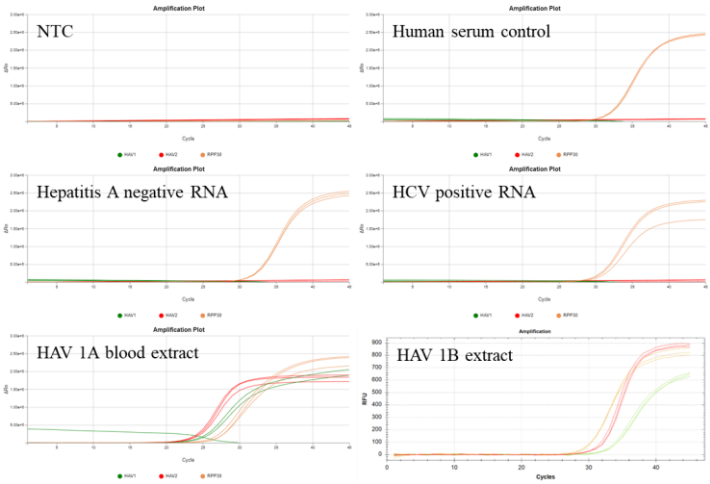

Figure 1: Extracts from human blood of patients infected with HAV types 1A and 1B are shown in the bottom panels with **red curves** for the site-1 assay (**TEX615**) and **green curves** for the site 2 assay (**FAM**). All sets of probes and primers are present in every reaction, but positive signal is only observed when the HAV is present, indicating that the amplification is specific. Verification experiments for the NTC show no signal (upper left), but the controls from human serum, HAV negative, and HCV positive samples show no signal from HAV assay, but do show amplification of endogenous RPP30 DNA (**HEX** in **orange**).

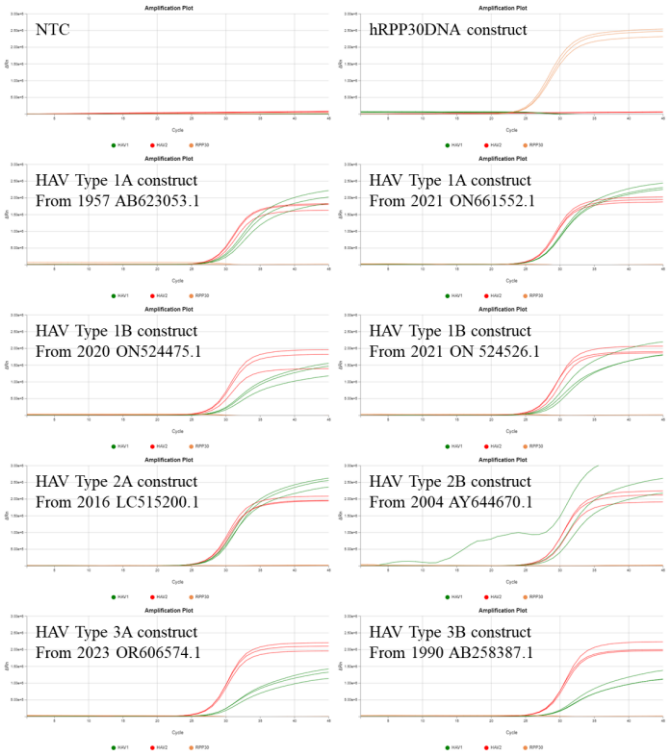

Figure 2: Verification experiments with 9 synthetic DNA UTR constructs (accessions given in each panel). **Red curves** for the site-1 assay (TexasRed615) and Green curves for the site-2 assay (FAM) amplify all of the samples, though the site-2 assay has a Cq shift of 2-3 cycles.

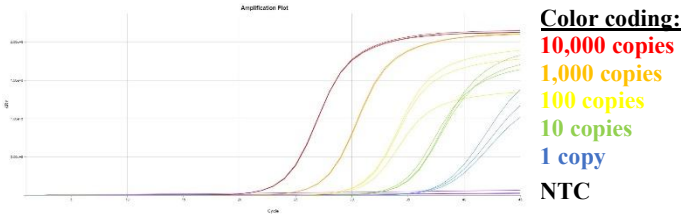

Figure 3: Serial dilution experiments show LOD <10 molecules for the transcribed RNA of HAV type 1A.

Conclusion: The data in **Figures 1** and **2** indicate that the HAV site-1 and site-2 primers and probe detect HAV RNA and are also compatible with the RPP30_DNA positive control primers and probe.

CONTACT US

For assistance, please contact DNA Software using the link: https://dnasoft.jira.com/servicedesk/customer/portals

Address: Michigan Life Science and Innovation Center, 46701 Commerce Center Dr, Plymouth, MI 48170

Phone: (734) 222-9080

NOTES

[1] FAM™ (Carboxyfluorescein), a trademark of Life Technologies Corp.

[2] BHQ-1™ (Black Hole Quencher) is a trademark of Biosearch Tech., Inc.

[3] TEX615™ is a trademark of Thermo Fisher Scientific.

[4] BHQ-2™ (Black Hole Quencher) is a trademark of Biosearch Tech., Inc.

[5] HEX™ (Hexachloro-fluorescein), a trademark of Thermo Fisher Scientific.

[6] TaqMan™ is a trademark of Roche Diagnostics, Inc.