PCRassays.com

ASSAY NAME: MENA1 (4-color)

Quantity: 100 x 20µL PCR reactions

4-plex assay: HIV, HCV, HBV, and human RPP30 RNA

SKU: PNP-MENA1-R-BR-100 (Bio-Rad)
PNP-MENA1-D-BR-100 (Bio-Rad)
PNP-MENA1-D-QS-100 (QuantStudio)
PNP-MENA1-D-MIC-100 (BMS MIC)

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

SCOPE OF THIS DOCUMENT

The oligonucleotide recipes are optimized for each instrument (BioRad, QuantStudio, MIC). The pre-validation data presented in this IFU were performed using PNP-MENA1-R-BR-100 on a BioRad CFX-96. The performance of the other SKUs on their corresponding instrument should be similar. Contact PCRassays.com if you are planning to use a different qPCR instrument.

CONTENTS

The MENA1 assay is a multiplexed real-time reverse transcription polymerase chain reaction (RT-qPCR) test intended for the qualitative detection of nucleic acid from human immunodeficiency virus type 1 (HIV-1), human hepatitis C virus (HCV), and human hepatitis virus B (HBV). The HIV assay detects the gene for "pol protein" (Protein ID: QJQ91623.1). The HCV assay detects the gene for "polyprotein" (Protein ID: ACJ04208.1). The HBV assay detects the gene for S protein (Protein ID: QJC70195.1).

Table of Dyes used in this kit:

Pathogen/Target	Dyes	Quencher	Refs.
HCV	FAM	BHQ-1	1,2
RPP30-RNA control	HEX	BHQ-1	6
HIV	TexasRED615	BHQ-2	3,4
HBV	Cy5	BHQ-2	5

The probes are designed as TaqMan[7] cleavage mechanism and thus the reaction requires a DNA polymerase with 5'-exonuclease activity.

KIT HANDLING AND CONTAMINATION

The MENA1 kit is shipped at ambient temperature, and should be stored at -20 °C. The kit 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.

Assay contents:

Tube 1: Primer/Probe mix (5X) for HIV, HCV, HBV, and hRPP30RNA.

Tube 2: (**Do NOT add to specimen unknowns**) Positive control: 5000 copies/µl positive controls of synthetic 500 bp DNA fragments of HIV, HCV, HBV, and hRPP30RNA.

Tube 3: InhibiTaq Standard 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.

Tube 4: (Optional) Spike-in control. 1.0E6 copies/uL of transcribed nt. human RPP30 spliced RNA (for SKUs with "R"). Alternatively a spike in synthetic DNA RPP30 is provided for SKU's with "D".

EXPERIMENTAL

(Optional) add 1 µL of spike-in control (Tube 4) to the specimen before extraction. **Do not add directly to the PCR reaction!** It serves as extraction and PCR reaction control.

Set up your reaction (20 µL) as follows on ice:

Component	Volume (µL)
InhibiTaq qPCR enzyme mastermix (2X)	10
Primer/Probe mix (5X)	4
Sample	2
Water (molecular biology grade)	4

Notes: To improve assay sensitivity, up to 6 µL of sample can be added (water volume adjusted accordingly) for a total reaction volume of 20 µL. For positive control rxns., add 2 µL of the solution from Tube 2 (i.e., the "sample").

A RT-PCR protocol was used for verification on a Bio-Rad CFX-96 instrument, 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 15 seconds
5	Plate Read
6	Go to Step 3, repeat 44× more

For QuantStudio and BMS MIC instruments, we recommend a Step 4 cycle times of 22 and 15 seconds, respectively.

RESULT INTERPRETATION

After running the qPCR reaction, use the instrument software to determine the quantification cycle, Cq (or use Ct if your instrument does not have the capability to compute a Cq). Fluorescence channels with a Cq < 38 cycles, and final RFU >Threshold is considered "positive" or "+" in the Table below. The "Threshold" value for calling a PCR positive is dependent on the instrument model, well size, and sample volume; thus the user must determine the threshold that is appropriate for their method. For our QuantStudio 7 Flex with 96 well plate with 100 µL wells and 20 µL reaction volume, the average RFU was approximately 1,000,000 and we used a threshold of 200,000 for calling positives.

HCV FAM™	HIV TexasRED 615™	HBV Cy5™	RPP30 HEX™	Recommended Interpretation
–	–	–	–	The PCR reaction may have failed, or no human RNA is present in the sample. Please repeat the experiment.
–	–	–	+	The sample does not contain HIV, HCV, or HBV.
+	–	–	–	The sample contains HCV RNA. The sample does not contain HIV or HBV. The sample may not contain spliced human RPP30 mRNA.
+	–	–	+	The sample contains HCV RNA and spliced human RPP30 mRNA. The sample does not contain HIV or HBV.
–	+	–	–	The sample contains HIV RNA. The sample does not contain HBV or HCV. The sample may not contain spliced human RPP30 mRNA.
–	+	–	+	The sample contains HIV RNA and spliced human RPP30 mRNA. The sample does not contain HBV or HCV.
–	–	+	–	The sample contains HBV DNA. The sample doesn't contain HIV or HCV RNA. The sample may not contain spliced human RPP30 mRNA.
–	–	+	+	The sample contains HBV DNA and spliced human RPP30 mRNA. The sample does not contain HIV or HCV RNA.
+	+	–	–	The sample contains both HCV and HIV RNA. The sample does not contain HCV RNA. The sample may not contain spliced human RPP30 mRNA.
+	+	–	+	The sample contains HCV, HIV, and spliced human RPP30 mRNA. The sample does not contain HBV RNA.
+	–	+	–	The sample contains both HCV RNA and HBV DNA. The sample does not contain HBV DNA. The sample may not contain spliced human RPP30 mRNA.
+	–	+	+	The sample contains HCV RNA, HBV DNA and spliced human RPP30 mRNA. The sample does not contain HIV DNA.
–	+	+	–	The sample contains HIV RNA and HBV DNA. The sample does not contain HIV RNA. The sample may not contain spliced human RPP30 mRNA.
–	+	+	+	The sample contains HIV RNA and HBV DNA, and spliced human RPP30 mRNA. The sample does not contain HCV RNA.
+	+	+	–	The sample contains HCV, HIV, and HBV. The sample may not contain spliced human RPP30 mRNA.
+	+	+	+	The sample contains HCV, HIV, HBV and spliced human RPP30 mRNA.

VERIFICATION EXPERIMENTS

The MENA1 kit verification was carried out as a 4-plex assay, which simultaneously detects RNA from HCV, RNA from HIV, DNA from HBV. Human RPP30 mRNA is detected in the HEX channel and serves as a positive control.

Experiments were performed in triplicate using the experimental procedure given above, but with different samples added to each reaction. The samples used for the verification experiments contained 1×10^4 copies/reaction of synthetic 500 bp DNA constructs (from Twist Biosciences) harboring the regions of interest from the 3 viral genomes. Some samples also contained human total brain RNA (Takata Cat#636530) to verify detection of the human RPP30 mRNA. **Figure 1** shows the results of these experiments, which indicate that the 4-plex specifically detects the different pathogens.

Extracted RNA samples of HIV (NATHIV1-LIN, BSL-1) and HCV (NATHCV-0005, BSL-1) from ZeptoMetrix and HCV extract (generously provided by the MI-DHHS) were employed to test the performance of the multiplex assay using extracted pathogen RNA samples. The results of these experiments are shown in **Figure 2**. The results indicate that the reverse transcription and amplification were successful.

The limit of detection (LOD) was estimated by performing serial dilution experiments in triplicate (**Figure 3**). For each dilution series only target construct was added. The results show a limit of detection (LOD) <10 copies/reaction.

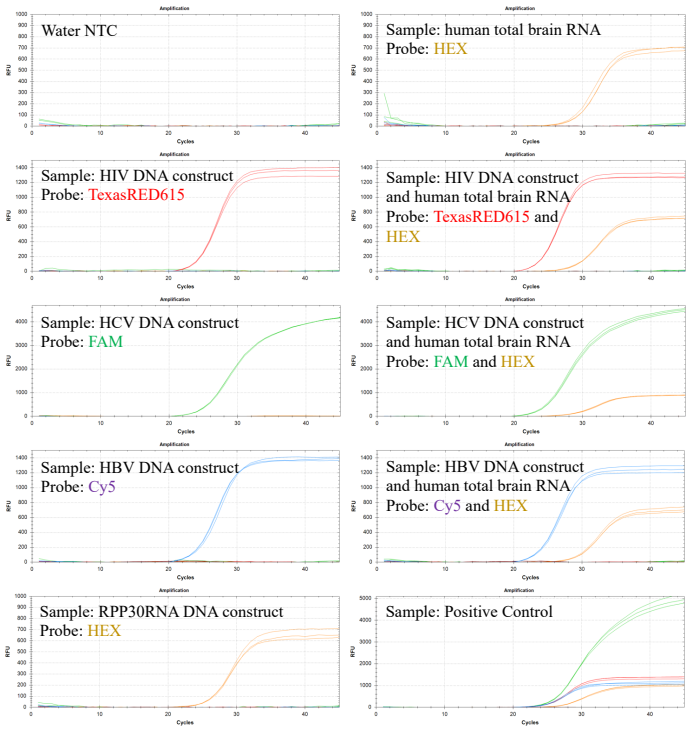

Figure 1: Verification experiments with single and multiple targets (given in text boxes for each panel). All sets of probes and primers are present in every reaction, but positive signal is only observed when the target(s) is present, indicating that the amplification is specific.

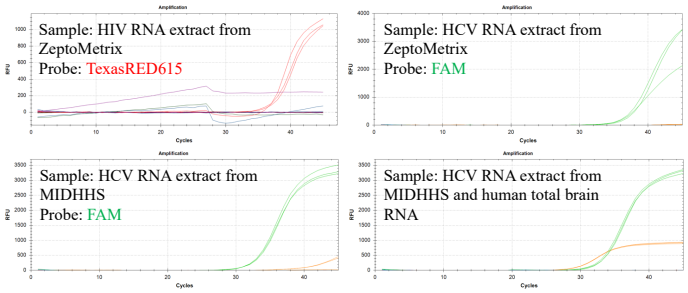

Figure 2: Multiplexed RT-qPCR detection of extracted pathogen RNA from HIV and HCV obtained from Zeptometrix, Inc., and HCV (generously provided by MI-DHHS) alone and spiked in human total brain RNA. The results indicate that the reverse transcription and amplification were successful. Note that samples from Zeptometrix are known to give late Cq values due to the stabilization process.

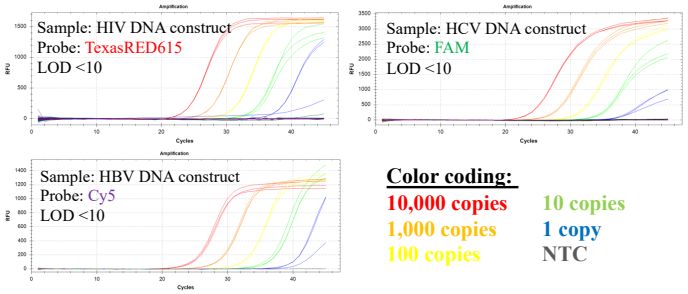

Figure 3: Serial dilution experiments show LOD <10 molecules for the synthetic DNA construct of each target. All NTC reactions show no signal.

Conclusion: The data in **Figures 1, 2, and 3** indicate that the 4-plex primers and probes specifically detect and differentiate the pathogens and are also compatible with RPP30_RNA positive control primers.

NOTES

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

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

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

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

[5] Cy5™, a trademark of GE Healthcare.

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

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

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