ASSAY NAME: *K. pneumo*

Quantity: 100 x 20µL PCR reactions

2-plex assay: *Klebsiella pneumoniae* and human RPP30 DNA

SKU#'s:
PNP-KPNE-D-BR (Bio-Rad with control assay)
PNP-KPNE-N-BR (Bio-Rad without control assay)
PNP-KPNE-D-QS (QuantStudio with control assay)
PNP-KPNE-N-QS (QuantStudio without control assay)
PNP-KPNE-D-MIC (BMS MIC with control assay)
PNP-KPNE-N-MIC (BMS MIC without control assay)

(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 document were performed using PNP-KPNE-D-BR-100 on a BioRad CFX96. 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 primers and probes in the *K. pneumo* kit are provided in Tube 1 as a 20X concentrated working solution. The same mix also contains primers/probe targeting human RPP30DNA Intron I as a qPCR positive control assay for human samples. The probes are designed as TaqMan[5] cleavage mechanism and thus the reaction requires a DNA polymerase with 5'-exonuclease activity (we recommend InhibiTaq Standard qPCR Master Mix).

Table of Dyes used in this kit:

Pathogen/Target	Dyes	Quencher	Refs.
RPP30-RNA control	HEX	BHQ-1	3, 4
K. pneumo	Cy5.5	BHQ-2	1, 2

Tube 2 "double positive control" contains a mixture of synthetic 500 bp DNA constructs containing the amplicon regions of all targets and human RPP30 DNA is provided as a positive extraction control. The concentration of each DNA construct is approximately 5,000 copies/µL. The Control DNA constructs are for validation purposes only and **Tube 2 should NOT be added to wells for specimen unknowns**.

Note: molecular biology grade water should be used to prepare the PCR reactions, which is NOT included in this kit.

Assay contents:

Tube 1: 20X Primer/Probe mix for *K. pneumo*. If you order SKU#: PNP-KPNE-D, then primers and probes for hRPP30 intron are also included.

Tube 2: (optional if ordered) 5000 copies/µl Positive controls of synthetic 500 bp DNA fragments of *K. pneumo* and hRPP30.

Tube 3: (optional if ordered) Spike-in control. 1.0E6 copies/uL of synthetic 500 BP regions of *K. pneumo* and human RPP30 intron.

Tube 4: (optional if ordered) InhibiTaq Standard 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.

KIT HANDLING AND CONTAMINATION

The *K. pneumo* assay 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.

Any contamination should be avoided by using appropriate personal protective equipment (PPE), powder free gloves, aerosol barrier pipette tips, and a clean hood.

EXPERIMENTAL

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

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

Notes: 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. For positive control rxns., add 2 µL of the solution from Tube 2 (i.e., the "sample").

A PCR protocol was used for verification on a Bio-Rad CFX96™ Real-Time System, with the following program:

Step	Thermocycling Protocol:
1	Incubate @ 95 °C for 2 minutes
2	Incubate @ 95 °C for 3 seconds
3	Incubate @ 55 °C for 15 seconds
4	Plate Read
5	Go to Step 3, repeat 44xmore

For QuantStudio instruments, we recommend a Step 3 cycle time of 22 seconds at 55 °C.

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 > "threshold" is considered "positive" or "+" in the Table below. The "threshold" is 2.0 on the BMS MIC, 200 on BioRad instruments and 200,000 on QuantStudio 5, 6, 7, 12K instruments.

K. pneumo Cy5.5™	hRPP30 HEX™	Recommended Interpretation
–	–	The PCR reaction failed. Please repeat the experiment.
–	+	The sample does not contain bacterial DNA of interest. The sample contains human RPP30 DNA.
+	–	The sample contains ***K. pneumo*** DNA. The sample may not contain human RPP30 DNA.
+	+	The sample contains ***K. pneumo*** DNA and human RPP30 DNA.

VERIFICATION EXPERIMENTS

The *K. pneumo* assay verification was performed as a 2-plex assay, which simultaneously detects DNA from *Klebsiella pneumoniae* and human RPP30 DNA, which serves as a positive extraction-control assay.

Experiments were performed in triplicate using the experimental procedure given above, but with different samples added to each reaction. The samples used for the validation experiments contained 1×10^4 copies/reaction of synthetic 500 bp synthetic DNA constructs (from Twist Biosciences) harboring the regions of interest from the target genome and the human RPP30 DNA gene, and human genomic DNA. The results of these experiments are shown in **Figure 1** below:

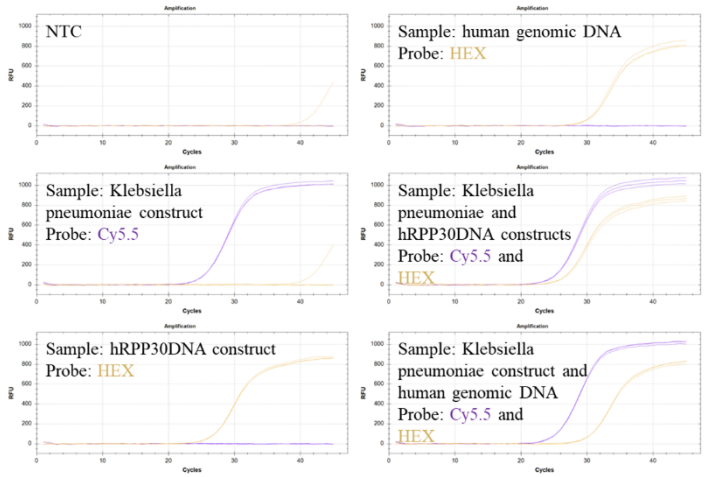
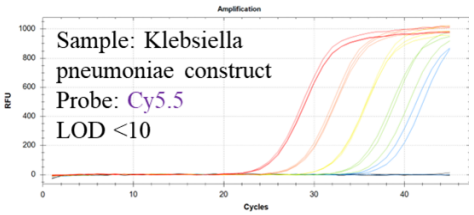


Figure 1: Verification experiments with single or double target(s) (given in text boxes for each panel). All sets of probes and primers are present in every reaction, but positive signal is only observed when the target(s) is present, indicating that the amplification is specific. The **CY5.5** probe detects *K. pneumo*. The **HEX** probe detects human RPP30 DNA. In the NTC one reaction (out if 3 replicates) shows **HEX** signal, possibly due to contamination, but the Cq >40 so it is not considered positive.



Color coding:
10,000 copies
1,000 copies
100 copies
10 copies
1 copy
NTC

Figure 2: Serial dilution experiments show LOD <10 molecules for the synthetic DNA construct of each target.

Conclusion: The data in **Figure 1** indicates that the *K. pneumo* primers and probe are compatible with DNAS RPP30 DNA positive control primers and probe in the human genomic DNA matrix.

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

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,
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Phone: (734) 222-9080

NOTES

[1] Cy5.5™ (Sulfo-Cyanine5.5) is a trademark of GE Healthcare.

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

[3] HEX™ (Hexachloro-fluorescein) is a trademark of Applera Corp.

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

[5] "TaqMan" is a trademark of Roche Molecular Systems, Inc.