PCRassays.com

ASSAY NAME: AMR-2A
(Antimicrobial Resistance Panel 2)

Quantity: 100 x 20µL PCR reactions

4-color assay: dfrA1, dfrA5, qnrA, qnrS, sul1, sul2, and human RPP30 DNA

SKU: BUN-AMR2A-D-BR384-100 (Bio-Rad)

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

SCOPE OF THIS PRODUCT INFORMATION SHEET (PIS):

The oligonucleotide recipes are optimized for each instrument (Bio-Rad, QuantStudio). The verification data presented in this PIS were performed using kit BUN-AMR2A-D-BR384-100 on a Bio-Rad CFX96 instrument using a white-bottomed plate. This kit is compatible with both CFX-96 and CFX-384 instruments. 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 AMR-2A assay are provided in Tube 1 as a 5X concentrated working solution that detects the 3 pathogens and a human extraction control. The probes are designed as TaqMan[7] cleavage mechanism and thus the reaction requires a DNA polymerase with 5'-exonuclease activity.

Table of Dyes used in this assay:

Pathogen/Target	Dyes	Quencher	Refs.
dfrA1, dfrA5	FAM	BHQ-1	1, 2
RPP30-DNA control	HEX	BHQ-1	3
qnrA, qnrS	TEX615	BHQ-2	4, 5
sul1, sul2	Cy5	BHQ-2	6

The primer and probes for each assay detect multiple alleles of each AMR gene (see Table bottom right) and confer resistance to multiple drugs and in different organisms (see Table below).

Table of AMR Genes, Drugs and Organisms:

AMR Gene	Drugs	Organisms
DfrA1	Trimetoprim	*Acinetobacter baumannii, Campylobacter jejuni, Escherichia coli, Salmonella enterica, Vibrio cholerae*
DfrA5	Trimetoprim	*Acinetobacter baumannii, Escherichia coli*
qnrA	Quinolone, Fluoroquinolone, Ciprofloxacin, Levofloxacin, Norfloxacin, Gatifloxacin, Cefpodoxime, Cefotaxime, Enrofloxacin, Trimethoprim- Sulfamethoxazole and Penicillin	*Enterobacter cloacae, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Shewanella algae*
qnrS	Quinolone, Fluoroquinolone, Ciprofloxacin, Levofloxacin, Norfloxacin, Gatifloxacin, Cefpodoxime, Cefotaxime, Enrofloxacin, Trimethoprim- Sulfamethoxazole And Penicillin	*Aeromonas hydrophila, Aeromonas sobria, Enterobacter hormaechei, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, Shigella flexneri*
sul1	Sulfonamide	*Acinetobacter baumannii, Aeromonas hydrophila, Aeromonas salmonicida, Corynebacterium diphtheriae, Corynebacterium striatum, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Morganella morganii, Pseudomonas aeruginosa, Salmonella enterica, Shigella flexneri*
sul2	Sulfonamide	*Haemophilus parasuis, Actinobacillus pleuropneumoniae, Actinobacillus porcitonsillarum, Aliivibrio salmonicida, Bibersteinia trehalosi, Mannheimia varigena, Pasteurella multocida, Pasteurella multocida, Salmonella enterica, Shigella sonnei, Vibrio cholerae*

Assay contents:

Tube 1: 5X Primer/Probe mix for dfrA1/5, qnrA/S, sul1/2, and hRPP30DNA.

Tube 2: (**Do NOT add to specimen unknowns**) Positive control: 5000 copies/µl positive controls of synthetic 500 bp DNA fragments of dfrA1, qnrA/S, sul1/2, and human RPP30DNA.

Tube 3: 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.

ASSAY HANDLING AND CONTAMINATION

The AMR-2A assay is shipped at ambient temperature, and 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 PCR reaction (20 µL) as follows on ice:

Component	Volume (µL)
InhibiTaq qPCR enzyme mastermix (2X)	10
Primer/Probe mix (5X)	4
Sample or Positive Control	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.
For 10 µL reactions, divide all of the amounts above by a factor of 2.

A PCR protocol was used for verification on a Bio-Rad CFX96 system using white plates, 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 20 seconds
4	Plate Read
5	Go to Step 2, repeat 44× more

Assay	Alleles Covered by PCRassays.com Test
DfrA1	DfrA1
DfrA5 group	DfrA5, DfrA30
QnrA group	QnrA1, QnrA2, QnrA3, QnrA4, QnrA5, QnrA6, QnrA7, QnrA9, QnrA10, QnrA11, QnrA12, QnrA13
QnrS group	QnrS1, QnrS2, QnrS4, QnrS5, QnrS6, QnrS7, QnrS8, QnrS9, QnrS10, QnrS11, QnrS12, QnrS13, QnrS14, QnrS15
sul1	sul1
sul2	sul2

RESULT INTERPRETATION

After running the qPCR reaction, use the instrument software to determine the quantification cycle, C_q (or use C_T if your instrument does not have the capability to compute a C_q). Due to low level contamination (~1-4 molecules per reaction on average) of sul1 and sul2 genes in the enzyme mastermix due to expression in *E. coli*, 10-30% of no template control (NTC) wells display Cy5 signal with Cq of 37-40 (note that qPCR mastermix from other vendors does not have this impurity). Thus, we consider true positives or "+" in the table below for reactions with Cq <34 cycles for sul1/2 and Cq <38 cycles for all other targets. In addition, a "positive" or "+" in the table below requires RFU > threshold. The RFU "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 Bio-Rad CFX96, using 96 well plates with 100µL wells and 20µL reaction volume, the average RFU was approximately 3,000 and we used a threshold of 300 for calling positives.

dfrA1/5 FAM™	qnrA/S TEX615™	sul1/2 Cy5™	RPP30 HEX™	Recommended Interpretation
–	–	–	–	The PCR reaction failed. Please repeat the experiment.
–	–	–	+	The sample contains human RPP30 DNA. The sample doesn't contain AMR DNA.
+	–	–	–	The sample contains dfrA1/5 DNA. The sample may not contain human RPP30 DNA.
+	–	–	+	The sample contains dfrA1/5 DNA and human RPP30 DNA.
–	+	–	–	The sample contains qnrA/S DNA. The sample may not contain human RPP30 DNA.
–	+	–	+	The sample contains qnrA/S DNA and human RPP30 DNA.
–	–	+	–	The sample contains sul1/2 DNA. The sample may not contain human RPP30 DNA.
–	–	+	+	The sample contains sul1/2 DNA and human RPP30 DNA.
+	+	+	–	The sample contains dfrA1/5, qnrA/S, and sul1/2 DNA. The sample may not contain human RPP30 DNA.
+	+	+	+	The sample contains dfrA1/5, qnrA/S, sul1/2 DNA, and human RPP30 DNA.

VERIFICATION EXPERIMENTS

The AMR-2A assay verification was carried out as a 7-plex assay, which simultaneously detects DNA from dfrA1, dfrA5, qnrA, qnrS, sul1, sul2, 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 verification experiments contained 1×10^4 copies/reaction of synthetic 500 bp DNA constructs (from Twist Biosciences) harboring the regions of interest from the AMR genomes, human RPP30 DNA gene, and human genomic DNA. **Figure 1** shows the results of these experiments, which indicate that the 7-plex specifically detects the different AMR genes in the human genomic DNA matrix.

The limit of detection (LOD) was estimated by performing serial dilution experiments in triplicate (**Figure 2**). For each dilution series only one target construct was added. The results show a limit of detection (LOD) <10 copies/reaction.

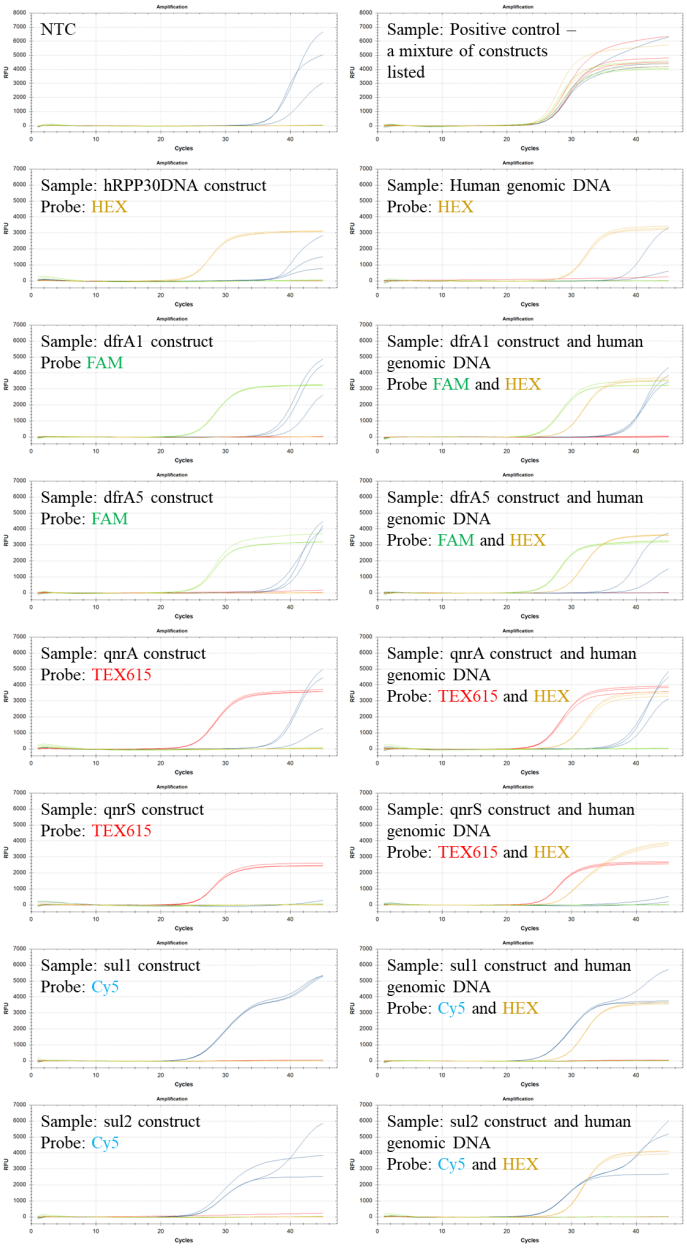

Figure 1: Verification experiments with single and multiple targets (given in text boxes for each panel). All sets of probes and primers are present in every reaction, but positive signal is only observed when the target(s) is present, indicating that the amplification is specific. Note the presence of late Cy5 signal in some of the panels. This is due to sul1 and sul2 gene contamination in the enzyme mastermix. The true positives for sul1 will sometimes show an additional transition which is due to contamination of sul2. The true positives for sul2 will sometimes show an additional transition which is due to contamination of sul1. Refer to "Result Interpretation" section for additional information.

Conclusion: The data in **Figure 1** indicate that the 7-plex primers and probes specifically detect and differentiate the AMR genes and are also compatible with RPP30 DNA positive control primers. Human genomic DNA doesn't interfere with the detection of the AMR genes.

NOTES

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

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

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

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

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

[6] Cy5™ is a trademark of GE Healthcare.

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

CONTACT US

For assistance, please contact DNA Software using the link:
https://www.pcrassays.com/contact/

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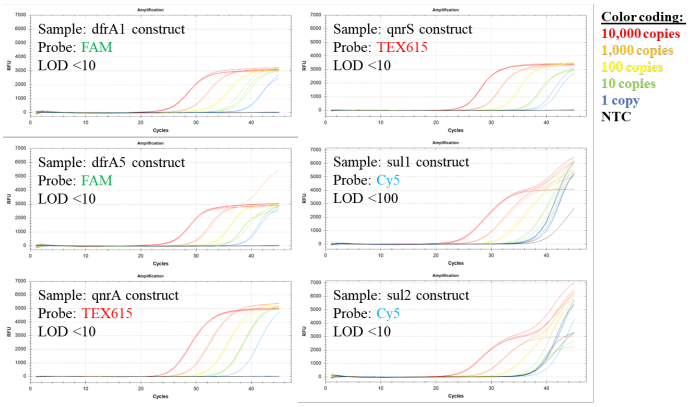

Figure 2: Serial dilution experiments show LOD <100 molecules for the synthetic DNA constructs of sul1 and sul2, and <10 molecules for synthetic DNA construct of other targets.