MANUAL

Urinary Tract Infection (UTI) Multiplex PCR Antibiotic Resistance Gene Profiling Kit

Code: PCR-UTI-2

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ISO 13485:2016

Introduction

RayBio® Urinary Tract Infection (UTI) Multiplex PCR Antibiotic Resistance Genes Profiling Kit is a ready to use real-time PCR assay for the detection of UTI Antibiotic Resistance Genes DNA in a liquid sample. The kit is based on Taqman™ DNA Polymerase amplification and fluorochrome-labeled specific-probe reporting. This kit detects 13 common Antibiotic Resistance Genes DNA long with Amplification Controls and RNase P (Internal Human DNA Amplification Control) across 5 multiplex real-time PCR panels with 4 different fluorescent probes in each panel.

Purpose

This kit is used for the qualitative in vitro detection of Urinary Tract Infection (UTI) Bacteria nucleic acid from purified DNA samples.

Kit Components (10-Sample Kit)

Component	Catalog Number	Ingredients	Specification	Quantity
2x PCR Master Mix	PCR-UTI-2-MM	Buffer, dNTP's, enzyme	965 µl / tube	1 tube
Primers and Probe Mix	PCR-UTI-2-PP-1	5 sets Primer and Probe Mix	40 µl / tube, 1 for each panel	1 tube
	PCR-UTI-2-PP-2			1 tube
	PCR-UTI-2-PP-3			1 tube
	PCR-UTI-2-PP-4			1 tube
	PCR-UTI-2-PP-5			1 tube
Amplification Control	PCR-UTI-2-AC-1	Antibiotic Resistance Gene DNA & RNase P	12 µl / tube, 2 for each panel	2 tubes
	PCR-UTI-2-AC-2			2 tubes
	PCR-UTI-2-AC-3			2 tubes
	PCR-UTI-2-AC-4			2 tubes
	PCR-UTI-2-AC-5			2 tubes
Non-Template Control	PCR-UTI-NTC	Nuclease-free water	500 µl / tube	2 tubes

Note: Do not mix reagents from different lots.

Storage

The kit can be stored at -20 °C for a period of 12 months prior to opening. After opening, the reagents are valid for at least 6 months if stored at -20 °C.

Required Materials (NOT INCLUDED)

1. Purified human urine DNA: DNA should be purified according to manufacturer protocols
2. DNA preservation solution
3. Fluorescence PCR instrument capable of reading FAM channel (494 nm maximum absorption, 518 nm maximum emission), VIC channel (520 nm maximum absorption, 558 nm maximum excitation), ROX channel (580 nm maximum absorption, 623 nm maximum excitation) and Cy5 (640 nm maximum absorption, 682 nm maximum excitation).
4. Vortex Mixer
5. Microcentrifuge
6. Pipettes
7. Sterile nuclease-free pipette tips (barrier tips recommended) and microfuge tubes
8. Compatible PCR Plate
9. Contact our technical support team for questions about compatibility: techsupport@raybiotech.com

Sample Requirements

1. All human urine specimens should be regarded as potentially infectious and handled with extreme caution. Measures should be taken to minimize the risk of laboratory transmission based on risk assessment when testing any samples. These precautions, at a minimum, should include proficiency and competency testing, appropriate PPE, avoiding aerosols, and using effective disinfectants (quaternary ammonium compounds and 0.5 % bleach).
2. We recommend centrifuging 1 ml of urine at 5000 g in a microcentrifuge for 10 minutes at room temperature and removing 800 µl of the supernatant. The remaining supernatant and pellet should be used for DNA extraction**. The final extracted DNA should be eluted in 100 µl of DNase, RNase-free water. The real-time PCR procedure requires 10µL of extracted DNA per reaction.
3. Extracted human urine DNA is the starting material for this kit. The DNA should be purified separately from the PCR assay set-up according to manufacturer protocols with a DNA preservation solution for inactivation of bacteria and preservation of the DNA.
   **The data shown in this manual was based on DNA extraction using a MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit from Thermo Fisher Scientific.

General Considerations

1. To prevent contamination of PCR reactions, clean and decontaminate all working surfaces, centrifuges, pipettes, and other equipment with 10 % bleach or DNA Away®, followed by 70 % Ethanol before every assay.
2. Conduct sample processing and DNA extraction in a separate area from the PCR assay setup (below termed the "**PCR Assay Setup Area**").
3. Care should be taken to avoid contamination of samples and reactions with DNA amplicons from used PCR plates. It is thus recommended to place used PCR plates in a sealed bag and discard them appropriately. Never open a used PCR plate or place it in the same area as the PCR instrument.
4. To minimize cross-contamination between experimental samples, disposable pipettes and filtered pipette tips are recommended.

Testing Method

1. **Assay Assembly (PCR Assay Setup Area)**
   1.1 Thaw reagents: Remove all components from the kit and fully thaw to room temperature. After thawing, mix gently by pipetting. Briefly centrifuge to collect contents at the bottom of each vial.

1.2 Prepare PCR Reaction Mix: the reaction mix for 1 test for each panel should contain 12.5 µl 2x PCR Master Mix and 2.5 µl Primers and Probe Mix, totally 15 µl. To calculate the total volume necessary for each panel, first calculate the total reaction for each panel (of samples + 1 Amplification Control + 1 Non-Template Control + 2 extra). Then, multiply the volumes of the 2x PCR Master Mix and the Primers and Probe Mix by the number of reactions and mix together to prepare a **PCR Reaction Mix** for each panel**.**

For example: for 10 samples, the total reactions per panel = 10 + 1 + 1 + 2 = 14 total reactions.

2. Sample Loading (PCR Assay Setup Area)

2.1. Add 15 µl of prepared **PCR Reaction Mix** from **1.2** above to each reaction well of a PCR plate.

2.2. Add 10 µl of extracted DNA (no more than 100 ng recommended), Amplification Control, or Non-Template Control for each panel to each reaction well of the PCR plate and pipette up and down at least 5 times to mix. Refer to Tables 1 and 2 for a summary of reaction components included in each well and an example plate layout.

Table 1: Reaction Components

Component	Amplification Control Reaction *(1 needed for each panel)*	Non-Template Control Reaction (*1 needed for each panel)*	Sample Reaction (*1 needed for each panel)*
PCR Master Mix	15 µl	15 µl	15 µl
Amplification Control	10 µl	--	--
Non-Template Control	--	10 µl	--
Extracted DNA	--	--	10 µl
Total Volume	**25 µl**	**25 µl**	**25 µl**

Table 2: Example layout on a 96-well plate

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10		
Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	Panel 1	NTC	AC1
Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	Panel 2	NTC	AC2
Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	Panel 3	NTC	AC3
Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	Panel 4	NTC	AC4
Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	Panel 5	NTC	AC5

2.3. Seal the plate tightly.

2.4. Centrifuge the plate to collect the contents at the bottom of the wells and run plate immediately.
Note: The sealed, prepared PCR plate can be stored at 2-8 °C for up to 4 hours before the "PCR Amplification" step 3 below.

3. PCR Amplification (PCR Assay Setup Area)

3.1. Instrument setup: Set the sample number, Non-Template Control, and Amplification Control accordingly to your 96-well plate setup. See Table 2 as reference. Each panel contains up to 4 targets including an Internal Amplification Control (IAC), which amplifies the RNase P gene from human samples.

3.2. Fluorescence Channel Selection:

3.2.1. Select FAM, VIC, and Cy5 (or equivalent channels) and set the targets based on Table 3.
3.2.2. Select ROX (or equivalent channel) and set the target name to “IAC”, this channel will detect the RNase P gene.
3.2.3. Ensure the “reference fluorescence dye” (passive reference) is set to “None”.

Table 3: Panels and fluorescence channel setup

	FAM	VIC	Cy5	ROX
Panel 1	AmpC		OXA-48	IAC
Panel 2	ErmA	MecA	ErmC	IAC
Panel 3	KPC	ErmB	OnrA	IAC
Panel 4	QnrB		VanB	IAC
Panel 5	VanS	VanA	VanC1	IAC

3.3 Set reaction conditions according to Table 4. Set the reaction volume to 25 µl.

Table 4: PCR Program

Step		Temperature (°C)	Time	Temperature Ramp Rate	Number of Cycles
UNG	Remove Carry-over Contamination	50	5 min	1.6 °C / sec	1
Stage 1	Initial Denaturation	95	5 min	1.6 °C / sec	1
Stage 2	Denature	95	10 s	1.6 °C / sec	40
	Anneal, extend, detect fluorescence	58	40 s	1.6 °C / sec	

3.4 Save the file, load the prepared plate, and run the program.

4. Results and Analysis

4.1. Open the data file and click ‘save as’ to save the file with a new name.
4.2. In the analysis settings, select automatic baseline, or select according to your lab validation.
4.3. Set the appropriate threshold values for each target as validated by your laboratory.
4.4. Determine Ct cutoff values for each target and controls.
Note: an example of established Ct target cutoffs is listed in Table 5 but each laboratory should establish their own cutoffs for the equipment and software in use.

Table 5. Example established Cutoff Ct values using a QuantStudio™ 5 Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA) and analyzed with Desing & Analysis Software 2.6.0.

Panel	Target	Cutoff Ct
Panel 1	AmpC	36.8
	OXA-48	34.4
Panel 2	ErmA	33.9
	MecA	34.4
	ErmC	30.9
Panel 3	KPC	35.6
	ErmB	33.4
	QnrA	34.0
Panel 4	QnrB	37.7
	VanB	35.9
Panel 5	VanS	36.1
	VanA	35.7
	VanC1	35.8

4.5. Interpretation of PCR reaction results.

Table 6. **Interpretation of PCR reaction results for kit QC parameters (AC and NTC)**

Amplification Control (AC)		Non-Template Control (NTC)	Interpretation
Ct < 40	and	No amplification, or Ct ≥ 40	Valid result
No amplification, or Ct ≥ 40	or	Ct < 40	Invalid result

Note: The Amplification Control and Non-Template Control are considered general controls for the sample preparation process. The PCR reaction results are considered valid if the Amplification Control and Non-Template Controls meet the established criteria listed in Table 6. The PCR reaction result is invalid if 1) the Ct of Amplification Control has no amplification or ≥ 40, or 2) the Ct of Non-Template Control < 40. If the reaction is invalid for a panel, the measurement of all DNA samples in this panel should be discarded, and the assay should be repeated. Result of IAC channel is excluded for QC interpretation.

Table 7. **Interpretation of PCR reaction results**

Target Gene Ct (FAM, VIC, CY5)	Interpretation
< Cutoff Ct – 0.5 Ct	+ (Positive)
= Cutoff Ct ± 0.5 Ct	Inconclusive
No amplification, or > Cutoff Ct +0.5	- (Negative)

Note: If the PCR reaction result is inconclusive, it is suggested to retest the sample. After retest, if the Ct ≤ Cutoff Ct, the PCR reaction result is considered as Positive. *If the Ct* > Cutoff Ct, the PCR reaction result is considered as Negative.

Table 8. Interpretation of individual PCR reaction channels within panels

Note: each channel should be interpreted independently of the other channels in the panel

	FAM	VIC	CY5	ROX (IAC)	Interpretation
Panel 1	+		+	+	Sample contains DNA of AmpC (FAM), OXA-48 (Cy5).
	+		+	-**	Sample contains DNA of AmpC (FAM), OXA-48 (Cy5).
	-		-	+	Sample does not contain Antibiotic Resistance Gene DNA in this panel
	-		-	-	Invalid Result

	FAM	VIC	CY5	ROX (IAC)	Interpretation
Panel 2	+	+	+	+	Sample contains DNA of ErmA (FAM), MecA (VIC), ErmC (Cy5).
	+	+	+	-**	Sample contains DNA of ErmA (FAM), MecA (VIC), ErmC (Cy5).
	-	-	-	+	Sample does not contain Antibiotic Resistance Gene DNA in this panel
	-	-	-	-	Invalid Result

	FAM	VIC	CY5	ROX (IAC)	Interpretation
Panel 3	+	+	+	+	Sample contains DNA of KPC (FAM), ErmB (VIC), QnrA (Cy5).
	+	+	+	-**	Sample contains DNA of KPC (FAM), ErmB (VIC), QnrA (Cy5).
	-	-	-	+	Sample does not contain Antibiotic Resistance Gene DNA in this panel
	-	-	-	-	Invalid Result

	FAM	VIC	CY5	ROX (IAC)	Interpretation
Panel 4	+		+	+	Sample contains DNA of QnrB (FAM), VanB (Cy5).
	+		+	-**	Sample contains DNA of QnrB (FAM), VanB (Cy5).
	-		-	+	Sample does not contain Antibiotic Resistance Gene DNA in this panel
	-		-	-	Invalid Result

	FAM	VIC	CY5	ROX (IAC)	Interpretation
Panel 5	+	+	+	+	Sample contains DNA of VanS (FAM), VanA (VIC), VanC1 (Cy5).
	+	+	+	- **	Sample contains DNA of VanS (FAM), VanA (VIC), VanC1 (Cy5).
	-	-	-	+	Sample does not contain Antibiotic Resistance Gene DNA in this panel
	-	-	-	-	Invalid Result

Positive Result: the sample contains the target DNA.

Negative Result: the sample does not contain the target DNA.

Invalid Result: the sample should be rerun with fresh samples and controls.

**if the IAC (ROX) control signal is not detected while one or more of the target specific signals (FAM, Cy5, VIC) are detected in the sample, the reaction result should be considered positive and the sample is considered to contain the target DNA. The IAC is designed to be a weak signal to ensure it does not compete with the target and lower the sensitivity of the assay.

Product performance index

1. **Limit of Detection:** The LOD of the assay is 2~13 copies/µl, or 20~130 copies per reaction (10 µl of sample volume).
2. **Repeatability:** Precision testing showed that the coefficient of variation (CV) of the precision Ct values within a kit lot are ≤ 1.2 %. Repeatability between lots of product are ≤ 10 %.

Specificity

For each panel target, no cross reactivity was identified when the kit was evaluated against the other included targets.