MANUAL

RayBio® Sexually Transmitted Disease (STD) Multiplex PCR Kit

ISO 13485:2016

Catalog #: PCR-STD

Introduction

RayBio® Sexually Transmitted Disease (STD) Multiplex PCR Kit is a ready to use real-time PCR assay for the detection of DNA and RNA from sexually transmitted pathogens in a liquid sample. This kit is based on Taqman™ DNA polymerase amplification and fluorochrome-labeled specific probe reporting. This kit detects 12 pathogens (Bacteria and Viruses) across 4 multiplex real-time PCR sub-panels with 3 different fluorescent probes in each sub-panel. An internal control, RNAse P, is also detected in each subpanel to monitor sample collection, DNA extraction, and amplification.

Purpose

This kit is used for the qualitative in vitro detection of bacteria and viruses comprising the most common Sexually Transmitted Diseases (STDs) from purified DNA samples.

Kit Components (22 Sample Kit)

Component	Catalog #	Ingredients	Specification	Quantity
2x PCR Master Mix	PCR-MM	Buffer, dNTPs, enzyme	1350 µL/tube	1 tube
RT Enzyme Mix	PCR-RT	Reverse Transcriptase	50 µL/tube	1 tube
Primers and Probe Mix	PCR-STD-PP-1	Primers and Probes	90 µL/tube, 1 for each sub-panel	1 tube
	PCR-STD-PP-2			1 tube
	PCR-STD-PP-3			1 tube
	PCR-STD-PP-4			1 tube
Amplification Control	PCR-STD-AC-1	Target pathogen DNA sequences for respective panel & internal amplification control (RNAse P)	50 µL/tube, 1 for each sub-panel	1 tube
	PCR-STD-AC-2			1 tube
	PCR-STD-AC-3			1 tube
	PCR-STD-AC-4			1 tube
Non-Template Control	PCR-STD-NTC	Internal Amplification Control (RNase P)	200 µL/tube	1 tube

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. If using kit over multiple tests, Amplification Controls and Non-Template Controls should be sub-aliquoted to prevent degradation from freeze-thaw cycles.

Required Materials (NOT INCLUDED)

1. Purified DNA from human Urine or Cervical Swab (sub-panels 1 and 2) and human Plasma (sub-panels 3 and 4)
2. DNA preservation solution
3. Fluorescence PCR instrument capable of reading FAM (494nm maximum absorption, 518nm maximum emission), VIC (520nm maximum absorption, 558nm maximum excitation), Cy5 (640nm maximum absorption, 682nm maximum excitation), and ROX (580nm maximum absorption, 623nm 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. Caution: All samples 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. DNA/RNA extracted from human Urine or Cervical Swabs (sub-panels 1 and 2) and plasma (sub-panels 3 and 4) is the starting material for this kit. DNA/RNA should be purified separately from the PCR assay set-up according to the manufacturer protocols with a DNA preservation solution for inactivation of bacteria and preservation of the DNA.

   ***Note**: HPV 16 and HPV 18 are undetectable in urine using this assay and require cervical swabs for accurate diagnosis.*

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. **Reagent preparation (PCR Assay Setup Area)**

1.1. **Thaw reagents**: Remove 2x PCR Master Mix, Amplification Control, and Non-Template Control from the kit and fully thaw to room temperature. After thawing, spin down at low speed, mix gently by pipetting, and spin again to collect contents at bottom of the vial.

1.2. **Calculate number of reactions needed**: The number of reactions to be prepared per PCR run is calculated as:

Reactions = n + 1 Amplification Control + 1 Non-Template Control + 2, where "n" is the number of samples (plus any replicates).

***Example**: For 22 samples with no replicates, total reactions = 22 + 1 + 1 + 2 = 26.*

1.3. **Prepare Separate PCR Reaction Mixes for Sub-Panels 1 to 4**: Mix 2x PCR Master Mix (PCR-MM), Primer and Probe Mix (PCR-PP-1 to 4), and RT Enzyme Mix (PCR-RT) per replicate according to table below. To calculate the total volume necessary for the run, multiply the volumes of each component by the number of reactions calculated in Step 1.2 above.

Per Replicate	Sub-Panel 1	Sub-Panel 2	Sub-Panel 3	Sub-Panel 4
2x PCR Master Mix (PCR-MM)	12.5 µL	12.5 µL	12.5 µL	12.5 µL
Primer and Probe Mix (PCR-PP-1 to 4)	2.5 µL	2.5 µL	1.5 µL	1.5 µL
RT Enzyme Mix (PCR-RT)	0 µL	0 µL	1 µL	1 µL

2. **Sample Loading (PCR Assay Setup Area)**

2.1. Add 15 µL of prepared PCR Reaction Mixes from step 1.3 to appropriate wells of a PCR plate (see Table 1 for representative plate layout).

***Note**: Sub-panels 1 and 2 should not be run on the same column as sub-panels 3 and 4 (and vice versa) due to the incompatible annealing temperatures.*

2.2. Add 10 µL of appropriate sample (Amplification Control, Non-Template Control, or extracted DNA from sample) to designated wells of a 96 well PCR plate or PCR tubes (see Table 1 for representative plate layout). Pipette up and down at least 5 times to mix.

***Note**: It is recommended that no more than 100 ng of extracted DNA be added to each reaction.*

2.3. Seal the plate or tubes tightly.

2.4. Centrifuge the plate or tubes for 30 seconds at low speed.

***Note**: The sealed PCR reaction tubes 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. **Sample setup**: Set the sample number, Non-Template Control, and Amplification Controls according to your 96-well plate setup. See Table 1 for a representative plate layout.

Table 1. Sample test layout on a 96-well PCR plate

Sub-Panel #											
1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Sample 1				Sample 9				Sample 17			
Sample 2				Sample 10				Sample 18			
Sample 3				Sample 11				Sample 19			
Sample 4				Sample 12				Sample 20			
Sample 5				Sample 13				Sample 21			
Sample 6				Sample 14				Sample 22			
Sample 7				Sample 15				Amplification Control			
Sample 8				Sample 16				Non-Template Control			

3.2. **Fluorescence channel selection**: Each sub-panel contains 4 targets including an Internal Amplification Control (IAC), which amplifies the RNAse P gene co-extracted from human samples. Select FAM, VIC, and Cy5 (or equivalent channels) and set the targets based on Table 2. Select ROX (or equivalent channel) and set the target name for "IAC." Set the reference fluorescence dye (or passive reference) to "None."

Table 2. Sub-Panels and fluorescence channel setup

	FAM	VIC	Cy5	ROX
Sub-Panel 1	HPV 16	Treponema pallidum	Chlamydia trachomatis	IAC
Sub-Panel 2	Trichomonas vaginalis	Neisseria gonorrhoeae	HPV 18	IAC
Sub-Panel 3	HSV 1	Hepatitis C	HIV 2	IAC
Sub-Panel 4	Hepatitis B	HIV 1	HSV 2	IAC

3.3. Set reaction conditions according to Table 3. Set the reaction volume to 25 µL. Ensure that program is set to detect fluorescence at the end of each cycle in step 3.

3.4. Save the file and run the program.

Table 3: PCR Program

Step		Temperature	Time	Temperature Ramp Rate	Number of Cycles
1	Remove Carry-over Contamination and Synthesize cDNA	25°C	2 min	2.74°C/sec	1
		50°C	10 min	2.74°C/sec	1
2	Initial Denaturation	95°C	5 min	2.74°C/sec	1
3	Denature	95°C	15 sec	2.74°C/sec	40
	Anneal, extend, and detect fluorescence	58°C & 60°C*	40 sec	2.12°C/sec	

**Set PCR columns for sub-panels 1 and 2 to 58oC and those for sub-panels 3 and 4 to 60°C. If using suggested plate map in Table 1, set columns 1, 2, 5, 6, 9, and 10 to 58°C and all other columns to 60°C.*

4. Results and Analysis

4.1. **Baseline and threshold determination**: Baseline and fluorescence thresholds (FAM, VIC, Cy5, and ROX) are determined automatically by the instrument. Extreme care should be taken when manually adjusting these settings.

4.2. Determine cutoff Ct values for each target.

***Note**: An example of established Ct target cutoffs is listed in Table 4, but each laboratory should establish their own cutoffs for the specific equipment and software in use.*

Table 4. Example established Cutoff Ct values using a QuantStudio™ 5 Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA) and analyzed with Desing & Analysis Software 2.6.0.

Panel	Target	Cutoff Ct
1	HPV 16	37
	Chlamydia trachomatis	35
	Treponema pallidum	37
	IAC (RNAse P)	36
2	HPV 18	35
	Neisseria gonorrhoeae	37
	Trichomonas vaginalis	37
	IAC (RNAse P)	36
3	HSV 1	35
	Hepatitis C	35
	HIV 2	35
	IAC (RNAse P)	36
4	HSV 2	34
	Hepatitis B	34
	HIV 1	34
	IAC (RNAse P)	36

***Note**:* All positive amplification curves should have an amplification status of “Amp” (or equivalent). If software reports “No amplification” or “Inconclusive amplification,” sample should be treated as negative.

4.3. **Interpretation of quality control standards**: Amplification control and Non-Template Controls are considered valid if they meet the pre-determined cutoff Ct criteria (See Table 5). If either FAM, VIC, Cy5, or ROX of the Amplification or Non-Template Control(s) fails to meet criteria, the reaction is invalid, all sample measurements in the experiment should be discarded, and the assay repeated.

4.4. **Interpretation of test results**: The PCR reaction sample results are explained according to Table 5. If test result is inconclusive or invalid for a given sample, that sample should be re-tested. All other samples should be considered valid and do not need to be re-tested.

Table 5. Interpretation of PCR reaction results

Target Gene Ct (FAM, VIC, Cy5, ROX)	Interpretation
Amplification And Ct < Cutoff – 0.5	Positive (+)
Amplification And Ct = Cutoff ± 0.5	Inconclusive
No amplification Or Ct ≥ Cutoff + 0.5	Negative (-)

Table 6: Interpretation of test results

Sub-Panel 1

FAM – HPV 16	VIC – Treponema pallidum	Cy5 – Chlamydia trachomatis	ROX – RNAse P	Interpretation
+	+	+	+ or -	Positive Result(s)*
-	-	-	+	Negative Result(s)
-	-	-	-	Invalid Result

Sub-Panel 2

FAM – Trichomonas vaginalis	VIC – Neisseria gonorrhoeae	Cy5 – HPV 18	ROX – RNAse P	Interpretation
+	+	+	+ or -	Positive Result(s)*
-	-	-	+	Negative Result(s)
-	-	-	-	Invalid Result

Sub-Panel 3

FAM – HSV 1	VIC – Hepatitis C	Cy5 – HIV 2	ROX – RNAse P	Interpretation
+	+	+	+ or -	Positive Result(s)*
-	-	-	+	Negative Result(s)
-	-	-	-	Invalid Result

Sub-Panel 4

FAM – Hepatitis B	VIC – HIV 1	Cy5 – HSV 2	ROX – RNAse P	Interpretation
+	+	+	+ or -	Positive Result(s)*
-	-	-	+	Negative Result(s)
-	-	-	-	Invalid Result

Positive Result: The sample contains the target gene(s).

Negative Result: The sample does not contain the target gene(s).

Invalid Result: The sample should be re-run with fresh samples and controls.

***The IAC is designed to be a weak signal to ensure that it does not interfere with the target and lower sensitivity of the assay. Strong positives can interfere with IAC amplification, resulting in elevated Ct values or no amplification at all. If ROX is undetected while one or more target specific signals are detected in the sample, the reaction result should be considered positive regardless of the ROX test result. The reverse is not true for negative samples.*

Product Performance Index

1. **Limit of Detection (LOD)**: The LOD for each target is detailed in Table 7.
2. **Repeatability**: Precision testing showed that the coefficient of variation (CV%) of the precision Ct values for all targets within a kit lot are ≤ 2.5%. CV% between kit lots are ≤ 10%.
3. **Specificity**: Cross- and within-panel specificity testing revealed cross-reactivity of HSV 2 with HSV 1 in sub-panel 4 (but not the reverse) and slight fluorescence bleed-through of the FAM channel (HPV 16) into the VIC channel (Treponema pallidum) of sub-panel 1. Positive results for HSV 2 and T. pallidum in samples co-positive for HSV 1 and HPV16, respectively, should be carefully compared against respective Amplification Controls and patient symptoms to reduce false positives.

Table 7. Assay Sensitivity

Pathogen	cp/µL*
HPV 16	2
HPV 18	2
Chlamydia trachomatis	4
Neisseria gonorrhoeae	2
Treponema pallidum	2
Trichomonas vaginalis	2
HSV 1	20
HSV 2	2
Hepatitis B	2
Hepatitis C	2
HIV 1	2
HIV 2	2

**Concentration in sample assuming 10 µL sample volume per reaction.*