Step 4: Multipex PCR protocol

Urodiag® Multiplex PCR Kit (OncoDiag)

50 Patient tests
Ref. No. UR50P

The Urodiag® Multiplex PCR Kit is an in vitro diagnostic test for the surveillance of patients with non-muscle-invasive bladder cancer (NMIBC).

The procedure for multiplex PCR reactions is carried out with the StepOnePlus Real-Time system (applied biosystems, Thermo Fisher Scientific). The kit can be stored in the freezer (-15-25°C) for up to 12 months. It is not recommended to freeze and thaw the kit more than 5 times.

Equipment and Reagents to Be Supplied by User

■ StepOnePlus Real-Time system

■ MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 ml (ThermoFisher Scientific_4346907) and Optical Adhesive Covers (ThermoFisher Scientific_4313663, 4311971) or MicroAmp Fast 8-Tube Strip, 0.1 ml (ThermoFisher Scientific_4358293) and MicroAmp Optical 8-Cap Strip (ThermoFisher ScientifIc_4323032)

■ Micropipets and pipet tips with aerosol barrier

■ Microcentrifuge for PCR plates

■ Vortexer

■ Cooling block

Kit contents

The kit is designed for 50 patients (minimum 10 patients/PCR run ⇒ 5 runs). It is intended for the qualitative detection of *FGFR3* somatic mutations (G372C, R248C, S249C, Y375C) and the quantification of three DNA methylation markers (*HS3ST2, SEPTIN9, SLIT2*) by multiplex PCR in urine DNA of patients.

The kit is composed of 8 tubes: 4 tubes for the Mutation assay (bleue and green cap), 3 tubes for the Methylation assay (red, orange and pink cap) and 1 tube with sterile water (white cap). Each tube contains all the components (PCR mastermix, primers and probes) necessary to carry out the Mutation and Methylation assays.

	Tube	Vol.	Description
Mutation assay	1	1040 µl	The tube contains all components (PCR master mix, primers and probes) necessary to detect S249C, Y375C mutations and the *GLOBIN* gene
	2	30 µl	The tube contains a mixture of two DNA solutions: (i) synthetic *FGFR3* sequences comprising the mutations S249C and Y375C placed in a plasmid vector, (ii) human control DNA to detect the globin gene (internal control)
	3	1040 µl	The tube contains all components (PCR master mix, primers and probes) necessary to detect R248C, G372C mutations and the *GLOBIN* gene
	4	30 µl	The tube contains a mixture of two DNA solutions: (i) synthetic *FGFR3* sequences comprising the mutations R248C and G372C placed in a plasmid vector, (ii) human control DNA to detect the globin gene (internal control)
Methylation assay	A	1040 µl	The tube contains all components (PCR master mix, primers and probes) necessary to detect and quantify, *ALBUMIN (unmethylated allele)* and *SEPTIN9 (methylated allele)*
	B	1040 µl	The tube contains all components (PCR master mix, primers and probes) necessary to detect and quantify, *HS3ST2* and *SLIT2 (methylated alleles)*
	C	132 µl	Control DNA (fully methylated human DNA)
	H_20	150 µl	Negative template control (NTC)

Protocol

Step 1: Sample preparation

Thaw all frozen reaction components to room temperature (15-25°C), then place them in a cooling block (recommended). Mix gentle, centrifuge briefly to collect solutions at the bottom of tubes.

- **Mutation assay**

Note: It is recommended to carry out the detection of *FGFR3* Mutations from 10 ng of DNA, i.e. 5 ng of DNA for the detection of S249C and Y375C mutations (Tube 1) and 5 ng of DNA for the detection of G372C and R248C mutations (Tube 2).

(For patient, the minimum quantity of DNA is 5 ng, i.e. 2.5 ng of DNA for the detection of S249C and Y375C mutations and 2.5 ng of DNA for the detection of G372C and R248C mutations).

Prepare samples according to recommendations in Table 1 below.

Tube	Control				Patient	
	Control 1		Control 2		Test 1	Test 2
	Positive	Negative	Positive	Negative		
1	16 µl	16 µl	/	/	16 µl	/
2	4 µl	/	/	/	/	/
3	/	/	16 µl	16 µl	/	16 µl
4	/	/	4 µl	/	/	/
DNA patient (~2.5-5 ng)	/	/	/	/	4 µl	4 µl
H_2O	/	4 µl	/	4 µl	/	/
Final volume	20 µl					

- **Methylation assay**

Note: It is recommended to carry out the methylation assay from 20 ng of bisulfite-converted DNA, i.e. 10 ng of bisulfite-converted DNA for the quantification of *ALBUMIN* et *SEPTIN9* (Tube A) and 10 ng of bisulfite-converted DNA for the quantification of *HS3ST2 and SLIT2* (Tube B).

(For patient, the minimum amount of DNA is 10 ng, i.e. 5 ng of bisulfite-converted DNA for the quantification of *ALBUMIN* et *SEPTIN9* and 5 ng of bisulfite-converted DNA for the quantification of *HS3ST2 and SLIT2*).

Prepare samples according to recommendations in Table 2 below.

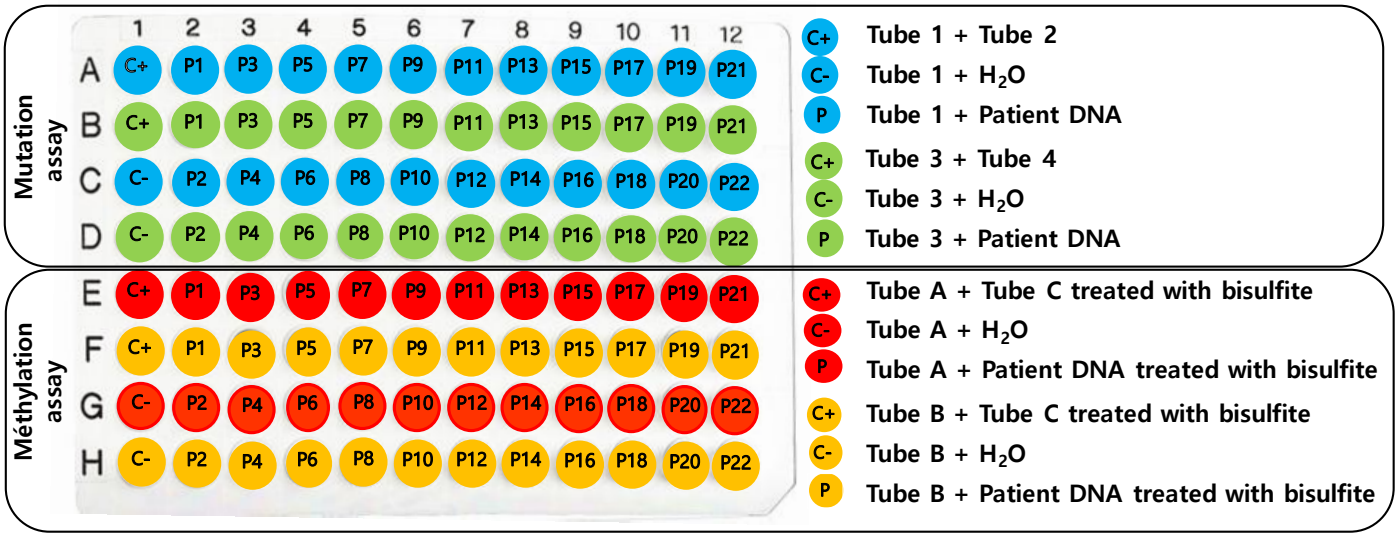
Tube	Control				Patient	
	Control 1		Control 2		Test 1	Test 2
	Positive	Negative	Positive	Negative		
A	16 µl	16 µl	/	/	16 µl	/
B	/	/	16 µl	16 µl	/	16 µl
C* (~10 ng)	4 µl	/	4 µl	/	/	/
DNA patient* (~5-10 ng)	/	/	/	/	4 µl	4 µl
H_2O	/	4 µl	/	4 µl	/	/
Final volume	20 µl					

* Bisulfite-converted DNA

Step 2: Sample deposition in MicroAmp Fast Optical 96-well plate or MicroAmp Fast Reaction (8-tubes/Strip)

As below, the position of the DNA samples on a 96-well PCR plate or PCR tubes (8-reaction tubes/strip) to carry out the URODIAG test of 22 patients.

1- Dispense appropriate volumes (reagents/samples) into corresponding wells or tubes, according Tables 1 & 2.



2- After covering the 96-well plate with the optical film or closing the PCR tubes with the caps, homogenize the reaction mixture by vortexing for 2 to 5 seconds.

3- Centrifuge the 96-well PCR plate or the PCR tubes using a microplate rotor for 10-15 seconds at approximately 1000 x g (3000 rpm).

Step 3: Program the StepOnePlus Real-Time system

The PCR reaction setup is saved in the file "URODIAG_TEMPLATE_PCR_edt.edt. Place the 96-well PCR plate or the PCR tubes in the real-time cycler, and start the cycling program (see below). Perform data analysis.

- **Parameters**

Mutation assay		
TaqMan reagents		
Quantitation –Comparative Ct		
Reaction volume per well		**20 µl**
Ramp speed		**Fast**
Ramp rate		**100%**
Threshold (ΔRn)	R248C G372C S249C Y375C *GLOBIN*	**0.24** **0.15** **0.15** **0.15** **0.15**
Baseline		**Auto**
Passive reference		**ROX**

Methylation assay		
TaqMan reagents		
Quantitation –Comparative Ct		
Reaction volume per well		**20 µl**
Ramp speed		**Fast**
Ramp rate		**100%**
Threshold (ΔRn)	*ALBUMIN* *HS3ST2* *SEPTIN9* *SLIT2*	**0.10** **0.10** **0.10** **0.10**
Baseline		**Auto**
Passive reference		**ROX**

- **Dye (Reporter)**

Mutation assay		
Detection	**Reporter**	**Quencher**
G372C	VIC	(aucun)
R248C	FAM	(aucun)
S249C	FAM	(aucun)
Y375C	VIC	(aucun)
GLOBINE	NED	(aucun)

Methylation assay		
Detection	**Reporter**	**Quencher**
ALBUMIN	VIC	(aucun)
HS3ST2	FAM	(aucun)
SEPTIN9	FAM	(aucun)
SLIT2	VIC	(aucun)

- **Multiplex pCR cycling conditions**

Step	**Number of cycles**	**Time**	**Temperature**	**Fluorescence data collection**
Initial PCR activation step	1	5 min	95°C	-
Denaturation	40	45 secs	95°C	-
Annealing/ extension		45 secs	60°C	✓

Place the PCR tubes or plate in the real-time cycler, and start the cycling program. Perform data analysis.

Step 4: Rendered results

- **Quality Control (QC)**

Mutation assay		**Ct values**	**Rendering**
Control 1	Positive control for *GLOBIN*	28-32	PASSED
	Positive control for S249C mutation	29-33	PASSED
	Positive control for Y375C mutation		PASSED
	Negative control	No amplification	PASSED
Control 2	Positive control for *GLOBIN*	28-32	PASSED
	Positive control for R248C mutation	29-33	PASSED
	Positive control for G372C mutation		PASSED
	Negative control	No amplification	PASSED
Patient	*GLOBIN*	28-32	PASSED

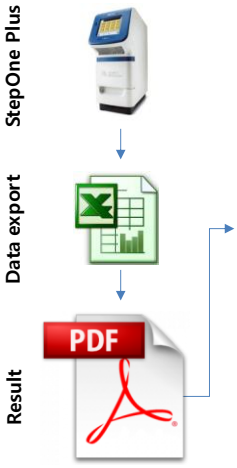
Methylation assay		Valeurs Ct	Interprétation
Control 1	Positive control for *ALBUMIN*	26-30	PASSED
	Positive control for *SEPTINE9*		PASSED
	Negative control	No amplification	PASSED
Control 2	Positive control for *HS3ST2*	26-30	PASSED
	Positive control for *SLIT2*		PASSED
	Negative control	No amplification	PASSED
Patient	*ALBUMIN*	26-30	PASSED

- **Patient**

Mutation assay		Ct values	Interpretation	Result
Patient	S249C mutation	25-40	DNA mutated for S249C	POSITIVE
	Y375C mutation		DNA mutated for Y375C	POSITIVE
	G372C mutation		DNA mutated for G372C	POSITIVE
	R248C mutation		DNA mutated for R248C	POSITIVE

Methylation assay		Ct values	Interpretation	
Patient	*HS3ST2*	25-40	Presence of methylated alleles of the *HS3ST2* gene	Determination of the methylation degree of the 3 target genes
	SEPTINE9		Presence ofmethylated alleles of *SEPTIN9* gene	
	SLIT2		Presence of methylated alleles of *SLIT2* gene	

After the run is complete, PCR data (Cts) are exported, in text file format, then into the Urodiag software for automatic rendering of results.



URODIAG		
MUTATION	METHYLATION SCORE	RESULT
POSITIVE	POSITIVE	POSITIVE
POSITIVE	NEGATIVE	
NEGATIVE	POSITIVE	
NEGATIVE	NEGATIVE	NEGATIVE