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Urodiag PCR Kit Handbook

Σ 50

Version 6

IVD

For use with the QuantStudio 5 qPCR System (Applied Biosystems_ThermoFisherScientific)

CE

REF UR50N

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Revision history : Revision history of the Urodiag PCR Kit Handbook v5

Révision	Description
B	Changing confidence intervals for the Mutation test
A	New user manual Urodiag Handbook_v6_2026_EN

MAIN TIPS FOR URODIAG TEST

Urodiag test is based on the simultaneous detection of mutations and methylations.

1 - Urine Collection (80-100 ml)

- The patient must not drink after 9 pm the day before the urine collection.
- The patient collects the first urine of the morning (in order to obtain a large number of bladder cells)
- The first urine collection must be done **at the time of diagnosis (reference or Ref).** The sample must be taken **before** the cystoscopy confirming the patient's cancer diagnosis. This initial test gives the mutation status of the FGFR3 gene and a methylation reference (sum of the meyhylation of 3 genes) for the next follow-up tests (T1, T2, …, Tn).

Note : We recommend that patients collect the urine sample (Ref) a few days before the diagnostic cystoscopy. This would allow a new Ref urine sample to be requested if the amount of DNA is lower than 25 ng (minimum requirement). It is possible to pool the DNA from the two Ref samples to obtain the required amount.

- At each follow-up test (**T1, T2, …Tn)**, a urine sample is collected. The test gives the follow-up status based on mutations (positive or negative) and the sum of the methylation percentages of 3 genes (for methylations, in reference with Ref result).

2 - Needed quantity of DNA

Urodiag test requires a quantity of **25 to 40 ng of DNA (Ref** or **T1, T2, … Tn)**

- Mutation assay requires **10 ng of DNA**

- Methylation assay requires **15 ng to 30 ng of DNA (30 ng are recommended)**

- **If the DNA concentration is above 1.25 ng/µl**, it must be diluted with elution buffer (The remaining DNA is stored at -20 °C).
- **If the DNA concentration is below 1.25 ng/µl**, it must be concentrated by heating to +50°C using a heating block or a DNA concentrator (speed vacuum). The volume of DNA to be evaporated is calculated to obtain a concentration close to 1.25 ng/µl

3 - Urodiag test result

The Urodiag software provides test results based on data obtained at Ref (initial diagnosis of NMIBC) and at each follow-up point in months (T1, T2, …, Tn).

Mutation test at Tn		Methylation SCORE	Result
Positive	and	≥ -0 .021	POSITIVE
Positive	and	< -0.021	POSITIVE
Negative	and	≥ -0 .021	POSITIVE
Negative	and	< -0.021	NEGATIVE

The Mutation test is Positive if at least one of the four mutations has been detected.

The Methylation score is determined by an equation that takes into account the sum of the methylation percentages calculated at the time of the initial diagnosis (**SUM_Ref**) and at the follow-up points Tn for the patient (**SUM_Tn**).

The Result is POSITIVE = Presence of bladder cancer recurrence.

The Result is NEGATIVE = Absence of bladder cancer recurrence

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Intended Use

The Urodiag PCR kit is an in vitro diagnostic test for the surveillance of Non-Muscle-Invasive Bladder Cancer (NMIBC). It is a ready-to-use kit for the detection of four somatic mutations in the FGFR3 gene and quantification of the methylation percentage of three genes. DNA will be extracted from urine and tested using real-time polymerase chain reaction (PCR) on QuantStudio 5 qPCR System (Applied Biosystems-ThermoFisher Scientific). The result of the Urodiag test is obtained on urine collected **at the time of diagnosis (Ref)** and **at each follow-up point (T1, T2…) in months**. The Urodiag test offers high sensitivity and specificity, even in patients at low risk of recurrence.

List of FGFR3 mutations

Mutation	**G372C**	**R248C**	**S249C**	**Y375C**
Base change	GGC > **T**GC	CGC > **T**GC	TCC > T**G**C	TAT > T**G**T

List of methylated genes

SEPTIN9	HS3ST2	SLIT2

Principle of the Procedure

The Urodiag PCR kit uses TaqMan MGB (minor groove binder) chemistry to detect four mutations in the FGFR3 gene and quantify the methylation percentage of three genes.

TaqMan MGB probe

It is based on the use of an oligonucleotide probe located between the two PCR primers and labeled with a fluorophore covalently attached to the 5'-end (reporter) and a quencher on the 3'end. The addition to the probe of an inclusive MGB moiety greatly increases the stability and specificity of probe hybridisation, and the use of an NFQ (Non-fluorescently quenched) enhances spectral performance. Because the quencher does not fluoresce, background is eliminated, and the signal-to-noise ratio is increased. The fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

Kit format

50 tests are supplied in the Urodiag PCR Kit. It includes two boxes with a box of filters for urine filtration and a box to perform multiplex PCR assays. The Urodiag Multiplex PCR kit is composed of eight tubes with five tubes for the Mutation assay (blue, green and white cap) and three tubes for the Methylation assay (red, orange and purple cap). Each tube contains all the components (PCR master mix, primers and probes) necessary to carry out the Mutation and Methylation assays.

Material provided

Urodiag Urine Filters	
Number of filters	**50**
▪ 50 disposable syringe filters ▪ Filters stored at 20°C ± 5°C ▪ Expiry time: 10 years from production date	

Urodiag Multiplex PCR Kit			
Number of reactions			**50**
Mutation Assay			
▪ Positive control mut 1 (C+ mut 1)	S249C + Y375C + GLOBIN	Blue	22 µl
▪ Master mix mut 1 (MM mut 1)	S249C + Y375C + GLOBIN	Blue	960 µl
▪ Positive control mut 2 (C+ mut 2)	R248C + G372C + GLOBIN	Green	22 µl
▪ Master mix mut 2 (MM mut 2)	R248C + G372C + GLOBIN	Green	960 µl
▪ Negative control mut (C- mut)	GLOBIN	Yellow	44 µl
Methylation Assay			
▪ Positive control meth (C+ meth)	Methylated DNA	Purple	126 µl
▪ Master mix meth A (MM meth A)	ALBUMIN + SEPTIN9	Red	960 µl
▪ Master mix meth B (MM meth B)	HS3ST2 + SLIT2	Orange	960 µl
All tubes are stored at -15°C to -30°C Expiry time: 12 months from production date.			

Urodiag sotware

The software allows the analysis of PCR data, interpretation, result management and rendering of Urodiag test to urologists

Materials Required but Not Provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Reagents

■ DNA Extraction using QIAamp DNA Mini Kit (Qiagen), catalog Nos. 51304 (50 rns) (see “Protocol for DNA Extraction and Purification”, page 7)

■ DNA conversion by sodium bisulfite using EZ DNA Methylation Kit (Zymo Research), catalog No. D5001 (55 reactions) (see “Protocol for DNA Conversion with Sodium Bisulfite”, page 9)

■ Qubit dosing tube, reference Q32851 (100), Q32856 (500) (Thermo Fisher Scientific) or equivalent

Equipement

■ QuantStudio 5 qPCR System (Applied Biosystems_ThermoFisherScientific)

■ QuantStudio Design & Analysis Software v1.5.3

■ Qubit 4 Fluorometer (Thermo Fisher Scientific) or equivalent

Protocol for Urine Collection

RECOMMENDATIONS

- **Do not drink after 9 p.m. on the evening before urine collection**

- **Collect the first morning urine**

- The <u>Ref urine sample</u> is collected <u>BEFORE</u> the <u>cystoscopic examination</u> is performed to confirm bladder cancer (NMIBC).

We recommend that patients collect a urine sample (Ref) 7-15 days before the cystoscopy. This would allow a new Ref urine sample to be requested if the amount of DNA is less than 25 ng (minimum requirement).

COLLECTION INSTRUCTIONS

Perform on STERILE CONTAINER

- Wash hands thoroughly with soap
- Make a careful local toilet
- Eliminate the 1st urine stream in the toilet
- **<u>Collect 80 ml to 100 ml (recommended) of urine</u>** in the sterile container (capacity of 120 ml)
- Close the bottle by screwing the lid on properly and identify it (name + first name + date and time of collection)

Sample storage time

- Transport the urine container from the patient's home to the competent service (laboratory, hospital), at room temperature and within 2 hours,

- After 2 hours, store the urine sample in the refrigerator (+4°C to +8°C) for up to 72 hours

Protocol for Urine Samples Filtration

Urodiag Urine Filters

50 Filters

Protocol

- Urodiag urine filter: Filters are stored at room temperature (15°C to 25°C).
- Expiry time: 10 years from production date.

Equipment and Reagents to Be Supplied by User:

■ Urine sample **Ref** and **T1, T2…Tn (80 ml to 100 ml)**

■ 50-100 ml syringe luer-lock (Terumo)

■ Phosphate-buffered saline (1X PBS solution), without Mg & Ca (stored at +4°C)

■ Waste bottle containing bleach

1. Remove the piston from the syringe and connect the **Filter (Ref** or **Tn)** to the syringe
2. Introduce the **urine sample (Ref** or **Tn)** and then reinsert the piston into the syringe
3. Apply gentle pressure on the syringe piston to ensure the filtration of the urine sample. The filtrate is collected in a waste container containing bleach

If you are using a 50 ml syringe, the operator should disconnect the Filter (Ref or **Tn) from the syringe after the first 50 ml of urine has been filtered and repeat steps 1 to 3 to filter the remaining volume of the sample.**

4. Remove the plunger from the syringe and connect the **Filter (Ref** or **Tn)** to the syringe
5. Introduce 5 ml of 1X PBS, reinsert the piston into the syringe
6. Apply gentle pressure on the syringe piston **to ensure complete filtration of the 5 ml of 1X PBS**. The filtrate is collected in the waste bottle containing bleach
7. Disconnect **the Filter (Ref** or **Tn)** from the syringe
8. **The Filter (Ref** or **Tn) is ready for the urinary DNA extraction procedure or can be stored one month at -20°C and shipped on dry ice.**

Note: Alternative protocol using the **QIAvac 24 Plus (Qiagen).**
Ensure that the main vacuum valve is closed (protocol in QIAvac 24 Plus Handbook). Switch on the vacuum pump by pressing the power switch. Adjust the needle of the vacuum between **-250 mbar** and **-300 mbar**. Insert the VacConnector into the luer slot, then the **Filter/syringe (without the piston) or Device,** on the QIAvac 24 Plus. Introduce the first 50 ml of the urine sample into the Device. Open the main vacuum valve and ensure that the needle is stabilized near -300 mbar. After all of urine sample has been filtered, switch off the vacuum pump. Remove the Device from the vacuum manifold, and discard the VacConnector.

Protocol for DNA Extraction and Purification

Protocol

The extraction and purification of DNA are carried out with the QIAamp DNA Mini Kit (QIAGEN). The kit can be stored at room temperature (+15°C to +25°C).

Expiry time: 12 months from delivery date.

Equipment and Reagents to Be Supplied by User:

- QIAamp DNA Mini Kit (Qiagen), catalog Nos. 51304 (50 rns)
- Absolute ethanol (96-100%) (stored at 4°C)
- Syringe 2.5 ml with needle 21 G x 25 mm (Terumo)
- Micropipets and pipet tips with aerosol barrier
- Microcentrifuge (with rotor for 1.5-2 ml tubes)
- Vortexer
- Water bath or heating block at +56°C
- Phosphate-buffered saline (PBS 1X), without Mg & Ca (stored at +4°C)

Buffer preparation:

Preparation of Wash Buffer- AW1 and **AW2** buffers are supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle. AW1 and AW2 buffers are stable for 1 year when stored closed at room temperature (+15°C to +25°C).

Preparation of Cell Lysis Buffer

1. Introduce 220 µl **Buffer PBS 1X** and 22 µl QIAGEN **Proteinase K** in 1.5 ml microcentrifuge tube.
2. Mix by pulse-vortexing for 1 second and then centrifuge briefly to collect the contents at the bottom of the tube.
3. Add 220 µl **Buffer AL**. Mix by pulse-vortexing for 2 seconds and then centrifuge briefly to collect the contents at the bottom of the tube.

   Note: *Mix Buffer AL thoroughly by shaking before use. Do not add Proteinase K directly to Buffer AL.*

4. Disconnect the needle and the piston from the syringe.

5. Connect the **Filter** to the syringe, then to the needle, and placed the system in 2 ml microcentrifuge tube.

   Note: *If the Filter has been stored at -20°C, leave it on the bench 5 min at room temperature before the lysis step*

6. Introduce 462 µl of **Cell Lysis Buffer** into the syringe, then reinsert the piston.

7. Pass the lysate 3 times through the **Filter (Ref** or **Tn)** by aspiration/discharge operation and pushing/pulling the piston slowly.

   Note: *If foaming occurs, we recommend centrifuging briefly the tube.*

8. Incubate the sample at 56°C for 15 minutes (water bath or heating block).

9. Briefly centrifuge the DNA sample **(Ref** or **Tn)** for 2 seconds to remove drops from the lid or sides.

10. Add 220 µl of **Ethanol** to the DNA sample **(Ref** or **Tn)** and mix by pulse-vortexing for 2 seconds. After mixing, briefly centrifuge the 2 ml microcentrifuge for 2 seconds to remove drops from the lid or sides.

11. Introduce the mixture (682 µl) onto the **QIAamp Mini spin column** (in a 2 ml collection tube). Close the cap, and centrifuge at full speed (14,000 rpm, 16,800 rcf) for 1 min. Discard the flow-through and collection tube.

12. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl **Buffer AW1.** Close the cap, and centrifuge at full speed (14,000 rpm, 16,800 rcf) for 1 min. Discard the flow-through and collection tube.

13. Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl **Buffer AW2.** Close the cap, and centrifuge at full speed (14,000 rpm, 16,800 rcf) for 3 min. Discard the flow-through and collection tube.

14. Place the spin column into a 1.5 ml microcentrifuge tube and centrifuge at full speed for 1 min to eliminate possible traces of Buffer AW2, which will be removed by pipetting.

15. Add 50 µl of **Buffer AE** directly to the column matrix, and incubate at room temperature for 5 minutes. Centrifuge at full speed for 1 min to elute the DNA **(Ref** or **Tn)**.

16. Determine the concentration (ng/µl) of DNA **(Ref** or **Tn)** by fluorometry (Qubit® fluorometer recommended)

17. The DNA solution (Ref or **Tn) is ready for immediately analysis or can be stored at -20°C. To carried out the Urodiag test, the DNA concentration (Ref or Tn) be close of 1.25 ng/µl.**

Urodiag test requires an amount of **25 to 40 ng of DNA (Ref** or **Tn)**

- Mutation assay requires **10 ng of DNA**
- Methylation assay requires **15 ng to 30 ng of DNA (30 ng are recommended)**

If the DNA concentration is ABOVE 1.25 ng/µl, it must be diluted **with Buffer AE** (The remaining DNA is stored at -20 °C).

If the DNA concentration is BELOW 1.25 ng/µl, it must be concentrated by heating to +50°C using a heating block or a DNA concentrator (speed vacuum). The volume of DNA to be evaporated is calculated to obtain a concentration close to 1.25 ng/µl

Protocol for DNA Conversion with Sodium Bisulfite

Protocol

The bisulfite conversion of DNA is carried out with the EZ DNA Modification kit (Zymo Research). The kit can be stored at room temperature (+15°C to +25°C).

Expiry date: 12 months from delivery date.

Equipment and Reagents to Be Supplied by User:

■ EZ DNA Methylation Kit (Zymo Research), catalog No. D5001 (up to 55 reactions)

■ Absolule ethanol (96-100%)

■ 1.5 ml microcentrifuge tubes

■ MicroAmp Reaction Tube with Cap, 0.2 ml (ThermoFisher Scientific, catalog No. N8010540), MicroAmp 8-Tube strip, 0.2 ml (ThermoFisher Scientific, catalog No. N8010580) and 8-Cap Strip (ThermoFisher Scientific, catalog No. N8010535)

■ Micropipets and Pipet tips with aerosol barrier

■ Microcentrifuge (with rotor for 1.5-2 ml tubes)

■ Vortexer

■ Heating block (+37°C)

■ Incubator at +50°C in thermocycler (recommended) or in heating block

■ Water, PCR grade (stored at +4°C)

Buffer preparation:

1. **Preparation of the conversion solution for <u>11 reactions using one CT-Conversion tube.</u>**

Centrifuge the CT-Conversion tube (solid mixture) briefly before the preparation of the conversion solution. The conversion solution must be prepared as follows:

- Add 750 µl sterile water and 210 µl of **M-Dilution Buffer** to a tube of **CT Conversion** tube
- Mix at room temperature (15°C to 25°C) with frequent vortexing for 5 to 10 minutes.

It is normal to see traces of undissolved reagent in the CT-Conversion tube. The CT-Conversion solution can be used immediately or stored for one week at +4°C or one month at -20°C.

2. **Preparation of M-Wash Buffer–** Add 24 ml of ethanol to the 6 ml **M-Wash Buffer** concentrate

3. **Preparation of DNA-** Introduce in a sterile microtube (1.5 ml) the different volumes indicated in the table below:

At each run	C+ meth (purple cap)* (1.25ng/µl)	Patient (Ref or Tn) (1.25 ng/µl)	
DNA sample	24 µl (30 ng)	12 µl to 23 µl(15 to 28.75 ng)	24 µl (30 ng)
Water, PCR grade	21 µl	Adjust the volume to 50 µl	21 µl
M-Dilution Buffer	5 µl	5 µl	5 µl
Total volume	50 µl	50 µl	50 µl
Mix the sample by pipetting up and down and centrifuged briefly the tube			

***Tube C+ meth of Urodiag® Multiplex PCR Kit (purple cap)** = Universal Methylated Human DNA Standard, Catalog No. ZD5011 (Zymo Research)

4. Incubate the sample in heating block for 15 minutes at +37°C.

5. Centrifuge briefly the tube for 2 seconds to remove drops from the lid or sides.

6. Add 100 µl of the conversion solution to DNA sample (50 µl) and mix by pipetting up and down 5 times.

7. Transfer the DNA sample (150 µl) contained in the 1.5 ml microtube to a MicroAmp tube, 0.2 ml.

8. Incubate the DNA sample in a thermocycler for 15 hours and 30 minutes at +50°C and then hold at +4°C.

9. The tube can be stored at +4°C for 10 minutes to 20 hours.

Alternative incubation condition. Step 6: The DNA sample (150 µl) contained in the 1.5 ml microtube is incubated in a heating block at 50°C for 15 hours and 30 minutes, then stored at +4°C for 10 minutes to 20 hours. Centrifuge the tube for 2 seconds to remove drops from the lid or sides.

10. Add 400 µl of **M-Binding Buffer** to a **Zymo-Spin IC Column** and place the column into a provided **Collection tube**.

11. Load the sample (~150 µl) into the Zymo-Spin IC Column containing the M-Binding Buffer. Close the column and mix by inverting 5 times the column.

12. Centrifuge the column at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds. Discard the flow-through.

13. Add 100 µl of **M-Wash Buffer** to the column. Close the column and centrifuge at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds.

14. Add 200 µl of **M-Desulphonation Buffer** to the column and let stand at room temperature for 15-20 minutes. After the incubation, centrifuge the column at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds.

15. Add 200 µl of **M-Wash Buffer** to the column. Close the column and centrifuge at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds. Add another 200 µl of **M-Wash Buffer** to the column and centrifuge at full speed (14,000 rpm, ≥10,000 x g) for an additional 30 seconds.

16. Place the column into a 1.5 ml microcentrifuge tube and centrifuge at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds to eliminate possible traces of M-Wash Buffer, which will be removed by pipetting.

17. Add **10 µl** of **M-Elution Buffer** directly to the column matrix, and let stand at room temperature for 5 minutes. Centrifuge the microcentrifuge tube at full speed (14,000 rpm, ≥10,000 x g) for 30 seconds to elute DNA.

The bisulfite-converted DNA solution is ready for immediately analysis or can be stored for one week at -20°C.

Multipex PCR Protocol

Urodiag Multiplex PCR Kit

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 QuantStudio 5 qPCR System (applied biosystems, ThermoFisher Scientific). The kit can be stored in the freezer (-15°C to -30°C) for 12 months. It is not recommended to freeze and thaw the kit more than 5 times.

Expiry time: 12 months from production date.

Equipment and Reagents to Be Supplied by User:

■ QuantStudio 5 qPCR System (ThermoFisher Scientific)

■ MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 ml_0.2 ml (No. 4346907, No. N8010560, ThermoFisher Scientific) with Optical Adhesive Covers (No. 4360954, ThermoFisher Scientific) or MicroAmp Fast Reaction Tubes, 0,1 ml_0,2 ml (8 tubes/strip, No. 4358293, No. 4316567, ThermoFisher Scientific) with MicroAmp Optical 8-Cap Strip, No. 4323032 (ThermoFisher Scientifc)

■ Micropipets and pipet tips with aerosol barrier

■ Microcentrifuge for PCR plates

■ Vortexer

■ Cooling block

■ PCR-grade water (nuclease-free)

Kit contents

The kit is designed for 50 tests of patients. A total of 240 PCR reactions can be performed for 5 runs (maximum 10 patients per run). Each run consists of 48 PCR reactions including 8 for Quality Control and 40 PCR reactions for 10 patients.

It is designed for the detection of 4 mutations of the FGFR3 gene (G372C, R248C, S249C and Y375C) and the quantification of 3 DNA methylation markers (HS3ST2, SEPTIN9, SLIT2) by multiplex PCR from urine DNA.

The kit is composed of 8 tubes: 5 tubes for the Mutation assay (blue, green and white cap), 3 tubes for the Methylation assay (red, orange and purple cap). Each tube contains all the components (PCR master mix, primers and probes) necessary to carry out the Mutation and Methylation assays.

	Cap	Label	Description
Mutation Assay		**MM mut 1** Lot 0000X Exp. Store at: -15 to -30°C Light sensitive Vol. 960 µL	**Master Mix mutation1:** The tube contains all components (PCR master mix, primers and probes) necessary to detect S249C, Y375C mutations and the GLOBIN gene (as internal control)
		**C+ mut 1** Lot 0000X Exp. Store at: -15 to -30°C Vol. 22 µL	**Positive control mutation 1:** The tube contains a plasmid (P1) with three synthetic sequences to detect the S249C and Y375C mutations of the FGFR3 gene and the GLOBIN gene
		**MM mut 2** Lot 0000X Exp. Store at: -15 to -30°C Light sensitive Vol. 960 µL	**Master Mix mutation 2:** The tube contains all components (PCR master mix, primers and probes) necessary to detect G372C, R248C mutations and the GLOBIN gene (as internal control)
		**C+ mut 2** Lot 0000X Exp Store at: -15 to -30°C Vol. 22 µL	**Positive control mutation 2:** The tube contains a plasmid (P2) with three synthetic sequences to detect the R248C and G372C mutations of the FGFR3 gene and the GLOBIN gene
		**C- mut** Lot 0000X Exp. Store at: -15 to -30°C Vol. 44 µL	**Negative Control mutation:** human DNA to detect the GLOBIN gene (internal control)
Methylation Assay		**MM meth A** Lot 0000X Exp. Store at: -15 to -30°C Light sensitive Vol. 960 µL	**Master Mix methylation A:** The tube contains all components (PCR master mix, primers and probes) necessary to detect and quantify, ALBUMIN (unmethylated allele) and SEPTIN9 (methylated allele)
		**MM meth B** Lot 0000X Exp. Store at: -15 to -30°C Light sensitive Vol. 960 µL	**Master Mix methylation B:** The tube contains all components (PCR master mix, primers and probes) necessary to detect and quantify, HS3ST2 and SLIT2 (methylated alleles)
		**C+ meth** Lot 0000X Exp. Store at: -15 to -30°C Vol. 126 µL	**Positive control methylation:** 100% methylated control DNA (human). The DNA solution is converted with sodium bisulfite before use.

Protocol

Step 1: Prepare the PCR reaction mix

1. Before each use, all reagent tubes need to be thawed at room temperature and then stored in a cooling block.

2. When the reagents are thawed, mix by inverting 10 times each master mix tube (MM mut 1, MM mut 2, MM meth A and MM meth B) and mix by tapping the DNA control tubes (C+ mut 1, C+ mut 2, C- mut and bisulphite-converted C+ meth) and the bisulphite-converted patient DNA tubes.

3. Centrifuge briefly to collect solutions at the bottom of the tube.

- **Mutation assay**

The Mutation Assay must be performed using **10 ng of DNA (Ref** or **Tn)** with 5 ng of DNA for the detection of S249C and Y375C mutations and 5 ng of DNA for the detection of G372C and R248C mutations.

Prepare samples according to Table 1 below.

Tube	**Control**				**Patient (Ref** or **Tn)**	
	Control 1		**Control 2**		**Test 1**	**Test 2**
	Positive	**Négative**	**Positive**	**Négative**		
MM mut 1	16 µl	16 µl	/	/	16 µl	/
C+ mut 1	4 µl	/	/	/	/	/
MM mut 2	/	/	16 µl	16 µl	/	16 µl
C+ mut 2	/	/	4 µl	/	/	/
C- mut	/	4 µl	/	4 µl	/	/
DNA patient (Ref or **Tn)**	/	/	/	/	4 µl	4 µl
Total volume	20 µl					

- **Methylation assay**

Prepare samples according to Table 2 below.

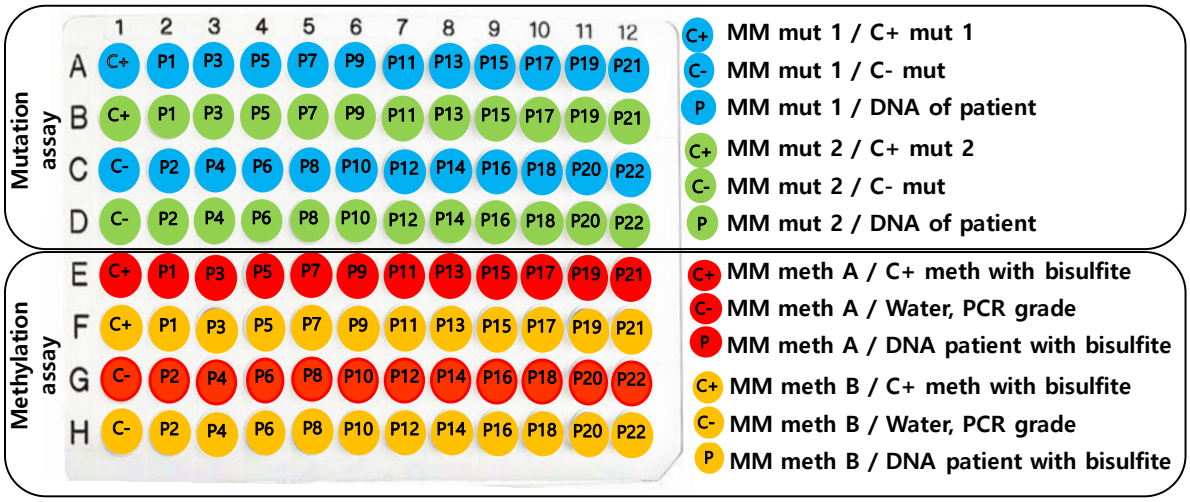
Tube	**Control**				**Patient (Ref** or **Tn)**	
	Control 1		**Control 2**		**Test 1**	**Test 2**
	Positive	**Negative**	**Positive**	**Negative**		
MM meth A	16 µl	16 µl	/	/	16 µl	/
MM meth B	/	/	16 µl	16 µl	/	16 µl
C+ meth*	4 µl	/	4 µl	/	/	/
Patient DNA (Ref or **Tn) ***	/	/	/	/	4 µl	4 µl
Water PCR grade	/	4 µl	/	4 µl	/	/
Total volume	20 µl					

* Bisulfite-converted DNA

Step 2: Loading of samples 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 (0.1 ml or 0.2 ml) or PCR tubes (8-reaction tubes/strip, 0.1 ml or 0.2 ml) to carry out the URODIAG test of 22 patients **(Ref** or **Tn)**.

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



2- Cover the reaction 96-well plate with the optical film or closing the PCR tubes, 0.1 ml or 0.2 ml with the caps,

3- Centrifuge the 96-well PCR or the PCR tubes using a microplate rotor for 10 seconds at approximately 1000 x g (3000 rpm) to collect reaction volumes at the bottom of the wells and remove air bubbles.

Step 3: Programming the QuantStudio 5 qPCR

The PCR reaction configuration is stored in the files QS5_BLOCK 0.1 ml_QUANTINOVA.edt and QS5_BLOCK 0.2 ml_QUANTINOVA.edt. The operator should import the file into the QuantStudio 5 qPCR System.

Place the 96-well PCR plate or PCR tubes in the PCR machine and start the PCR program by selecting RUN.

- **Parameters**

Mutation assay		
TaqMan reagents		
Mode Quantitation –Comparative Ct		
Reaction volume per well)		**20 µl**
Ramp speed		**Fast**
Ramp rate		**100%**
Threshold (ΔRn)	R248C	**0.190**
	G372C	**0.100**
	S249C	**0.045**
	Y375C	**0.080**
	GLOBIN	**0.100**
Baseline		**Auto**
Passive reference		**ROX**

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

- **Dye (Reporter)**

Mutation assay		
Detection	**Reporter**	**Quencher**
G372C	VIC	(none)
R248C	FAM	(none)
S249C	FAM	(none)
Y375C	VIC	(none)
GLOBIN	NED	(none)

Methylation assay		
Detection	**Reporter**	**Quencher**
ALBUMIN	VIC	(none)
HS3ST2	FAM	(none)
SEPTIN9	FAM	(none)
SLIT2	VIC	(none)

- **Multiplex PCR cycling conditions**

Steps	**Number of cycles**	**Time**	**Temperature**	**Fluorescence data collection**
Initial PCR activation step (hot start)	1	03:00 min	95°C	-
Denaturation	40	00:05 min	95°C	-
Annealing/ extension		00:30 min	60°C	✓

Step 4: Rendered results

Confidence intervals (min and max Ct values) as well as threholds (∆Rn) were set for a highest accuracy of the Urodiag test in terms of sensitivity and specificity, respectively.

- **Quality Control (QC)**

Mutation assay			**Ct values (CI*)**		**Rendering**
			Min	**Max**	
Control	**Positive**	GLOBIN	27	33	PASSED
		FGFR3 mutations: G372C, R248C, S249C and Y375C	27	34	PASSED
	Negative	GLOBIN	27	33	PASSED
		FGFR3 mutations: G372C, R248C, S249C and Y375C	No amplification		PASSED
Patient (Ref or **Tn)**	**Positive**	GLOBIN	27	33	PASSED

*CI = Confidence Interval

Methylation assay			Ct values (CI)		Rendering
			Min	Max	
Control	**Positive**	ALBUMIN, HS3ST2, SEPTIN9 and SLIT2	26	32	PASSED
	Negative	ALBUMIN, HS3ST2, SEPTIN9 and SLIT2	No amplification		PASSED
Patient (Ref or **Tn)**	**Positive**	ALBUMIN	26	32	PASSED

- **Patient (T0** or **T1, T2…)**

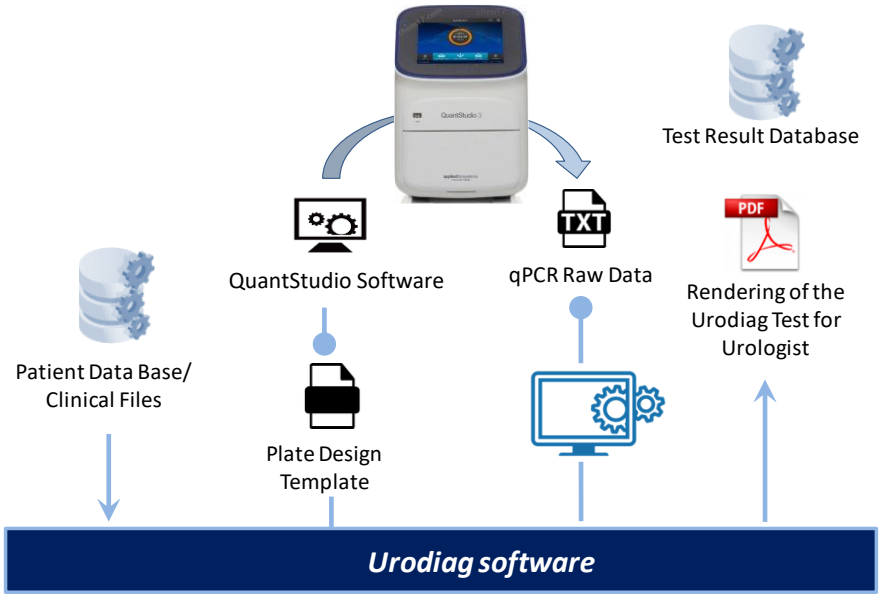
Mutation assay		Ct values (CI)		Interpretation	Result
		Min	Max		
Patient (Ref or **Tn)**	S249C mutation	25	40	DNA mutated for S249C	POSITIVE
	Y375C mutation			DNA mutated for Y375C	POSITIVE
	G372C mutation			DNA mutated for G372C	POSITIVE
	R248C mutation			DNA mutated for R248C	POSITIVE

Methylation assay		Ct values (CI)		Interpretation	
		Min	Max		
Patient (Ref or **Tn)**	HS3ST2	25	40	Presence of methylated alleles of the *HS3ST2* gene	Determination of the methylation degree of the 3 target genes
	SEPTIN9			Presence of methylated alleles of the *SEPTIN9* gene	
	SLIT2			Presence of methylated alleles of the *SLIT2* gene	

Urodiag Test Results

The Urodiag software was co-developed by Oncodiag and Biomaneo (https://biomaneo.fr). The software allows the analysis of PCR data, interpretation, result management and rendering of Urodiag test to urologists. The result of the Urodiag test is obtained from data obtained at **Ref** (initial diagnosis of NMIBC) and those obtained at each follow-up point in months **(Tn).**

Streamlined representation of the workflow is shown below:



It will be provided to customer:

- Urodiag software
- User's manual
- Installation and training
- Maintenance

- **Urodiag test results**

The Urodiag software provides test results based on data obtained at Ref (initial diagnosis of NMIBC) and at each follow-up point in months (T1, T2, …, Tn).

Mutation test at Tn		Methylation SCORE	Result
Positive	and	≥ -0 .021	POSITIVE
Positive	and	< -0.021	POSITIVE
Negative	and	≥ -0 .021	POSITIVE
Negative	and	< -0.021	NEGATIVE

- **The Mutation test is Positive** if at least one of the four mutations has been detected.
- **The Methylation score** is determined by an equation that takes into account the sum of the methylation percentages calculated at the time of the initial diagnosis (**SUM_Ref**) and at the follow-up points Tn for the patient (**SUM_Tn**).

- **Rendering of the Urodiag test for Urologist**

Address

Urologist
Street
City

Date :

Subject: Rendering of the test result for patient 01

The result of the Urodiag test on July 01, 2019 is as follows:

NEGATIVE

For information, below is the table giving the Urodiag results during the patient monitoring:

Date	Mutation	Methylation	Urodiag result
01/01/2019	POSITIVE	1st analysis serving as reference	
01/04/2019	NEGATIVE	NEGATIVE	NEGATIVE
01/07/2019	NEGATIVE	NEGATIVE	NEGATIVE
01/10/2019	NEGATIVE	NEGATIVE	NEGATIVE
01/01/2020	NEGATIVE	NEGATIVE	NEGATIVE
01/04/2020	POSITIVE	NEGATIVE	POSITIVE
01/07/2020	NEGATIVE	NEGATIVE	NEGATIVE

Lab manager

Clinical Performances

Risk of recurrence	Sensitivity	Negative Predictive Value (NPV)
Overall	**95%**	**99%**
High	**97%**	**99%**
Low	**93%**	**98%**

Sensitivity: Proportion of patients having a recurrence with the Urodiag test is positive

Negative Predictive Value: Probability that patients with a negative Urodiag test truly do not have a recurrence

Performance Characteristics

Limit of detection (LOD)

- Mutation test

S249C	Y375C	R248C	G372C
2.5%	1%	1%	0.5%

- Methylation test

SEPTIN9	SLIT2	HS3ST2
10 pg	10 pg	>10 pg

Analytical specificity

The oligonucleotide sequences of each target were defined using NCBI database (National Center for Biotechnology Information).

Stability of components of the Urodiag Kit

- Urodiag Urine Filter Kit: 10 years from production date
- Urodiag Multiplex PCR Kit: 12 months from production date
- DNA extraction using the QIAamp DNA Mini Kit (QIAGEN): 12 months from delivering date
- DNA modification using the EZ DNA Modification kit (Zymo Research): 12 months from delivering date

Disposing of waste from the Urodiag Kit

After you have used the Urodiag kit, you must dispose of the waste in the correct way. This will help to protect the people involved, the environment and keep patient data private.

Tube (box 1) and filter (box 2): Dispose of these items in a DASRI (Déchets d'Activités de Soins à Risque Infectieux) container. This is in accordance with the French Public Health Code (articles R1335-1 to R1335-8). Make sure that the tubes are tightly closed to avoid any biological risk.

Packaging: Sort the cardboard packaging according to local recycling channels. Follow the sorting symbols on the materials.

General precautions: If a urine sample shows signs of bacterial contamination (turbidity, unusual odour), dispose of it immediately as DASRI and ask for a new sample to avoid false results. If in doubt, contact your waste manager or consult the local authorities to ensure compliance with regulatory obligations.