*ampli*Cube STD Panel 2.1

MIKROGEN DIAGNOSTIK

IVD

Instructions for use (English)

1 Intended Purpose

The *ampli*Cube STD Panel 2.1 is a qualitative *in-vitro* test for specific detection of the DNA of *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* (and their differentiation) in urine samples (preferably first pass urine) or urogenital smears of human origin.

This test is not intended for use on other real-time PCR thermocyclers (see Section 8 for more information). Do not use a LightCycler® 480 II (Roche) for this test.

2 Field of Application

Trichomonas vaginalis is an anaerobic protozoan parasite that belongs to the Trichomonadidae family and is transmitted primarily by sexual intercourse. In men, the disease is typically without symptoms but those affected can develop urethritis. In women, symptoms manifest as an inflammation of the mucous membranes of the sexual organs (trichomoniasis). The urethra can also be affected in women, leading to inflammation.
The bacteria *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum* are all members of the Mycoplasmataceae family. A mycoplasma infection is a highly infectious and commonly occurring sexually transmitted disease. The pathogens are often present in the genitals without harming the host but they can occasionally cause local inflammation. Depending on the localisation of the inflammation (ureter, bladder, prostate, kidneys, renal pelvis, vagina, fallopian tubes or ovaries), the symptoms vary. The most common symptoms are an increased urge to urinate, burning on urination, yellowish discharge (urethritis) and pain around the kidneys. In men *U. urealyticum* is the pathogen that causes non-gonococcal urethritis and prostatitis.

3 Test Principle

The test is a real-time PCR system. It uses specific primers and marked probes for amplifying and detecting DNA from *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Ureaplasma parvum*.
To ensure that the nucleic acids isolated from the patient sample do not contain any substances that inhibit PCR, an internal control (IC) is added to the sample during DNA isolation. This IC is amplified and detected in the same PCR batch. This can rule out false negative test results due to inhibition of the PCR reaction. At the same time, the IC serves as evidence of nucleic acid extraction from the patient sample.
Probes for detecting pathogen-specific DNA are marked with the reporter dyes FAM (*Trichomonas vaginalis*), HEX (*Mycoplasma hominis*), ATTO Rho12 (*Ureaplasma urealyticum*) and Quasar 705 (*Ureaplasma parvum*) and the probes for detecting the internal control are marked with ATTO 647N. This allows simultaneous detection of all target sequences in a single reaction mixture.
The Ct value (cycle threshold) describes the part of the curve in which the fluorescence rises exponentially above the background value for the first time.

4 Reagents

4.1 Package contents

The reagents in one pack are sufficient for 50 assays.
Each set of reagents contains:

P&P MIX	**150 µl** Primer & Probe Mix for *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Ureaplasma parvum* and internal control (**green lid**)
ENZYME	**600 µl** enzyme mix (**white lid**) Contains DNA polymerase. (**Component is stained blue.**)
CONTROL INT	**250 µl internal control (transparent lid)**
CONTROL +	**170 µl positive control (red lid)**
CONTROL -	**2× 1800 µl** negative control (**blue lid**)
INSTRU	**1** instructions for use

4.2 Additional reagents, materials and equipment required

- Depending on the real-time PCR cycler used, MIKROGEN provides reagents for dye calibration:
  MIKROGEN *ampli*Cube Color Compensation (cobas z 480 Analyzer (Roche), 5-plex, article no. 50503), dye calibration set for QuantStudio 5 (Applied Biosystems, article no. 50504) or the dye calibration set for the CFX96 (Bio-Rad, article no. 50505). For processing on a MIC (bms) PCR cycler, MIKROGEN provides validated MIC assay templates.
- Nucleic acid extraction: The following nucleic acid extraction systems are recommended: MagNA Pure® System, Total Nucleic Acid Isolation Kit (Roche) or *alpha*Clean Mag RNA/DNA Kit (MIKROGEN) with processing on the M32, M48 or M96 extractor (Biocomma)
- Real-time cycler: cobas z 480 Analyzer (Roche), CFX96™ (Bio-Rad), QuantStudio 5 (Applied Biosystems), Mic (bms), Rotor-Gene Q (Qiagen)
- 96-well PCR plates and films or reaction tubes (PCR-clean): Follow the recommendations of the manufacturer of the real-time PCR cycler
- Micropipettes with single-use tips with filter 10 µl, 20 µl, 100 µl and 1000 µl
- Vortex mixer with high rotational speed (recommended 3200 rpm)
- Mini centrifuge
- If necessary, plate centrifuge
- Single-use gloves, powder-free
- Cooling block

5 Shelf life and handling

- Store reagents between −25°C and −18°C before and after use.
- Repeated thawing and freezing of the components (more than ten times) must be avoided. It is recommended to aliquot the test components after the first thawing.
- Always appropriately cool reagents during the working steps (+2°C to +8°C).
- Protect the kit components from direct sunlight throughout the test procedure.
- Before starting the test, completely thaw, mix (by briefly vortexing) and centrifuge all reagents.
- The packages have an expiry date; after this date has passed, no further guarantee of quality can be given.
- The test may only be performed by trained, authorised and qualified personnel.
- Substantial changes made by the user to the product or the directions for use may compromise the intended purpose of the test specified by MIKROGEN.
- Cross-contamination can lead to false test results. Add patient samples and controls carefully. Ensure that the reaction mixtures are not transferred to other wells.

6 Warnings and safety precautions

- Only use for *in-vitro* diagnostics.
- All patient samples must be treated as potentially infectious.
- Suitable single-use gloves must be worn throughout the entire test procedure.
- All reagents and materials that come into contact with potentially infectious samples must be treated with suitable disinfectants or be disposed of according to laboratory guidelines. The concentrations and incubation times specified by the manufacturer must be followed.
- Do not replace or mix the reagents with reagents from other kit batches, other MIKROGEN PCR kits, or with reagents from other manufacturers.
- Read through and carefully follow all instructions before performing the test. Deviations from the test protocol described in the instructions for use can lead to false results.

7 Sampling and reagent preparation

7.1 Sample material and sample preparation

The starting material for the *ampli*Cube STD Panel 2.1 is DNA extracted from urine samples (preferably first pass urine) or urogenital smears human origin. The quality of the nucleic acid preparation affects the test result. Ensure that the extraction method selected is compatible with the real-time PCR technology.

7.2 Extraction of nucleic acids

Extract the nucleic acids from the patient sample and the negative control (NC). We recommend a starting volume for the extraction of 200 µl and an elution volume of 50 µl or 100 µl depending on the extraction system. Follow the instructions from the manufacturer of the extraction kit.

1. Thaw the internal control (IC) (transparent lid) and the negative control (NC) (blue lid).
   Completely thaw the IC and the NC. Mix the IC and the NC before use by briefly vortexing and then briefly centrifuge.
2. For the extraction, add 5 µl IC to each patient sample and the NC. Add the IC to the sample/lysis buffer mix and not directly to the sample material.
3. Extract the patient sample and the NC. (Note: the NC cannot be used in the PCR without extraction.)
4. Do not extract the positive control.

The following nucleic acid extraction systems were used for the performance assessment:

Extraction system	Sample volume	Elution volume
MagNA Pure® 24 (Roche) Total NA Isolation Kit	200 µl	50 µl
M96 Nucleic Acid Extraction Systems (biocomma) *alpha*Clean Mag RNA/DNA Kit (MIKROGEN)	200 µl	100 µl
M32 Nucleic Acid Extraction Systems (biocomma) *alpha*Clean Mag RNA/DNA Kit (MIKROGEN)	200 µl	100 µl

If you would prefer to use extraction methods other than those listed in section 4.2 and for the performance assessment, please contact the manufacturer to clarify the compatibility.

7.3 Preparing the master mix

1. Thaw the Primer & Probe mix (green lid) and the enzyme mix (white lid). Protect the reagents from light while doing so.
   Completely thaw the reagents. Mix the reagents before use by vortexing and then briefly centrifuging.
2. Prepare the master mix using the following pipetting scheme:

Components	Master mix for 1 reaction
Primer & Probe mix	3 µl
Enzyme mix	12 µl
Total volume	15 µl

3. Mix the entire master mix by vortexing and then briefly centrifuging.
4. Set up 15 µl master mix for each PCR reaction.

7.4 Preparing the PCR reaction

1. Thaw the positive control (PC) (red lid).
   Completely thaw the reagents. Mix the reagents before use by vortexing and then briefly centrifuging.

Components	1 reaction
Master mix from 7.3	15 µl
Sample eluate or eluate of the NC or the PC	10 µl

2. Pipette 10 µl of the sample eluate into 15 µl master mix.
3. Pipette 10 µl of the positive control (not prepared) into 15 µl of the master mix.
4. Pipette 10 µl of the eluate of the negative control into 15 µl master mix.

Every run must include a positive and a negative control.

Seal the PCR plate with an optically clear adhesive film or seal the test tube with the lid provided.

⚠ Vortex the PCR plates or test tubes for <u>at least 5 sec</u> at maximum speed and then briefly centrifuge them.

⚠ Vortex the PCR test tubes for the <u>Mic PCR cycler</u> for <u>at least 10 sec</u> at maximum speed.

8 Programming the real-time cycler

The *ampli*Cube STD Panel 2.1 was evaluated with the CFX96™ (Bio-Rad) and validated on the QuantStudio 5 (Applied Biosystems), cobas z 480 Analyzer (Roche), Mic (bms) and Rotor-Gene Q (Qiagen).

8.1 Setting the detection channels

CFX96™ (Bio-Rad)

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour	Green	Yellow	Orange	Red	Dark red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N	Quasar 705
Mode	all channels				

The data are recorded and analysed on the CFX96™ using calc / data acquisition mode: all channels. The CFX96™ (Bio-Rad) must be calibrated first for ATTO Rho12 and ATTO 647N. The dye calibration set (Bio-Rad, article no. 50505) is available from MIKROGEN.

QuantStudio 5 (Applied Biosystems)

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour	Green	Yellow	Orange	Red	Dark red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N	Quasar 705
Excitation	X1 / 470 nm	X2 / 520 nm	X4 / 580 nm	X5 / 640 nm	X6 / 662 nm
Emission	M1 / 520 nm	M2 / 558 nm	M4 / 623 nm	M5 / 682 nm	M6 / 711 nm
Quencher	[none]	[none]	[none]	[none]	[none]

Under the settings, select 1. Run mode ‘standard’, 2. Reference dye ‘none’, 3. Experiment type ‘custom’. The QS5 (QuantStudio 5) must be calibrated first for ATTO 647N and Quasar 705. The dye calibration set (Applied Biosystems, article no. 50504) is available from MIKROGEN.

cobas z 480 Analyzer (Roche)

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour	Green	Yellow	Orange	Red	Dark red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N	Quasar 705
Excitation	465 nm	540 nm	540 nm	610 nm	680 nm
Emission	510 nm	580 nm	610 nm	670 nm	700 nm
Quencher	[none]	[none]	[none]	[none]	[none]

For the cobas z 480 Analyzer (Roche), first use the Color Compensation kit (5-plex, article no. 50503) that is provided by MIKROGEN.

Mic (bms)

When using a Mic (bms), only four dyes can be used due to the limit of four channels.

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour	Green	Yellow	Orange	Red	No detection

MIKROGEN provides validated MIC assay templates for processing. Only use the MIKROGEN Mic assay templates.

Rotor-Gene Q (Qiagen)
When using a Rotor-Gene Q (Qiagen), only four dyes can be used.

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour	Green	Yellow	Orange	Red	Dark red
Reporter dye	FAM	HEX	ATTO Rho12	ATTO 647N	No detection
Excitation	470 nm	530 nm	585 nm	625 nm	No detection
Emission	510 nm	555 nm	610 nm	660 nm	No detection
Quencher	[none]	[none]	[none]	[none]	No detection

8.2 PCR program

Reverse transcription	50°C	8 minutes
Denaturation	95°C	3 minutes
Amplification	**45 cycles**	
• Denaturation	95°C	10 seconds
• Annealing/elongation	60°C	45 seconds

Essential information about programming the particular real-time PCR cycler can be found in the instructions for the cycler. For specific information about programming the real-time PCR cycler when using the *ampli*Cube STD Panel 2.1, please contact the manufacturer.

9 Results

9.1 Validation

1. The negative control must be below the threshold. The internal control (IC) must have a positive curve for the negative control. If the negative control has a positive curve (contamination) or if the IC is not valid in the negative control, the test run cannot be analysed.
2. The positive control must have a positive curve.
   The Ct value for the positive control must be <33. A positive control outside this range indicates that there is a problem with the amplification.
3. The internal control (IC) must have a positive curve for negative samples.
   The signal for the IC for a patient sample must be compared to the signal of the IC in the extracted negative control. A difference of >+3 for the Ct value of the IC of a sample compared to the IC of the negative control or the absence of an IC signal for the sample may indicate significant inhibition of the RT-PCR reaction. In these cases, a negative test result is invalid.

9.2 Analysis

The data can be analysed with the corresponding PCR cycler software or a software solution for automated PCR analysis and interpretation specifically supported by MIKROGEN. Additional information and corresponding instructions are available from MIKROGEN upon request.

		Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Colour		Green	Yellow	Orange	Red	Dark red
Reporter dye		FAM	HEX	ATTO Rho12	ATTO 647N	Quasar 705
Analysis with	**CFX96™**	FAM	HEX	ROX	Cy5	Quasar 705
	QS5	FAM	VIC	ROX	ATTO 647N	Quasar 705
	Mic	FAM	HEX	ROX	Cy5	No detection
	RG Q	Green	Yellow	Orange	Red	No detection

Amplification signals above the threshold are evaluated as positive results. Empty fields in the table are considered negative results.

Colour	*Trichomonas vaginalis*	*Mycoplasma hominis*	*Ureaplasma urealyticum*	Internal control (IC)	*Ureaplasma parvum*
Green	Positive				
Yellow		Positive			
Orange			Positive		
Red				Positive*	
Dark red					Positive

* For positive signals in the detection channels for the pathogens, the signal for the internal control is not required for test interpretation. A high pathogen load in the patient sample can lead to a reduced or missing signal for the internal control.

10 Limits of the method, restrictions

- Test results must always be viewed in the context of the clinical situation. Therapeutic consequences of the findings must be linked to the clinical data.
- A negative *Trichomonas vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* or *Ureaplasma parvum* test result cannot rule out infection with the particular pathogens.

11 Performance Characteristics

11.1 Diagnostic sensitivity and specificity

The sensitivity and specificity were determined using defined positive and defined negative samples.

Table 1: Defined positive clinical samples

*ampli*Cube STD Panel 2.1	*Trichomonas vaginalis* (n=33)	*Mycoplasma hominis* (n=5)	*Ureaplasma urealyticum* (n=6)	*Ureaplasma parvum* (n=19)
Negative	0	0	0	0
Positive	33	5	6	19
Sensitivity [%]	**100**	**100**	**100**	**100**
95% CI* [%]	89.57–100	56.55–100	60.97–100	83.18–100

* CI = confidence interval

Table 2: Defined negative clinical samples

*ampli*Cube STD Panel 2.1	*Trichomonas vaginalis* (n=66)	*Mycoplasma hominis* (n=66)	*Ureaplasma urealyticum* (n=66)	*Ureaplasma parvum* (n=66)
Negative	66	66	66	66
Positive	0	0	0	0
Specificity [%]	**100**	**100**	**100**	**100**
95% CI* [%]	94.50–100	94.50–100	94.50–100	94.50–100

* CI = confidence interval

11.2 Analytical sensitivity

The limit of detection (LoD) of the *ampli*Cube STD Panel 2.1 was determined using a dilution series of purified genomic DNA (Vircell Standard) of known concentration in a CFX96™ (Bio-Rad). The 95% limit of detection was determined using probit regression analysis with CombiStats™ Version 6.0 software (Council of Europe).

Table 3: Limit of detection (LoD)

	Trichomonas vaginalis	*Mycoplasma hominis*	*Ureaplasma urealyticum*	*Ureaplasma parvum*
LoD 95% limit of detection [Copies/PCR]	6.41	15.50	5.64	8.90
95% CI* [Copies/PCR]	2.90–25.28	9.53–37.67	3.28–18.69	4.80–27.51

* CI = confidence interval

11.3 Analytical specificity

The BLAST trial (www.ncbi.nlm.nih.gov/blast/) showed that the selected primers and probes of the *ampli*Cube STD Panel 2.1 specifically detect the selected pathogens.
Furthermore, the specificity was determined by studying genomic DNA/RNA of other human pathogenic bacteria and viruses.

Table 4: Bacteria and viruses that were tested in order to demonstrate the analytical specificity of the *ampli*Cube STD Panel 2.1.

Bacteria
Aerococcus urinae
Campylobacter coli
Campylobacter jejuni
Candida albicans
Candida glabrata
Candida krusei
Candida parapsilosis
Chlamydia trachomatis
Citrobacter freundii
Clostridium difficile
Clostridium perfringens
EHEC stx+
Enterococcus faecalis
Mycoplasma genitalium
Mycoplasma pneumoniae
Neisseria gonorrhoeae
Proteus mirabilis
Proteus vulgaris
Providencia stuartii
Treponema pallidum
Yersinia enterocolitica

Viruses
Adenovirus
Cytomegalovirus
Herpes simplex virus 1
Herpes simplex virus 2
Mumps virus
Varicella zoster virus

None of these samples generated a positive signal. The primers and probes used in the *ampli*Cube STD Panel 2.1 showed no cross-reactions with the pathogens listed in Table 4. The internal control (IC) was valid in all tests.

11.4 Equivalence of different sample material

The coefficient of variation (CV) was determined for the Ct value between water and the extract of the particular sample material after the addition of purified genomic DNA (Vircell Standard) of known concentration.

Table 5: Equivalence of different sample material

	Trichomonas vaginalis	***Mycoplasma hominis***	***Ureaplasma urealyticum***	***Ureaplasma parvum***
CV [%] (urine, H_2O)	0.57	0.95	1.24	0.75
CV [%] (swab, H_2O)	0.73	1.11	1.32	0.81

The coefficient of variation (CV), based on the Ct value (cycle threshold) between water and the DNA extracts (obtained for the various sample materials), was ≤1.32% for all target genes.

12 Literature

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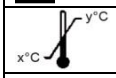
We will be pleased to send you additional literature on request.

13 Explanation of symbols

	Content is sufficient for <n> assays Number of assays
P&P MIX	Primer & Probe mix
ENZYME	Enzyme mix
CONTROL INT	Internal control
CONTROL +	Positive control
CONTROL -	Negative control
INSTRU	Instructions for use
	Follow the instructions for use
CONT	Content, contains
IVD	*In-vitro* diagnostic medical device
LOT	Batch/version number
REF	Order number
	Use by Expiry date
	Store between x°C and y°C
	Manufacturer

14 Manufacturer and version data

***ampli*Cube STD Panel 2.1**	Article no. 50322
Instructions for use Valid from	GAACSD2102EN 2023-04

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