*ampli*Cube STD Panel 3

MIKROGEN DIAGNOSTIK

IVD

Instructions for use (English)

1 Purpose

The *ampli*Cube STD Panel 3 is a qualitative in-vitro test for specific detection of the DNA of Herpes simplex virus (type 1/2) and *Treponema pallidum* in urogenital smears of human origin.

2 Field of Application

The Herpes simplex virus and the bacterium *Treponema pallidum* can cause genital blisters (ulcers).
The Herpes simplex virus types (HSV-1, HSV-2) can cause both oral and genital herpes. During close mucosal membrane contact, the viruses are transmitted in the extremely infectious fluid inside the herpes blisters. Herpes around the mouth and lips is usually caused by HSV-1. Herpes around the sexual organs and anus is caused by the Herpes simplex virus 2 (HSV-2) in 80% of cases and HSV-1 in 20% of cases. Worldwide, the herpes viruses are the most common cause of genital ulcers in both sexes.
Treponema pallidum subsp. *pallidum* is the pathogen that causes syphilis and is a member of the Spirochaetaceae family. Transmission is through direct contact with mucosal membranes during sexual intercourse. The highest numbers of the bacterium are detected in the secretion from the chancre, the primary lesion of syphilis. DNA detection is very important because the pathogen cannot yet be cultured.

3 Test Principle

The test is a real-time PCR system. It uses specific primers and marked probes for amplifying and detecting DNA from Herpes simplex virus (type 1/2) and *Treponema pallidum*.
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 enables false negative test results due to inhibition of the PCR reaction to be ruled out. 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 (Herpes simplex virus), HEX (*Treponema pallidum*) 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 of one pack are sufficient for 50 assays.
Each set of reagents contains:

P&P MIX	**150 µl** primer & probe mix for STD Panel 3 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 (**colourless lid**)
CONTROL +	**170 µl** positive control (**red lid**)
CONTROL -	**2 × 1800 µl** negative control (**blue lid**)
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4.2 Additionally required reagents, materials and equipment

- MIKROGEN *ampli*Cube Color Compensation for Light Cycler® 480 II (Roche)
- Commercial nucleic acid isolation kit. The following nucleic acid extraction system is recommended:
  MagNAPure® Compact, Total Nucleic Acid Kit I (Roche)
- Real-Time Cycler. The following cycler is recommended:
  Light Cycler® 480 II (Roche)
- 96 well PCR plates and films or reaction tubes (PCR-clean), dependent on the cycler
- Micropipettes with single-use tips with filter 10 µl, 20 µl, 100 µl and 1000 µl
- Vortex mixer
- Mini centrifuge
- Possibly 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 chill reagents during the working steps (+2°C – +8°C).
- Keep the kit components away from direct sunlight throughout the test procedure.
- Before starting the test, all reagents must be completely thawed, mixed (briefly vortex) and centrifuged.
- The packages have an expiry date, after which no further guarantee of quality can be given.
- The test must 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 coming into contact with potentially infectious samples must be treated with suitable disinfectants or be disposed of according to laboratory guidelines. The concentration specifications and incubation times of the manufacturer must be taken into consideration.
- 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

The starting material for the *ampli*Cube STD Panel 3 is DNA extracted from urogenital smears of human origin. The quality of the nucleic acid preparation affects the test result. It must be ensured 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. Follow the instructions from the manufacturer of the extraction kit.

1. Thaw the internal control (IC) (colourless lid) and the negative control (NC) (blue lid).
   Ensure that the IC and the NC are completely thawed. 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. The IC should be added to the sample/lysis buffer mix and not directly to the sample material. (Note: the IC cannot be used in the PCR without extraction!)
3. Extract the patient sample and the NC. (Note: the NC cannot be used in the PCR without extraction!)
4. The positive control is not extracted.

The following nucleic acid extraction system is recommended and was used for the performance assessment:

Extraction system	Sample volume	Elution volume
MagNAPure Compact (Roche) Total Nucleic Acid Kit I	200 µl	50 µl

If you would prefer to use other extraction methods, 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. **Ensure that the reagents are completely thawed. Mix the reagents before use by briefly vortexing and then briefly centrifuge.**
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 centrifuge.
4. Introduce 15 µl master mix for each PCR reaction.

7.4 Preparing the PCR reaction

1. Thaw the positive control (PC) (red lid). **Ensure that the reagents are completely thawed. Mix the reagents before use by briefly vortexing and then briefly centrifuge.**

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

Every run must include a positive and a negative control.
Seal the PCR plate with an adhesive optically clear film or the reaction tube with the lid provided.

⚠ **PCR plates and tubes must be vortexed for at least 10 sec at maximum speed, followed by brief centrifugation.**

8 Programming the real-time cycler

The *ampli*Cube STD Panel 3 was evaluated with the LightCycler® 480 Instrument II (Roche).

8.1 Setting the detection channels

	Herpes Simplex Virus (type 1/2)	*Treponema pallidum*	Internal control (IC)
Reporter dye	FAM	HEX	ATTO 647N
Colour	Green	Yellow	Red
Emission	510 nm	580 nm	660 nm
Quencher	[none]	[none]	[none]

Details of the wavelengths for the detection channels refer to the LightCycler® 480 II.
For the LightCycler® 480 II, it is necessary to first use colour compensation, which is provided by Mikrogen.

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 different real-time cyclers can be found in the instructions for the cycler used. For specific information about programming the real-time PCR cycler when using the *ampli*Cube STD Panel 3, please contact the manufacturer.

9 Results

The data analysis for the LightCycler® 480 II uses the *Abs Quant/2nd Derivative Max* method.

9.1 Validation

1. The negative control must be below the *threshold*. If this control is contaminated (positive curve), the test run cannot be evaluated.
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 there is a problem with the amplification.
3. The internal control must have a positive curve for negative samples and the negative control. A deviation in the curve progression of the IC in negative samples in comparison with the negative control indicates that there is a problem in the extraction of the nucleic acids or inhibition of the PCR.

9.2 Evaluation

Signals above the *threshold* are evaluated as positive results. Empty fields in the table are considered a negative result.

	Herpes Simplex Virus (type 1/2)	*Treponema pallidum*	Internal control (IC)
Colour			
Green	Positive		
Yellow		Positive	
Red			Positive*

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

10 Limitations and Restrictions of the Method

- 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 Herpes simplex virus (type 1/2) and/or *Treponema pallidum* test result cannot rule out an infection with the particular pathogen.

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 samples

*ampli*Cube STD Panel 3	Herpes Simplex Virus (type 1/2) (n=19)	*Treponema pallidum* (n=10)
Negative	0	0
Positive	19	10
Sensitivity	**100%**	**100%**

Table 2: Defined negative samples

*ampli*Cube STD Panel 3	Herpes Simplex Virus (type 1/2) (n=10)	*Treponema pallidum* (n=10)
Negative	10	10
Positive	0	0
Specificity	**100%**	**100%**

11.2 Analytical sensitivity

The limit of detection (LoD) of the *ampli*Cube STD Panel 3 was determined using a dilution series of plasmid DNA of known concentration on a LightCycler® 480 II System (Roche). The 95% limit of detection was determined using probit analysis with CombiStats™ Version 5.0 software (Council of Europe).

Table 3: Limit of detection (LoD)

	Herpes Simplex Virus (type 1/2)	*Treponema pallidum*
LoD 95% limit of detection Genome/PCR	11.92 (6.26 – 32.33)	7.16 (4.23 – 16.51)

11.3 Analytical specificity

The BLAST trial (www.ncbi.nlm.nih.gov/blast/) shows that the selected primers and probes of the *ampli*Cube STD Panel 3 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 to demonstrate the analytical specificity of the *ampli*Cube STD Panel 3.

Bacteria
Aeromonas hydrophila
Bordetella pertussis
Campylobacter coli
Campylobacter jejuni
Chlamydia trachomatis
Clostridium difficile
Clostridium perfringens
Enterococcus faecalis
E. coli Shigatoxin positive
E. coli EIEC
E. coli ETEC LT
E. coli ETEC ST
E. coli VTEC/EHEC stx1
E. coli VTEC/EHEC stx2
Haemophilus influenzae
Klebsiella pneumoniae
Moraxella catarrhalis
Neisseria gonorrhoeae
Proteus mirabilis
Pseudomonas aeruginosa
Salmonella enteritidis
Salmonella typhimurium
Serratia marcescens
Shigella sonneii
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
Yersinia enterocolitica

Viruses
Adenovirus A
Adenovirus Serotype 1(C)
Adenovirus Serotype 3 (B)
Astrovirus
Coronavirus 229E
Coronavirus NL63
Coronavirus OC43
Cytomegalovirus
Enterovirus 68
Epstein-Barr Virus
Human Metapneumovirus A
Influenza A virus
Influenza B virus
Measles virus
Mumps virus
Norovirus G1
Norovirus G2
Parainfluenza 1
Parvovirus B19
Respiratory syncytial virus A
Respiratory syncytial virus B
Rotavirus
Varicella zoster virus

None of these samples generated a positive signal. The primers and probes used in the *ampli*Cube STD Panel 3 showed no cross-reaction with the pathogens listed in Table 4. The internal control (IC) was valid in all tests.

12 Literature

1. P. French (2007): Syphilis. BMJ 2007; 334:143-7
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3. Journal of Clinical Microbiology October 2014 Volume 52 Number 10 pp. 3693–3700
4. E. W. Hook (2016): Syphilis. the lancet Vol 389 April 15, 2017
5. D. Jaishankar et al (2016): Genital Herpes: Insights into Sexually Transmitted Infectious Disease. Microbial Cell, September 2016 Vol. 3 No. 9 pp: 438-450
6. C. Johnston et al (2016): Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. Clinical Microbiology Reviews: January 2016 Volume 29 Number 1 pp: 149-161
7. V. Lee et al (2008): Syphilis: an update. Clinical Medicine Vol 8 No 3 June 2008 pp: 330-333
8. J. Le Goff et al (2014): Diagnosis of genital herpes simplex virus. infection in the clinical laboratory. Virology Journal 2014, 11:83
9. J. Radolf et al (2016): *Treponema pallidum*, the syphilis spirochete: making a living as a stealth pathogen. Nat Rev Microbiol. 2016 December 14(12): 744–759.
10. L.V. Stamm (2016): Syphilis: Re-emergence of an old foe. Microbial Cell September 2016 Vol. 3 No. 9 pp: 363-370

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

13 Explanation of symbols

Σ	Content is sufficient for <n> formulations Number of formulations
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	Contents, contains
IVD	In vitro diagnostic agent
LOT	Batch/version number
REF	Order number
	Use by Expiry date
y°C x°C	Store between x°C and y°C
	Manufacturer

14 Manufacturer and version data

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MIKROGEN GmbH
Anna-Sigmund-Str. 10
82061 Neuried
Germany
Tel. +49 89 54801-0
Fax +49 89 54801-100
Email mikrogen@mikrogen.de
Internet www.mikrogen.de

CE

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