IVD CE 2962

bosphore

INSTRUCTIONS FOR USE

Bosphore CT/NG/MG
Panel Kit v1

For *In Vitro* Diagnostic Use

MB534v6f
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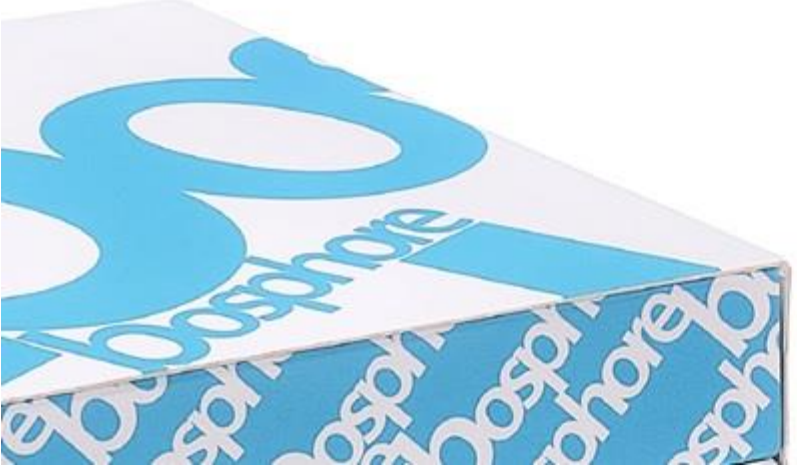

Anatolia geneworks

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1. PRODUCT DESCRIPTION AND INTENDED USE

Bosphore CT/NG/MG Panel Kit v1 is a Real-Time-Polymerase Chain Reaction kit designed for the qualitative detection of *Chlamydia trachomatis* (*C. trachomatis*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Mycoplasma genitalium* (*M. genitalium*) bacterial DNA. The kit is intended for use with samples extracted from vaginal swabs, endocervical swabs, urethral swabs, urine, and eye swabs from individuals suspected of being infected with these bacteria, as determined by health authorities.

This multiplex format kit aims to diagnose the presence of CT/NG/MG bacterial DNA, thereby aiding in the diagnosis of the associated diseases. The device is suitable for use on the general population, including adults, the elderly, pregnant individuals, children, and neonates suspected of Chlamydia (*C. trachomatis*), Gonorrhea (*N. gonorrhoeae*) and Mgen (*M. genitalium*) infections.

Positive results indicate the presence of CT/NG/MG bacterial DNA. However, a positive result does not exclude the possibility of infection by other pathogens. Negative results should not be solely relied upon to rule out CT/NG/MG infection and must be interpreted in conjunction with clinical observations, patient history, and epidemiological information.

Bosphore CT/NG/MG Panel Kit v1 is intended for use by qualified and trained clinical laboratory personnel who have received the necessary training in analyzing Real-Time PCR data and performing in vitro diagnostic procedures.

This kit detects three pathogens, namely *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*, in a single tube using a single specimen. The conserved regions of the *OmpA* gene for *C. trachomatis*, *Porin* gene for *N. gonorrhoeae* and *mgpC* gene for *M. genitalium* are targeted to specifically identify each pathogen. Fluorescence detection is achieved using FAM, HEX, Texas RED and Cy5 filters. The kit includes an internal control, a synthetic DNA molecule, which ensures the quality of DNA extraction, identifies PCR inhibition and helps prevent application mistakes. The internal control can be added during either the DNA extraction or PCR step.

If DNA extraction is to be performed using Bosphore STD EX-Tract Kit, the internal control can only be added from the PCR step.

FAM (Gene)	**HEX** (Gene)	**Texas RED** (Gene)	**Cy5**
Chlamydia trachomatis (*OmpA*)	*Neisseria gonorrhoeae* (*Porin*)	*Mycoplasma genitalium* (*mgpC*)	Internal Control

Table 1: Channels by Which Pathogens are Controlled and Related Genes

The device is intended to be compatible and for use with the following Real-Time PCR and DNA extraction systems.

- Montania 4896 Real-Time PCR Instrument (Anatolia Geneworks), CFX96 (BioRad), LightCycler 480 Instrument II (Roche), Rotorgene Q (Qiagen), Quant Studio 5 (Thermo Fisher Scientific), Q Real-Time Quantitative PCR Instrument (QuantaBio)/MIC qPCR Cycler (Biomolecular Systems).
- Bosphore STD EX-Tract Kit (Anatolia Geneworks), Unio Bacterial DNA Extraction Kit – Unio B24/B48 Extraction Systems (Anatolia Geneworks), Unio 96 Extraction & PCR Setup System (Anatolia Geneworks) – Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks).

The kit has an integrated internal control to check DNA extraction, PCR inhibition, or application problems. The amplification data of the internal control is detected with the Cy5. The internal control can be added either during DNA extraction or the PCR step.

2. CONTENT

Bosphore CT/NG/MG Panel Kit v1 consists of the following dH_2O, Real-Time PCR Master Mix, positive control, and internal control.

Component	Reagent	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1,000 µL)	(1,000 µL)	(1,000 µL)
2	PCR Master Mix	(1,650 µL)	(825 µL)	(413 µL)
3	Internal Control	(550 µL)	(275 µL)	(275 µL)
4	Positive Control	(176 µL)	(88 µL)	(88 µL)

Table 2: Bosphore CT/NG/MG Panel Kit v1 Content

Prior to initial usage, please carefully inspect the product and its components to ensure they are complete in terms of quantity, type and content. Do not utilize a defective or incomplete product, as it may compromise the performance of the kit.

3. STORAGE

PCR reagents for Bosphore CT/NG/MG Panel Kit v1 should be stored at -20 °C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR, the components should not be exposed to room temperature and the PCR master mix components should not be exposed to light or air more than necessary. Vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the PCR master mix in a closed container.

If the components are stored according to the recommended conditions, they will remain stable until the expiry dates on the labels.

4. REQUIRED MATERIALS AND DEVICES

- Montania 4896 Real-Time PCR Instrument (Anatolia Geneworks), CFX96 (BioRad), LightCycler 480 Instrument II (Roche), Rotorgene Q (Qiagen), Quant Studio 5 (Thermo Fisher Scientific), Q Real-Time Quantitative PCR Instrument (QuantaBio)/MIC qPCR Cycler (Biomolecular Systems),
- 0.1 mL or 0.2 mL thin wall PCR tubes, PCR plates or strips,
- Bosphore STD EX-Tract Kit (Anatolia Geneworks), Unio Bacterial DNA Extraction Kit – Unio B24/B48 Extraction Systems (Anatolia Geneworks), Unio 96 Extraction & PCR Setup System (Anatolia Geneworks) – Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks),
- Deep freezer (-20 °C),
- Desktop centrifuge with rotor for 1.5 mL or 2 mL microcentrifuge tubes,
- Calibrated adjustable micropipettes,
- DNase, RNase, pyrogen-free micropipette tips with filters,
- DNase, RNase, pyrogen free 1.5 mL or 2 mL microcentrifuge tubes,
- Disposable laboratory gloves and laboratory coats.

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Delivery and Expiration Dates:

- The product should be delivered on dry ice. Check for its presence upon arrival.
- Check the expiration dates on the box and tube labels upon arrival. Do not use expired products or components.

Handling and Contamination Prevention:

- Handle the product components and samples properly to avoid contamination and maintain optimal performance.
- Perform nucleic acid extraction, PCR setup and PCR analysis in different compartments. Store samples separately to avoid contact with kit components.
- Avoid interchanging vials or bottle caps.
- Store samples and contaminated materials separately from the kit components.
- Refrain from opening the PCR plates and/or tubes after amplification.

- Use separate/assigned micropipette sets for PCR setup and nucleic acid extraction operations.
- Wear lab coats, caps, masks, and clean pairs of gloves specific/assigned for PCR setup, nucleic acid extraction and PCR analysis operations.

Component Usage:

- Do not combine components from different kit lots, as it may compromise product performance.
- Only use the specified sample types provided with this kit. The use of other sample types can compromise product performance.
- Ensure proper centrifugation of product components after thawing to avoid contamination with reagent residues in the lids, which could compromise product performance.

PCR Setup and Analysis:

- The presence of PCR inhibitors may result in false negative or invalid results.
- Follow the specified volumes for component setup as outlined in the instructions for use. Using different volumes affects product performance.

	Exercise caution to avoid mixing up samples or sample IDs during PCR setup or transfer to the PCR instrument, as the incorrect assignment of samples can lead to false positive or false negative results.

- Adhere to the specified cycling conditions provided in the instructions for use. Using other cycling conditions may compromise product performance.
- Use only the control settings specified in the instructions for use for data analysis. Using different control settings can yield incorrect results.

Equipment and Thawing:

- Use calibrated or verified micropipettes, DNase, RNase and pyrogen-free micropipette tips with filters and DNase, RNase, and pyrogen-free microcentrifuge tubes.
- Thoroughly thaw all components before starting the test procedure. After thawing, centrifuge briefly (spin-down for 3-5 seconds) and mix well to ensure homogeneity before use.
- Keep the kit components on ice or a cooling block until the reaction is prepared and quickly return them to -20 ºC.

Safety Precautions and Waste Disposal:

- Review pathogen information to be aware of health-related risks.

- Review cleaning/disinfection procedures suitable for the relevant pathogens.

⚠	Handle biological samples with extreme caution in a microbiological safety cabinet of the appropriate class. Avoid physical contact with pathogens by wearing lab coats, goggles, caps, masks and gloves. Do not eat or drink within the workspace and prevent unauthorized individuals' access to the working area.

- Discard all pathogenic wastes produced during the nucleic acid extraction step, including swab and urine samples and materials contact with them, into medical waste in compliance with local regulations for safe disposal.
- Kit packaging materials and plastic disposables can have environmental effects. Follow appropriate recycling or disposal protocols to minimize environmental impact. Laboratories must comply with local regulations for the safe disposal of packaging materials and plastic disposables.

5.1. Residual Risks

While this Real-Time PCR kit has been designed to facilitate the accurate detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium*, it is important to be aware of certain residual risks associated with its usage. In compliance with regulations, the following limitations, contraindications, precautions, or warnings have been provided to ensure user safety and reliable results:

5.1.1. Sample and Detection Limitations

This kit is specifically intended for use with the following sample types: vaginal swabs, endocervical swabs, urethral swabs, urine, and eye swabs. It may not yield accurate results when used with other sample types.

The kit is designed for the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium* only.

5.1.2. Contraindications

There are no specific contraindications associated with the use of this PCR kit. It can be used in all age groups, provided that appropriate samples are collected.

5.1.3. Precautions

For detailed precautions to be taken when using the kit, please read the entire "REQUIRED MATERIALS AND DEVICES" and this section of the user manual. These precautions cover aspects such as sample collection, reagent handling and safety measures to ensure accurate and reliable results.

It is important to note that when the kit is used within its shelf life, under appropriate storage conditions, in suitable laboratory environments and by competent technical personnel, there are no anticipated problems. However, it is crucial to adhere to the precautions and instructions provided in the user manual to minimize any potential risks associated with the use of the kit.

Please carefully review and understand all the information provided, including the limitations, precautions and guidelines outlined in the user manual, to ensure safe and effective usage of this Real-Time PCR kit.

	This information serves as an addition to the user manual and should be considered an essential part of the instructions for use.
	For the Summary of Safety and Performance (SSP) document, please visit: https://anatoliageneworks.com/SSP/SSP_Bosphore_CT_NG_MG_Panel_Kit_v1_v00.pdf

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for *in vitro* diagnostic.
- This product should be used by this user manual.
- This product is to be used by personnel specially trained to perform molecular genetics laboratory techniques.

7. INFECTION

7.1. Structures of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Mycoplasma genitalium*

Chlamydia trachomatis, *Neisseria gonorrhoeae and Mycoplasma genitalium* are bacterial pathogens that can cause sexually transmitted infections. *C. trachomatis and N. gonorrhoeae* are gram-negative bacteria, while *M. genitalium* is a gram-positive bacterium.

C. trachomatis has a spherical shape with a diameter of approximately 300 to 1,000 nm. It is an obligate intracellular bacterium that has a complex life cycle involving two distinct developmental forms, the elementary body (EB) and the reticulate body (RB) (Kuo et al., 1995). The EB is the infectious form of the bacterium and can survive outside of the host cell.

N. gonorrhoeae has a bean-shaped morphology and measures approximately 0.6 to 1.0 µm in width and 0.9 to 1.5 µm in length (Unemo & Shafer, 2014). It is a facultative intracellular bacterium that can survive and replicate inside host cells, such as neutrophils.

M. genitalium is a small bacterium with a diameter of approximately 200 to 300 nm. It lacks a cell wall and a distinct membrane containing cholesterol (Borgogna et al., 2020). It is an obligate intracellular bacterium that can only replicate inside host cells.

Transmission of these pathogens can occur through sexual contact and symptoms can vary depending on the specific pathogen and the location of infection.

Symptoms for *C. trachomatis and N. gonorrhoeae* infections may include discharge, painful urination, and pelvic pain, while *M. genitalium* infections may cause urethritis or cervicitis (Falk et al., 2005). Further details on transmission and symptoms will be discussed in the Performance Evaluation Report.

7.2. Structural Components of *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*

C. trachomatis is a Gram-negative bacterium composed of several structural components. The chromosome is a small, circular, non-methylated DNA molecule that lacks histones. The outer membrane contains porin proteins and lipopolysaccharides, while the peptidoglycan layer is absent. *C. trachomatis* also possesses a type III secretion system, which aids in host cell invasion and nutrient acquisition. Inclusion bodies, which are dense, membrane-bound structures where the bacterium replicates, are also present.

N. gonorrhoeae is a Gram-negative bacterium with several key structural components. The chromosome is a large, linear DNA molecule that lacks histones. The outer membrane contains porin proteins, lipooligosaccharides and a variety of outer membrane proteins. The peptidoglycan layer is present but thin and located between the inner and outer membranes. *N. gonorrhoeae* also possesses a type IV secretion system, which is involved in host cell invasion, nutrient acquisition, and DNA transfer. Pili, hair-like structures that aid in adherence and host cell invasion, are also present.

M. genitalium is a small bacterium with unique structural components. The genome is a small, circular, double-stranded DNA molecule that lacks a cell wall. The membrane contains cholesterol and glycolipids, but no peptidoglycan layer. Adhesins, which are surface proteins that aid in adherence to host cells, are present.

Bacteria	Structural Component	Description
C. trachomatis	Elementary bodies (EBs)	Rigid, spherical shape with a diameter of about 300-400 nm; composed of a rigid outer membrane, a peptidoglycan layer and an inner membrane.
	Reticulate bodies (RBs)	Larger and more pleomorphic than EBs; contain multiple nucleoids and a more flexible outer membrane.
	Unique cell wall	Made up of lipopolysaccharides and peptidoglycan; lacks muramic acid and has a unique muropeptide composition.
	Inclusion bodies	Contain host-cell-derived nutrients and can be visualized using Giemsa or iodine stains; formed from host-cell membrane invaginations.
	Small genome	Consisting of a single circular chromosome of about 1.04 Mb, lacking RNA polymerase or metabolic enzymes for de novo biosynthesis of nucleotides or amino acids.

	Type III secretion system	A specialized protein secretion system that delivers effector proteins into host cells.
	Serovar diversity	There are at least 15 different serovars of *C. trachomatis*, each with distinct antigenic properties.
N. gonorrhoeae	Outer membrane	Contains lipopolysaccharides, outer membrane proteins and phospholipids; has a unique lipid A structure that contributes to the pathogenesis.
	Type IV pilus	Major virulence factor and target for immune evasion, involved in attachment to host cells and DNA uptake during natural transformation.
	Fimbriae	Involved in attachment to host cells and antigenic variation; there are multiple types of fimbriae, including types 1, 2 and 5.
	Complex genome	Consisting of a single circular chromosome of about 2.25 Mb and multiple plasmids; with many genes encoding for cell surface proteins and adhesins.
	Two-component regulatory system	Controls gene expression in response to environmental stimuli; consists of a sensor kinase and a response regulator.
	Opa proteins	Outer membrane proteins mediate attachment to host cells and immune evasion; there are multiple types of Opa proteins with distinct antigenic properties.
	LOS variation	Lipooligosaccharide (LOS) structures vary among different strains, contributing to antigenic diversity.
M. genitalium	Simple cell membrane	Lacks a cell wall and is composed of lipids and proteins; contains cholesterol for stability.
	Cytoskeleton	Made up of actin-like proteins that form a network of filaments within the cell, providing structural support and playing a role in cell division.
	Adhesins	Including P140 and P110, involved in attachment to host cells and pathogenesis; P140 has a tip structure that can interact with host cell surface receptors.
	Small genome	Consisting of a single circular chromosome of about 580 kb, lacking genes for many metabolic pathways.
	Unique tRNA system	Uses a non-canonical initiation codon for translation initiation; the initiator tRNA contains a C-U base pair in the anticodon stem.

Table 3: Summary of structural components of the bacteria *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*.

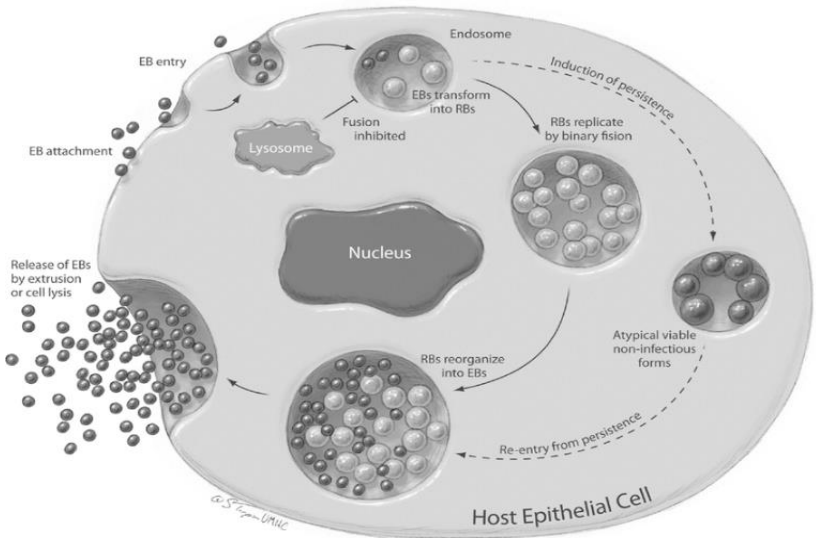

Figure 1: Developmental cycle of *C. trachomatis* (Schust et al., 2012)

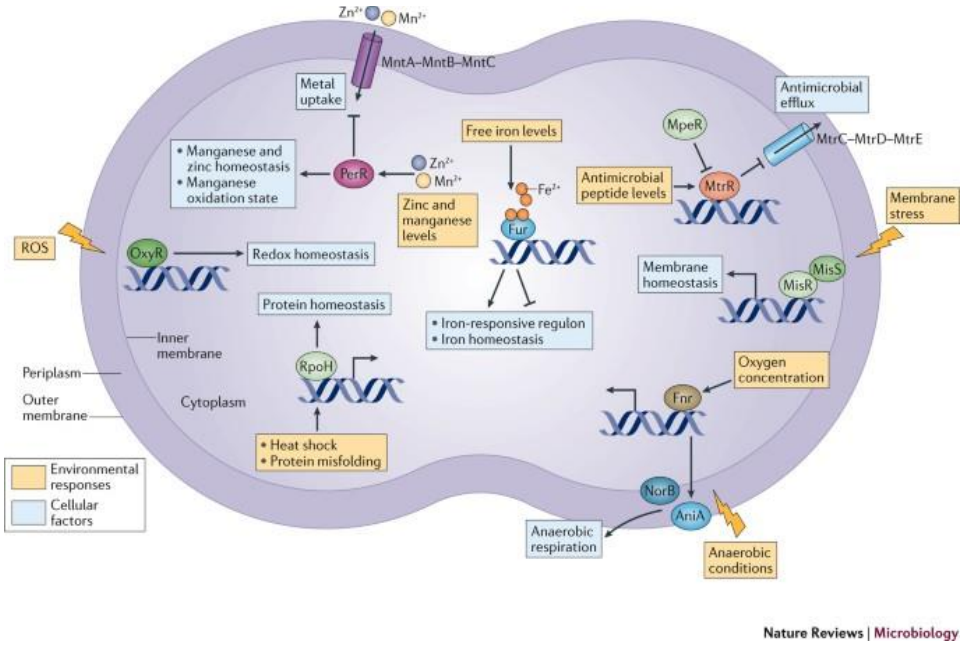

Figure 2: Schematic structure of *N. gonorrhoeae* (Quillin & Seifert, 2018)

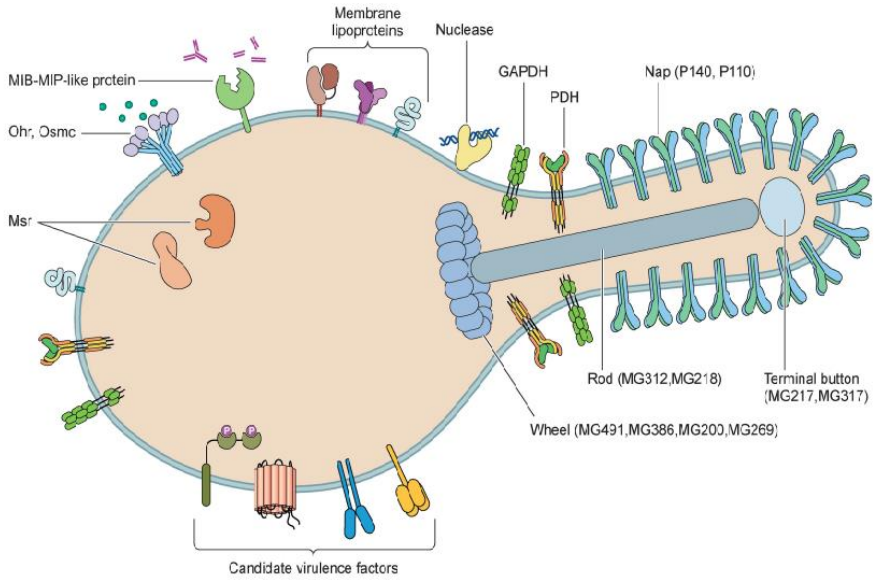

Figure 3: Schematic structure of *M. genitalium* (Wu et al., 2022)

7.3. Global Prevalence and Rate of Incidence

According to the World Health Organization (WHO), more than 1 million sexually transmitted infections (STIs) are acquired every day worldwide, the majority of which are asymptomatic.

Each year, there are an estimated 374 million new infections with one of three curable STIs: chlamydia, gonorrhea and trichomoniasis.

In 2020, WHO estimated 367 million new infections with one of three STIs, including chlamydia (129 million), gonorrhea (82 million) and trichomoniasis (156 million). STIs (like gonorrhea) can increase the risk of HIV acquisition. STIs such as gonorrhea and chlamydia are major causes of pelvic inflammatory disease and infertility in women (WHO, 2022).

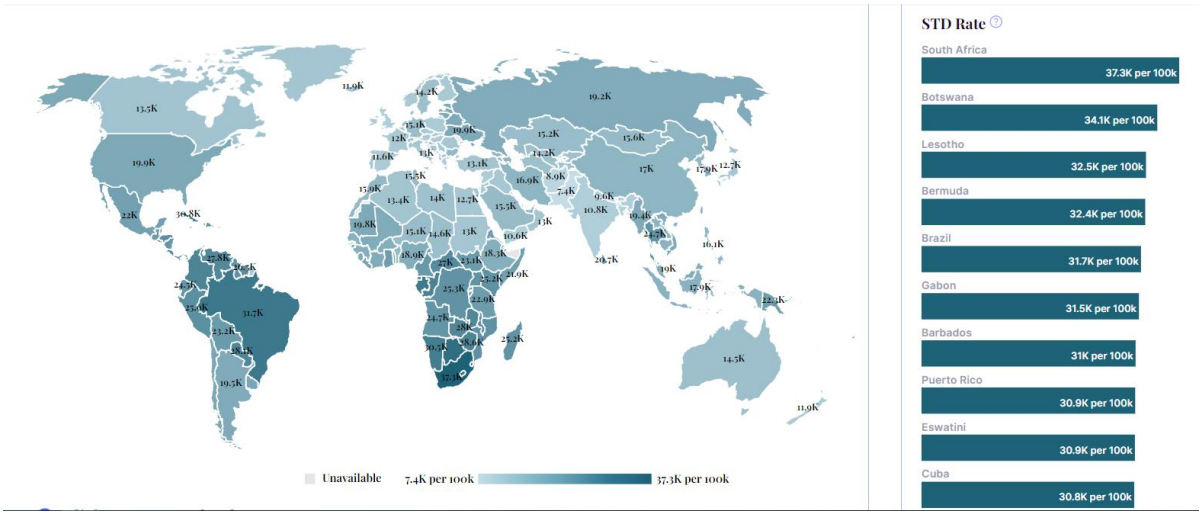

Figure 4: STD rates by country, 2023

7.4. Population at High Risk for *C. trachomatis, N. gonorrhoeae* and *M. genitalium*

C. trachomatis:

Young people: Chlamydia is most common among sexually active individuals under the age of 25, with rates highest among adolescents and young adults aged 15-24 years.

Women: Women are more likely than men to be diagnosed with chlamydia. This may be due in part to the fact that the infection can be asymptomatic in up to 70% of women.

Men who have sex with men (MSM): MSM is at increased risk for chlamydia, likely due to the higher rates of unprotected anal sex in this population.

People with multiple sexual partners: Having multiple sexual partners can increase the risk of chlamydia and other STIs.

N. gonorrhoeae:

Young people: As with chlamydia, gonorrhea is most common among sexually active individuals under the age of 25, with rates highest among adolescents and young adults aged 15-24 years.

Men who have sex with men (MSM): MSM is at higher risk for gonorrhea than heterosexual men, likely due to the higher rates of unprotected anal sex in this population.

African Americans: African Americans have higher rates of gonorrhea than other racial and ethnic groups in the United States. This may be due in part to factors such as poverty, limited access to healthcare and stigma around STIs.

People with multiple sexual partners: Having multiple sexual partners can increase the risk of gonorrhea and other STIs.

M. genitalium:

Men who have sex with men (MSM): MSM is at increased risk for *M. genitalium* infection, likely due to the higher rates of unprotected anal sex in this population.

Women: *M. genitalium* has been identified as a cause of cervicitis (inflammation of the cervix) and other genital infections in women.

People with multiple sexual partners: As with chlamydia and gonorrhea, having multiple sexual partners can increase the risk of *M. genitalium* and other STIs (WHO, 2016).

In conclusion, while anyone sexually active can be at risk for *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*, certain populations are at elevated risk due to a variety of factors such as age, sexual orientation, and several sexual partners. Individuals in these high-risk groups need to get tested regularly for STIs (Kreisel, K et al., 2021).

7.5. Current Practice

C. trachomatis, *N. gonorrhoeae* and *M. genitalium* are three of the most common sexually transmitted infections (STIs) worldwide. They can cause a wide range of symptoms, from mild to severe and can lead to serious long-term health consequences, such as infertility, ectopic pregnancy, and pelvic inflammatory disease. Therefore, early, and accurate diagnosis of these infections is crucial for effective treatment and prevention of further transmission.

There are several testing methods available for the detection of these STIs, each with its advantages and limitations. The most used testing methods include nucleic acid amplification tests (NAATs), enzyme-linked immunosorbent assays (ELISAs), culture and point-of-care tests (POCTs).

NAATs are highly sensitive and specific tests that amplify the genetic material of the bacteria, either DNA or RNA, to detect their presence in a patient sample. These tests can detect even small amounts of bacterial DNA or RNA and can be performed on various types of specimens, including urine, vaginal, cervical, rectal, and pharyngeal swabs. There are different types of NAATs available, such as polymerase chain reaction (PCR), transcription-mediated amplification (TMA) and strand displacement amplification (SDA), with varying sensitivity and specificity.

ELISAs are antibody-based tests that detect the presence of specific antibodies against the bacteria in a patient's serum or plasma. These tests are less sensitive and specific than NAATs and can only detect the presence of past or current infections, not early or asymptomatic infections. ELISAs are mainly used for epidemiological studies and to monitor the spread of infections in a population.

Culture involves growing the bacteria in a laboratory setting from a patient sample. The culture was the gold standard for STI testing in the past, but it is now less commonly used due to its lower sensitivity and longer turnaround time compared to NAATs. Culture can also be challenging to perform, requiring specialized laboratory facilities and experienced personnel. However, culture remains a valuable tool for the detection of antibiotic resistance, which is an increasing problem in the treatment of *C. trachomatis* and *N. gonorrhoeae* infections. Culture is also the only method available for the detection of *M. genitalium* that is not FDA-cleared.

POCTs are rapid diagnostic tests that can provide results within minutes, usually in a clinical setting, such as a physician's office or a community-based clinic. POCTs can be performed by non-laboratory personnel and require minimal training, making them suitable for use in resource-limited or remote areas.

However, POCTs for *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* are less sensitive and specific than NAATs and false-positive and false-negative results are common (Muralidhar S.,2015).

Currently, there are several POCTs available for the detection of *C. trachomatis* and *N. gonorrhoeae*. However, there are no FDA-cleared POCTs for the detection of *M. genitalium* (Reinton et al., 2013).

In conclusion, NAATs are the most sensitive and specific testing method for the detection of *C. trachomatis*, *N. gonorrhoeae,* and *M. genitalium* and are widely used in clinical practice. Enzyme-linked immunosorbent assays (ELISAs) and cultures are less commonly used but remain valuable tools for epidemiological and antibiotic resistance monitoring, respectively. Point-of-care tests (POCTs) provide rapid results but are less sensitive and specific than NAATs and should be used with caution. The choice of testing method depends on various factors, such as the patient population, the clinical setting, the resources available and the test performance characteristics. It is important to select the appropriate testing method to ensure accurate diagnosis and appropriate treatment of these common STIs (Calas et al., 2021).

7.6. Patient Sample Type

C. trachomatis, *N. gonorrhoeae* and *M. genitalium* are bacterial pathogens that cause STIs in humans. Real-Time PCR is a powerful molecular tool used to detect these pathogens in clinical samples with high sensitivity and specificity, even at low levels of bacterial DNA in complex samples.

The clinical samples used for qPCR analysis of *C. trachomatis, N. gonorrhoeae* and *M. genitalium* include swabs, urine, and other genital samples, which depend on the site of infection and the patient's symptoms.

Swab samples are the most used samples for qPCR analysis of these pathogens as they are minimally invasive and can be easily collected by trained healthcare professionals. For the detection of *C. trachomatis* and *N. gonorrhoeae*, swab samples are usually collected from the cervix, urethra, rectum, or pharynx, depending on the site of infection. For *M. genitalium*, swab samples are typically collected from the urethra or cervix.

Urine samples are another common sample type for qPCR analysis of *C. trachomatis, N. gonorrhoeae and M. genitalium*. First-catch urine, which contains the highest concentration of bacteria, is typically used for the detection of *C. trachomatis and N. gonorrhoeae*. Any urine sample can be used for the detection of *M. genitalium*.

Other genital samples, such as vaginal swabs, can also be used for qPCR analysis of these pathogens. Vaginal swabs are typically used for the detection of *C. trachomatis* and *N. gonorrhoeae* in women, while endocervical samples are used for the detection of *M. genitalium*. Urine samples are generally used to detect these pathogens in men.

In summary, qPCR is a highly sensitive and specific method for detecting *C. trachomatis, N. gonorrhoeae* and *M. genitalium* in clinical samples. The choice of sample type should be based on clinical judgment and the specific requirements of the qPCR assay used.

Chlamydia is considered the most common bacteria causing genital infections in the industrialized world and may lead to serious conditions such as pelvic inflammatory disease (PID), with consequences including infertility, ectopic pregnancy, and chronic pelvic pain. Screening women at risk for STIs can reduce half of the cases of PID (Welsh et al., 1997). For the diagnosis of *C. trachomatis* by culture, only specimens from the site of infection are recommended for diagnosis.

The quality of cervical specimens' cells from the columnar region significantly affects the positivity rates of *C. trachomatis* diagnosis by culture, molecular assays, and direct fluorescent antibody assay (DFA). These variations in specimen quality and diagnostic assay sensitivity significantly impact determining prevalence in a population. Reports of cervical specimen adequacy rates ranging from 50% to 64% indicate the necessity of training and periodic retraining of clinicians obtaining cervical samples for chlamydia testing.

Nucleic acid amplification methods have improved the sensitivity of diagnostic tests for the detection of a genital chlamydial infection.

The ligase chain reaction (LCR) and the polymerase chain reaction (PCR) are amplification techniques that use oligonucleotide probes or primers to amplify target sequences. Ribosomal RNA amplification methods, such as Qβ replicase amplification and TMA, have also been developed.

Recent studies have demonstrated that nucleic acid amplification methods represent a highly sensitive and specific approach for the detection of *C. trachomatis* in genital specimens of men and women when compared to cell culture.

Comparison studies between PCR, LCR and TMA have demonstrated high sensitivity in detecting *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* from genital and urine swabs in both men and women (Lee et al., 1995). Unlike culture-based methods, transport and storage conditions are less critical for amplifying assays and transport media are typically provided by the manufacturer. While cell culture-based diagnosis of genital chlamydial infections requires samples from the site of infection with enough elementary bodies (EBs), amplifying assays have shown high efficacy in identifying genital chlamydial infections in both men and women, including urine as a non-invasive specimen.

Automated LCR and PCR assays on first-void urine (FVU) in women have detected up to 30% more chlamydia-infected women than endocervical swab culture (Bass et al., 1993, Stary et al., 1999). Urine testing by amplifying methods is recommended for both men and women and the sensitivity of FVU is like that of cervical samples (Quinn et al., 1996, Jensen et al., 1997). However, male urine has shown a better performance pattern compared to female urine. Amplifying methods such as TMA have lower sensitivity, likely due to a larger number of inhibitory substances and a lower load of organisms (Lee et al., 1995, Smith & Weed, 1975). Since most organisms contaminating urine are no longer viable, urine as a non-invasive sample is not appropriate for chlamydial diagnosis by cell culture, which has a sensitivity of only about 30% (Taylor-Robinson, 1996).

Enzyme immunoassays (EIAs) have been used to perform antigen testing of urine sediment for *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*. However, in asymptomatic persons with a low number of elementary bodies (EBs), the sensitivity of these tests is rather poor (Stary et al., 1996, Gaydos et al., 1998). The use of non-invasive specimens, such as urine, vulval smears and vaginal swabs, has a major impact on the effectiveness of control programs for both males and females (Gaydos et al., 1998, Kacena et al., 1998, Stary et al., 1997, Limberger et al., 1992).

Furthermore, vulval smears may also serve as suitable non-invasive specimens in women and their processing for laboratory testing is uncomplicated and does not appear to be influenced by inhibition problems or by a low load of EBs (Hook et al., 1997).

However, like urine, vulval swabs cannot be recommended for culture tests due to the low number of viable organisms, resulting in a sensitivity of up to 30%. Neither can vulval or vaginal swabs be used for EIAs, due to the low number of EBs, resulting in a sensitivity of 40.7% (Hook et al., 1997, Limberger et al., 1992).

Vaginal swabs can be used as semi-invasive samples instead of cervical specimens and provide a high sensitivity of 91.8% (Limberger et al., 1992). Vulval or vaginal sampling may even be performed by the women themselves.

Recent studies indicate that different non-invasive specimen types are suitable for highly sensitive amplifying assays such as LCR, polymerase chain reaction (PCR) and transcription-mediated amplification (TMA) and may be used especially for chlamydia testing for screening of asymptomatic population groups.

The presumptive diagnosis of *N. gonorrhoeae* is based on the presence of typical intracellular Gram-negative diplococci with a sensitivity ranging between 40% and 98%, depending on the symptomatic status of the disease in men and women. Culture for gonococci is still regarded as the gold standard, requiring the presence of viable bacteria, exact specimen collection and transport conditions and a suitable culture medium.

However, the diagnosis of *N. gonorrhoeae* by an oligonucleotide directed against rRNA specific for gonococci had a sensitivity and specificity of up to 100% when compared with the culture technique (Whiley, D, 2006).

In a study with a total of 502 men and women, all infected individuals were detected by a DNA probe assay (Gen-Probe PACE 2 system) for gonococcal diagnosis in cases of unreliable culture conditions (Limberger R.J. et al., 1992).

Borderline results were confirmed by a probe competition assay to avoid false-positive results, especially in women. Nucleic acid amplification methods such as PCR or LCR have been reported to have high sensitivity and specificity for detecting *N. gonorrhoeae* and *C. trachomatis* in urogenital samples. LCR is effective in identifying *N. gonorrhoeae* in FVU of infected women and provides a practical alternative to culture for screening high-risk populations. Multiplex PCR and LCR are highly sensitive for detecting both *C. trachomatis and N. gonorrhoeae* from a single urine or genital swab, making it a more cost-effective way to screen multiple pathogens.

7.7. Overview of Alternative Devices that Measure the Target Analyte

Real-Time PCR is a commonly used method for detecting pathogens, such as *C. trachomatis and N. gonorrhoeae*, in clinical specimens. However, there are also other devices and techniques available for measuring these target analytes.

One alternative method is culture-based detection. This method involves the growth of pathogens in a laboratory setting to identify and confirm their presence (Hassanzadeh, P et al., 2013).

Although this method is highly specific and can provide valuable information on antimicrobial susceptibility, it can take several days to obtain results, which may delay treatment and increase the risk of transmission. Furthermore, culture-based detection may not always be sensitive enough to detect low levels of pathogens.

Another option is the use of antigen detection assays, which detect specific proteins associated with the pathogens. These assays can provide rapid results, often within 15 minutes and are relatively inexpensive compared to other methods. However, they may not be as sensitive or specific as Real-Time PCR, particularly for detecting asymptomatic infections. Additionally, the presence of non-specific cross-reactive antigens can lead to false positive results, while the absence of antigens in certain samples can lead to false negatives. (Wi, T. E. et al., 2019).

NAATs are another option for detecting the target analytes. NAATs use different methods to amplify and detect the genetic material of the pathogens. Examples of NAATs include transcription-mediated amplification (TMA), strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP). Each of these methods has its advantages and disadvantages in terms of sensitivity, specificity, cost, and ease of use.

TMA is a highly sensitive and specific NAAT that can detect low levels of pathogens, including those that may not be detected by culture-based methods. TMA is particularly useful for detecting asymptomatic infections and is FDA-approved for the detection of *C. trachomatis* and *N. gonorrhoeae*. However, TMA requires specialized equipment and trained personnel, which can make it more expensive than other methods.

SDA is another NAAT that is highly specific and can detect low levels of pathogens. SDA is less commonly used than TMA and Real-Time PCR, but it is effective for the detection of *N. gonorrhoeae*. Like TMA, SDA requires specialized equipment and trained personnel.

LAMP is a novel NAAT that can amplify DNA rapidly under isothermal conditions. LAMP is highly sensitive and specific and can be performed with minimal equipment, making it a potential alternative to Real-Time PCR for resource-limited settings. However, LAMP may be more susceptible to contamination and false positives than Real-Time PCR (Cosentino, L. A. et al., 2012).

It is important to note that the choice of detection method depends on several factors, including the availability of resources, the urgency of the situation and the specific needs of the patient population. Healthcare providers must carefully consider the benefits and limitations of each device or technique before deciding. In some cases, a combination of methods may be necessary to maximize sensitivity and specificity.

In conclusion, while Real-Time PCR is a highly sensitive and specific method for detecting *C. trachomatis, N. gonorrhoeae,* and *M. genitalium*, there are also several alternative devices and techniques available for measuring these target analytes. Healthcare providers must carefully evaluate the pros and cons of each method before selecting the best option for their patients.

7.8. Consensus Expert Opinions / Positions from Relevant Professional Associations

***C. trachomatis*:**

WHO recommends the use of NAATs, including PCR, TMA, and SDA, for the detection of *C. trachomatis*. These tests can be performed on various specimen types such as urine, vaginal swabs and endocervical swabs (WHO, 2016).

CDC also recommends the use of NAATs as the preferred method for *C. trachomatis* testing, with urine specimens being the preferred sample type (CDC, 2014).

***N. gonorrhoeae*:**

WHO recommends the use of NAATs, including PCR and TMA, for the detection of *N. gonorrhoeae* (WHO, 2016).

CDC recommends the use of NAATs as the preferred method for *N. gonorrhoeae* testing, with urine specimens being the preferred sample type (CDC, 2014).

***M. genitalium*:**

CDC also recommends the use of NAATs as the preferred method for *M. genitalium* testing, with vaginal swabs being the preferred sample type. (CDC, 2021)

8. METHOD

Bosphore CT/NG/MG Panel Kit v1 is a qualitative diagnostic tool designed to detect specific DNA regions using the Real-Time PCR method. Real-Time PCR, also known as quantitative PCR (qPCR), is a powerful technique that allows monitoring of the PCR reaction in Real-Time, eliminating the need for additional analysis methods such as gel electrophoresis.

The Real-Time PCR reaction begins with an initial denaturation step. During this step, the PCR tube containing the reaction mixture is heated to a temperature of 95 °C and this high temperature is maintained for a duration of 5 minutes. The purpose of the initial denaturation is to separate the double-stranded DNA template into single strands, providing the starting point for subsequent amplification. This step ensures that the DNA strands are available for binding with specific primers.

Following the initial denaturation, the reaction proceeds through a series of cycles, each consisting of denaturation and annealing steps. The denaturation step occurs during each cycle, where the reaction mixture is heated to a temperature of 97 °C for 15 seconds. This high temperature ensures that the DNA strands separate completely, providing single-stranded templates for the subsequent steps. Denaturation involves the disruption of the hydrogen bonds between the DNA strands, leading to the separation of the double-stranded DNA into individual single-stranded DNA templates.

After denaturation, the reaction temperature is lowered to 58 °C for 50 seconds during the annealing step. At this lower temperature, the primers designed to be complementary to the target regions of the DNA template bind to their specific sequences. The primers are short DNA sequences that are designed to hybridize, or anneal, to the complementary sequences on the single-stranded DNA template. This step allows the primers to find their target regions and bind to them, indicating the presence of the target DNA sequences.

During the cycles, the denaturation and annealing steps repeat for a total of 42 repetitions. Each cycle consists of a denaturation step to separate the DNA strands and an annealing step to allow the primers to bind to their target sequences. These repeated cycles increase the chances of detecting the target DNA sequences, indicating a positive result.

Bosphore CT/NG/MG Panel Kit v1 utilizes Real-Time PCR with an initial denaturation step followed by repeated cycles of denaturation and annealing and hold step. The denaturation step ensures complete separation of the DNA strands, while the annealing step facilitates the binding of primers to their target sequences, indicating the presence of the target DNA regions.

Threshold Cycle (C_T) and Result Interpretation:

During the Real-Time PCR reaction, the fluorescence generated by the reporter molecule is monitored. The point at which the fluorescence signal rises above the background level and becomes distinguishable is known as the threshold cycle (C_T). The samples that cross the threshold are regarded as positive. The samples that do not cut the threshold must be regarded as negative or having a bacterial load below the detection limit of the assay.

Bosphore CT/NG/MG Panel Kit v1 is a qualitative assay, meaning it provides a presence or absence result for the target DNA sequences. A positive result indicates the presence of the target DNA sequences, while a negative result indicates their absence or a bacterial load below the detection limit of the assay.

Multiplex qPCR and Internal Control:

Bosphore CT/NG/MG Panel Kit v1 employs multiplex qPCR, allowing simultaneous detection and amplification of multiple target DNA regions. Additionally, internal control is included in the system to monitor the extraction procedure, PCR inhibition and potential issues during the application.

The internal control ensures the reliability of the PCR results by validating the overall PCR process and identifying any potential issues that may affect the interpretation of the results.

9. PROCEDURE

9.1. Sampling & Storage

To ensure accurate and reliable results, it is essential to follow the appropriate sampling and storage procedures when using Bosphore CT/NG/MG Panel Kit v1 for Real-Time PCR analysis. Please adhere to the guidelines provided in this section.

Swab Selection:

It is recommended to use swabs immersed in Universal Transport Medium (UTM), such as Copan Universal Transport Medium (UTM-RT) System or Copan Liquid Amies Elution Swab (ESwab) Collection and Transport System. These swabs are compatible with Bosphore CT/NG/MG Panel Kit v1. However, swabs with wooden shafts, calcium alginate and aluminum swabs should not be used as they may cause PCR inhibition (Wadowsky et al., 1994; Cloud et al., 2002).

Specimen Collection and Handling:

All specimens must have been collected according to the WHO document named: "Laboratory Diagnosis of sexually transmitted infections, including human immunodeficiency virus".

Instructions for Collecting an Endocervical Swab Specimen (performed by a trained healthcare provider):

- Use a cleaning swab to remove excess mucus before sample collection.
- Insert a swab 2–3 cm into the cervical os and rotate gently for 5–10 seconds.

Please note that endocervical samples should not be taken in prepubertal girls or women who have had a hysterectomy. Instead, specimens should be sampled from the vestibule of the vagina. Additionally, a urine specimen should be collected for nucleic acid amplification testing (NAAT) diagnostics.

Instructions for Collecting a Vaginal Swab Specimen (performed by a trained healthcare provider):

- Rotate the swab against the posterior vaginal walls for 5 seconds.

Instructions for Collecting a Urine Specimen:

- Do not have the patient clean the genital area.
- Collect 10–20 mL of the first void urine in a sterile collection container at least 1 hour after the patient has urinated.

Instructions for Collecting an Eye Swab Specimen (performed by a trained healthcare provider):

- Retract the inferior eyelid.
- Move a thin swab across the surface of the inferior palpebral conjunctiva towards the inner corner of the eye.

Instructions for Collecting a Urethral Swab Specimen (performed by a trained healthcare provider):

- Take urethral specimens at least 1 hour after the patient has urinated.
- Collect discharge directly on a swab. If no discharge is evident:

a. In men, the urethra should be stripped towards the orifice to evacuate the exudate.

b. If no exudate is obtained, insert a thin swab 2–3 cm into the urethra and gently rotate for 5–10 seconds.

c. In women, massage the urethra against the pubic symphysis and use the same technique as for men.

Specimen Stability

The stability of the specimens plays a crucial role in ensuring accurate and reliable results in Real-Time PCR analysis. It is essential to properly handle and store the samples to maintain their integrity throughout the testing process. The following information summarizes the specimen stability based on the analysis of various pathogens in different storage conditions:

For urine samples:

- Samples stored at +4 °C remained stable for up to 5 days.

For swab samples in UTM-RT:

- Samples stored at room temperature remained stable for up to 48 hours.
- Samples stored at +4 °C remained stable for up to 5 days.
- Samples stored at -20 °C remained stable for up to 30 days.

For swab samples in ESwab:

- Samples stored at room temperature remained stable for up to 48 hours.
- Samples stored at +4 °C remained stable for up to 7 days.
- Samples stored at -20 °C remained stable for up to 30 days.

Sample transport:

Clinical urine samples can be transported within 5 days at +4 ºC temperature (e.g., with cold-packs); swab samples in UTM-RT and ESwab can be transported at room temperature within 2 days at the latest.

Additionally, freeze and thaw cycle experiments were conducted:

The samples underwent three freeze-thaw cycles, it was observed that 3 freeze-thaws did not affect the results in urine and swab samples in UTM-RT and swab samples in ESwab. It is crucial to adhere to the recommended storage conditions and timeframes to ensure sample stability and obtain accurate PCR results.

Bacterial DNA Extraction:

Bacterial DNA extraction should be performed following the instructions provided by the extraction kit manufacturer. Ensure that all steps of bacterial DNA extraction are conducted within a biological safety cabinet.

9.2. DNA Extraction

For efficient DNA extraction, we recommend using the following kits:

- Unio B24/B48 Extraction Systems - Unio Bacterial DNA Extraction Kit (Anatolia Geneworks).
- Unio 96 Extraction & PCR Setup System – Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks).
- Bosphore STD EX-Tract Kit (Anatolia Geneworks).

Please ensure that the bacterial DNA extraction is performed in accordance with the instructions provided by the manufacturers.

9.3. Kit Components

9.3.1. Negative Control

The negative control is an essential component of Bosphore CT/NG/MG Panel Kit v1 for Real-Time PCR. It consists of double-distilled water (dH_2O) and serves as a reference sample to ensure accurate results by detecting any contamination or errors that could lead to false-positive outcomes.

To use the negative control:

- Label a separate tube or well as "Negative Control."
- Add the provided dH_2O to the labeled tube or well.
- Treat the negative control sample the same way as your experimental samples.
- Include the negative control in each PCR run.

Throughout the reaction, monitor the negative control closely. It should remain negative, showing no amplification signals (No C_T). Any amplification in the negative control indicates potential contamination.

The negative control plays a vital role in Bosphore CT/NG/MG Panel Kit v1, ensuring the reliability of your results. Including the negative control and carefully monitoring it will enable you to confidently interpret your data.

9.3.2. PCR Master Mix

The Real-Time PCR Master Mix is a comprehensive solution designed for efficient and accurate Real-Time PCR amplification. It combines a highly specific and accurate Taq DNA Polymerase with hot-start property, PCR buffers, a balanced mix of dNTPs, specific forward and reverse primers, dual-labeled probes, and essential additives. This master mix has been carefully formulated to enable simultaneous detection of *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Mycoplasma genitalium* (MG) and internal control within a single reaction. To prepare the reaction, simply add 10 µL of extract nucleic acid to 15 µL of the PCR Master Mix.

9.3.3. Internal Control

The kit includes an internal control that serves multiple purposes. The internal control is a synthetic DNA molecule specifically designed to assess the efficiency of DNA extraction, identify application errors, and detect any potential PCR inhibition.

This control is added to the sample-proteinase K mixture at the beginning of the DNA extraction process to evaluate the extraction efficiency and identify any application errors that may occur.

While adding the internal control during nucleic acid extraction, it is recommended to add 5 µL per sample. Conversely, when directly adding the internal control to the PCR Master Mix to monitor PCR inhibition, only 0.2 µL is required. For a comprehensive evaluation of the extraction system's efficiency, we suggest adding internal control to the negative control.

	Please note that if the internal control has already been included during the extraction step, there is no need to incorporate it into the PCR Master Mix.

In negative samples, the absence of internal control amplification in the target channels may indicate issues with extraction or application, or potential PCR inhibition. In such cases, it is advisable to repeat both the extraction and PCR steps.

It is important to be aware that in samples with a high bacterial load, including the positive control, the internal control's fluorescent signal may be suppressed, making it difficult to detect an increase in the signal level.

9.3.4. Positive Control

The provided kit encompasses a synthetic DNA positive control, which encompasses synthetic DNA from *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium*. To assess the efficiency of the PCR reaction, it is imperative to incorporate this positive control into the testing process.

To determine the acceptability of the PCR results, please refer to the acceptance criteria table outlined in Section 10 of this manual. The table specifies the threshold C_T value of the positive control. If the threshold C_T value of the positive control exceeds the upper limit specified in the criteria table, it could indicate a potential decrease in the reaction's yield. Therefore, monitoring the C_T value of the positive control aids in assessing the overall efficiency of the reaction.

9.4. Preparing the PCR

Before starting the Real-Time PCR, it is important to prepare the PCR reaction mix accurately. Each PCR should include at least one negative control and positive control, and the samples with internal control. Please follow the detailed instructions below:

- Ensure that all kit components are properly dissolved before use. Thoroughly mix the reagents by gentle inversion or vortexing.
- Use the table provided below to calculate the volumes of each component required for the PCR reaction mix. The volumes mentioned in the table are for one reaction only.
- To determine the volumes needed for the master mix, multiply these volumes by the total number of samples.

If preparing the mix for more than 5 samples, add 10% to the total number of samples and adjust the volumes accordingly.

Components	Volume
PCR Master Mix	15 µL
Internal Control*	0.2 µL*
Sample DNA (Negative / Positive Control)	10 µL
Total Volume	25 µL

*Internal control should not be added to the reaction if it has already been added during the extraction step.

Table 4: Bosphore CT/NG/MG Panel Kit v1 Components and Volumes

- Prepare PCR tubes or strips for each reaction. Pipette 15 µL of the PCR Master Mix into each PCR tube or well of the strip/plate.
- Add 10 µL of the appropriate DNA to each designated well or tube. Ensure that the DNA samples, positive control, and negative control are added to separate wells or tubes.
- Close the tube caps/seal the PCR plate carefully to prevent contamination and evaporation.
- Ensure that the mix in each tube is at the bottom of the tube. If necessary, briefly spin down the tubes to collect the contents at the bottom.
- Once the PCR reaction mix is prepared, it is ready for the next steps in the PCR process. Follow the provided protocol to proceed with the Real-Time PCR analysis.

9.5. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore CT/NG/MG Panel Kit v1 consists of initial denaturation for activation of the Taq DNA Polymerase (with hot-start property), a two-step amplification cycle and a terminal hold. The Real-Time data is collected at the second step of the amplification cycle. The thermal protocol to be applied for the reaction is indicated on below:

Steps	Temperature	Time	
Initial Denaturation	95 °C	05:00 min	
Denaturation	97 °C	00:15 min	42 Cycles
Annealing (Data Collection)	58 °C	00:50 min	
Hold	32 °C	00:20 min	

Table 5: Bosphore CT/NG/MG Panel Kit v1 Thermal Protocol

Before starting to work with Bosphore CT/NG/MG Panel Kit v1, the following steps must be completed and checked:

- Choose all the filters to be used (FAM, HEX, Texas RED, and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol,
- Start the experiment.

9.5.1. Experiment Setup for Montania 4896 Real-Time PCR Instrument

Preparation:

- Ensure that the Montania 4896 Real-Time PCR Instrument is set up correctly and connected to a computer installed by the manufacturer or authorized technical service.
- Verify that all the necessary reagents and components for the PCR reaction are available and stored under appropriate conditions for the experimental setup.

Sample and Reagent Setup:

- Prepare the PCR reaction mixture according to the specifications mentioned above.
- Distribute the PCR Master Mix homogeneously into PCR tubes.

Adding Samples:

- Add the DNA template containing the target sequence of interest to each PCR tube. Make sure to include appropriate positive and negative controls in the tubes.
- Gently mix the contents to ensure proper mixing of the template with the reaction mixture.

Loading the PCR Instrument:

- Open the software interface for the Montania 4896 Real-Time PCR Instrument on the connected computer.
- Create a new experiment or select a pre-existing experiment template suitable for your PCR reaction.
- Choose in which block the experiment will be performed.
- Define the necessary parameters such as reaction volume, cycling conditions and fluorescence detection channels.

Loading Samples:

- Transfer the prepared PCR tubes to the appropriate sample block of the Montania 4896 Real-Time PCR Instrument. Ensure that the lids are tightly closed, and the samples are properly aligned in the block to avoid potential issues due to sudden temperature changes during operation.

Initiating the PCR:

- Double-check that all the required settings and parameters have been accurately entered into the software interface.

i	It is recommended to label the experiment at this stage to avoid mixing up the order of sample, positive control, and negative control in the mixes.

- Start the PCR run using the software interface to initiate the cycling program and data collection process.
- Monitor the progress of the PCR run in the software interface by observing the amplification curves and fluorescence signals.

Data Analysis and Interpretation:

- Please refer to the "ANALYSIS" tab for Cross Talk settings.
- After the completion of the PCR run, the Montania 4896 Real-Time PCR system will automatically generate amplification curves for each sample.
- Interpret the generated data based on the information provided in this user manual.
- Analyze the target amplification accordingly if the negative and positive controls are by the acceptance criteria.

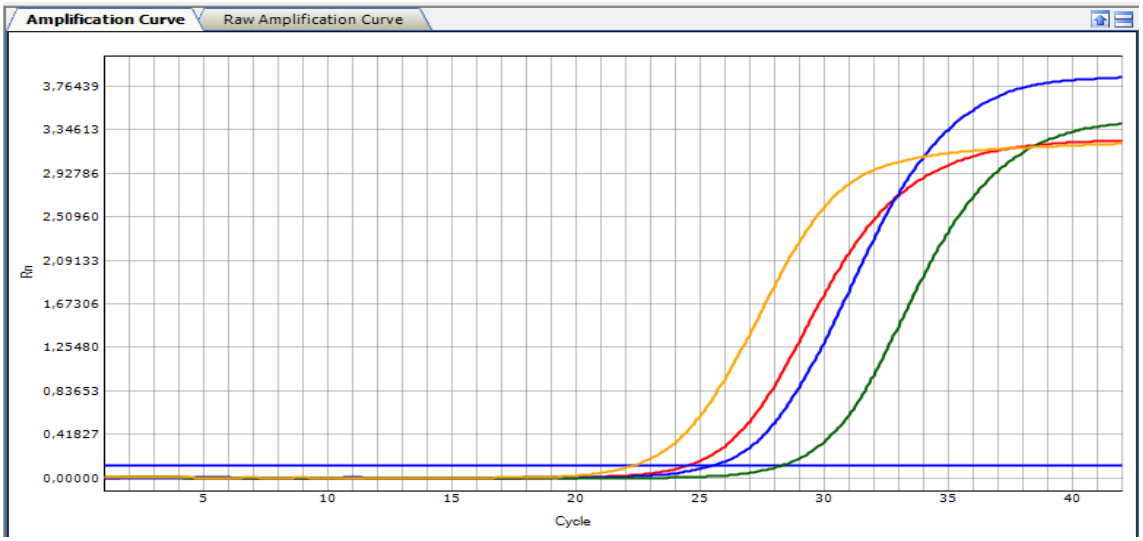

Figure 5: Amplification Curve for Montania 4896

9.5.2. Experiment Setup for CFX96 Real-Time PCR Detection System

Instrument Preparation:

- Ensure that the CFX96 instrument is properly connected to a power source and turned on.
- Allow the instrument to warm up to the desired temperature (if applicable) following the manufacturer's instructions.
- Open the CFX Manager software on the connected computer.

Sample Preparation:

- Prepare your PCR reaction mix according to the instructions provided with Bosphore CT/NG/MG Panel Kit v1.
- Add your DNA sample to the PCR Master Mix.
- Mix the reaction thoroughly by pipetting gently and ensure that all components are well combined.

Plate Setup:

- Prepare a white PCR plate compatible with the CFX96 system. Ensure that the wells are labeled correctly to identify the samples.
- Transfer the PCR reaction mix into the appropriate wells of the PCR plate using a micropipette. Include positive and negative controls.
- Cover the PCR plate with an optically clear adhesive film or seal to prevent contamination and evaporation.

Instrument Setup:

- Launch the CFX Manager software on the connected computer if not already open.
- Place the prepared PCR plate into the CFX96 instrument.
- Create a new experiment in the software and specify the experiment parameters (e.g., target, reaction volume, etc.).
- Set up the thermal cycling conditions, including the initial denaturation temperature and time, amplification cycles and annealing temperatures.

Running the Experiment:

- Start the experiment by clicking the "Run" button in the CFX Manager software.

- The CFX96 instrument will perform the specified thermal cycling program while simultaneously monitoring fluorescence signals in real time.
- Once the run is complete, the CFX Manager software will generate a Real-Time PCR amplification plot and provide data analysis options.

Data Analysis:

- Analyze the Real-Time PCR amplification plot using the CFX Manager software. Interpret the results according to the experimental objectives and acceptance criteria communicated in this manual.

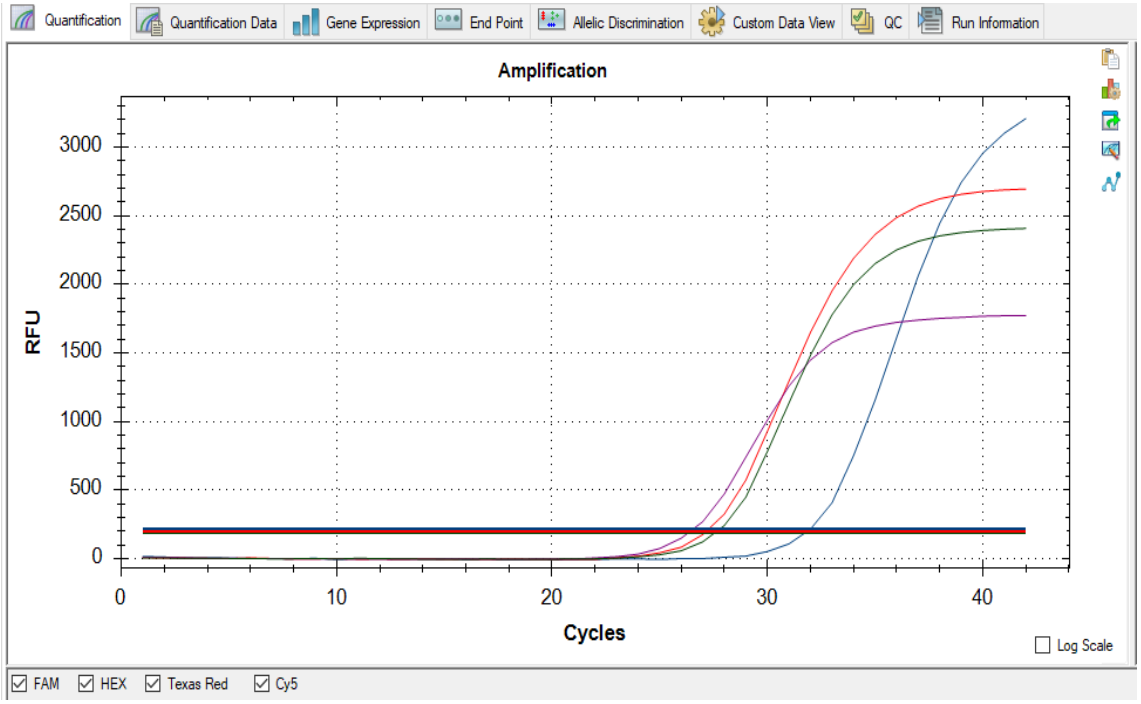

Figure 6: Amplification Curve for CFX96

Applying Fluorescence Drift Correction and Baseline Setting:

- In the CFX Manager software, navigate to the experiment settings.
- Enable the Fluorescence Drift Correction feature to compensate for any potential variations in fluorescence intensity during the run.
- Set the Baseline Setting to the appropriate parameters to ensure accurate baseline calculations during data analysis.

9.5.3. Experiment Setup for LightCycler 480 Instrument II

Instrument Preparation:

- Ensure that the LightCycler 480 Instrument II system is properly connected to a power source and turned on.
- Make sure a white plate compatible with the Roche LightCycler 480 Instrument II is used.

Reagent Preparation:

- Prepare your PCR Master Mix according to the instructions provided with Bosphore CT/NG/MG Panel Kit v1.
- Ensure that your PCR Master Mix is properly aliquoted into individual wells of the white plate.

Sample Preparation:

- Prepare your samples and controls in appropriate volumes.
- Add your samples to the PCR Master Mix, making sure to include positive and negative controls.

Performing the Experiment

Open the LightCycler 480 Instrument II software on the connected computer.

Create a New Experiment:

- Set up the experiment by specifying the type of experiment (quantification).
- Enter the necessary experimental details, such as the target channels, sample names and reaction conditions.
- Define the appropriate cycling protocol for your experiment, including denaturation, annealing and extension temperatures and times.

Set up the Plate Layout:

- Assign the sample names to the corresponding wells in the white plate.
- Include appropriate control wells (positive and negative controls) for result interpretation.

Load the White Plate:

- Carefully load the white plate with the prepared PCR Master Mix and samples, following the plate layout defined in the software.

- Ensure that the plate is properly sealed to prevent contamination and evaporation.

Perform Color Compensation Protocol:

- Perform a color compensation protocol using Bosphore Color Compensation Set B or Set D specifically designed for the LightCycler 480 Instrument II system.
- Follow the instructions provided with the kit to perform the color compensation protocol accurately.

Start the Experiment:

- Place the white plate into the LightCycler 480 Instrument II system multiwell plate loader.
- Retract the loader with the inserted PCR plate into the instrument.
- Start the run using the predefined cycling protocol.

Data Analysis and Interpretation

Monitor the amplification in Real-Time:

- During the experiment, the LightCycler 480 Instrument II system will continuously measure the fluorescence emitted by the samples.
- The instrument software will display real time amplification curves for each well.

Analyze the data:

- Once the run is complete, open the LightCycler 480 Instrument II software for data analysis.
- Choose "Abs Quant/Fit Points" as the analysis type to ensure appropriate threshold level selection.
- Perform the analysis using the "Cycle Range" option.

Interpret the results:

- Refer to the acceptance criteria contained in this manual for result interpretation.

Remember to refer to the LightCycler 480 Instrument II system user manual for detailed instructions on instrument setup, operation, and data analysis. Additionally, follow the instructions provided with Bosphore Color Compensation Set B or D for accurate color compensation protocol execution.

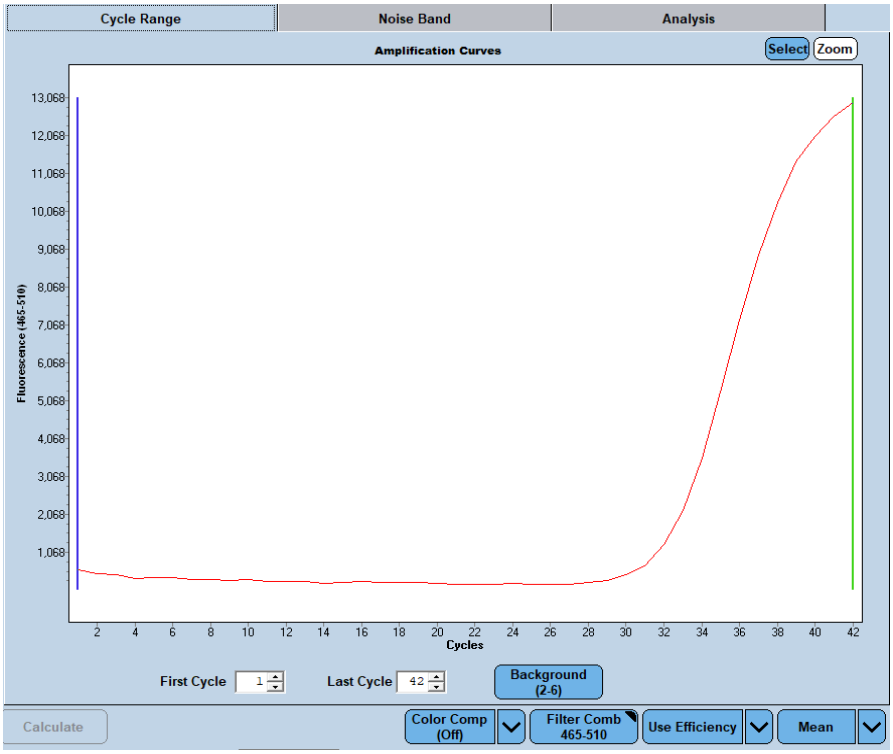

Figure 7: Amplification Curve for LightCycler480 Instrument II

i	The amplification curve is for a single channel. Please refer to the LightCycler 480 Instrument II user manual for detailed instructions specific to your kit and instrument model.

9.5.4. Experiment Setup for Rotorgene Q

Instrument Setup:

- Ensure that the RotorGene Q instrument is placed on a stable and level surface.
- Connect the instrument to a power source and switch it on.
- Allow the instrument to warm up according to the manufacturer's instructions.

Sample Preparation:

- Collect and prepare the samples according to Bosphore CT/NG/MG Panel Kit v1 guidelines.

Reaction Setup:

- Dispense the PCR Master Mix into PCR tubes or strips, taking care to avoid any contamination.
- Add the DNA templates and positive/negative controls to the appropriate wells or tubes, ensuring proper sample identification.

Load the Strips/Tubes:

- Carefully load the strips/tubes with the prepared PCR Master Mix and samples, following the proper sample identifications defined in the software.
- Ensure that the strip/tube caps properly closed to prevent contamination and evaporation.

Thermal Cycling Conditions:

- Set up the appropriate thermal cycling conditions on the RotorGene Q instrument according to Bosphore CT/NG/MG Panel Kit v1 guidelines.
- Disable automatic gain optimization and manually setup the gain for each channel to 10.
- Optimize the cycling parameters, including denaturation temperature, annealing temperature, and the number of cycles, for your specific assay.

Data Analysis:

- After the Real-Time PCR run is complete, analyze the data using the RotorGene Q software.
- Utilize the "Slope Correct" and "Dynamic Tube" options for analysis, as recommended by Bosphore CT/NG/MG Panel Kit v1.
- If the sigmoidal curve is not observed, attempt analyzing the sample without the "Slope Correct" option.
- Set up appropriate analysis parameters, including threshold settings, baseline correction and normalization.

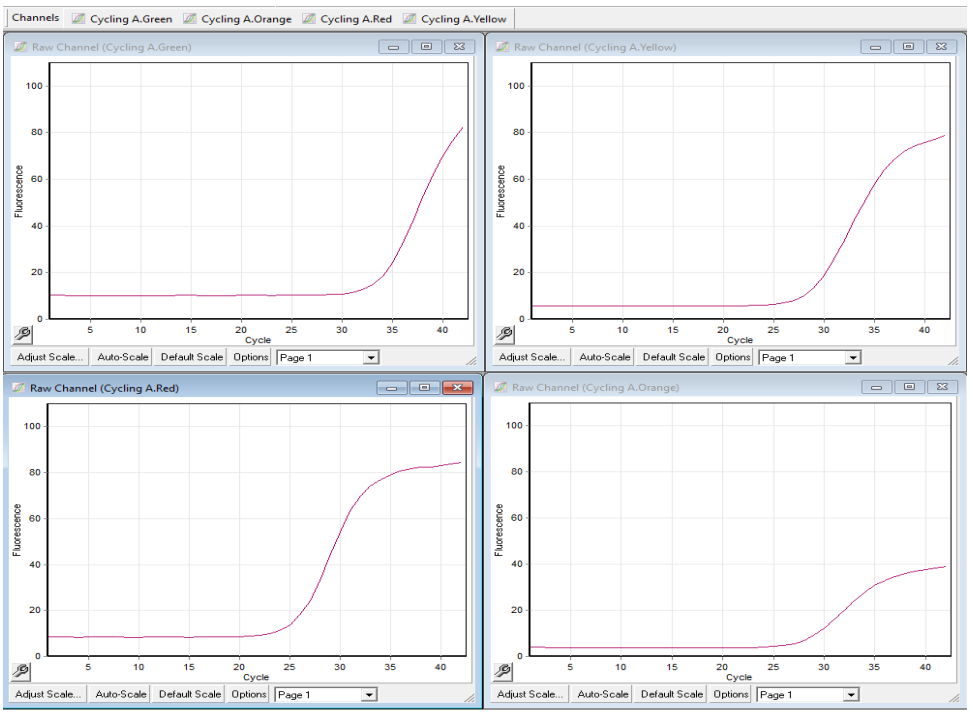

Figure 8: Amplification Curve for Rotorgene Q

9.5.5. Experiment Setup for Quant Studio 5 Real-Time PCR Instrument

Instrument Preparation:

- Ensure that the Quant Studio 5 instrument is powered on and connected to a computer with the appropriate software installed.
- Check that the instrument is clean and free from any contamination. Clean the instrument following the manufacturer's guidelines if necessary.

Plate Setup:

- Prepare a 96-well plate. Label the plate accordingly to track your samples and controls.
- Add the PCR Master Mix and the DNA templates and positive/negative controls to the appropriate wells, ensuring accurate and precise pipetting.

Instrument Setup:

- Open the Quant Studio Design and Analysis Software on your computer and establish a connection with the Quant Studio 5 instrument.
- Select the Chemistry: TaqMan protocol for Bosphore CT/NG/MG Panel Kit v1 based on the kit's specifications. Consult the software user manual for detailed instructions on protocol selection.
- Set the desired cycling parameters, including the number of cycles, denaturation, annealing and hold temperatures and times, as specified by Bosphore CT/NG/MG Panel Kit v1.
- Set the passive reference dye as "none".
- Choose the appropriate fluorescence channels for the probes used in Bosphore CT/NG/MG Panel Kit v1.

Run the Experiment:

- Load the prepared 96-well plate into the Quant Studio 5 instrument, ensuring it is properly aligned.
- Start the experiment from the software, initiating the Real-Time PCR run according to the selected Chemistry: TaqMan protocol and the defined cycling parameters.
- Monitor the progress of the run in Real-Time on the software interface. The instrument will perform the necessary temperature cycling and fluorescence detection for each cycle.
- Once the run is completed, the software will provide you with data, including amplification curves, C_T values and other relevant information.

Data Analysis:

- Interpret the results based on Bosphore CT/NG/MG Panel Kit v1 guidelines provided in the kit's manual.

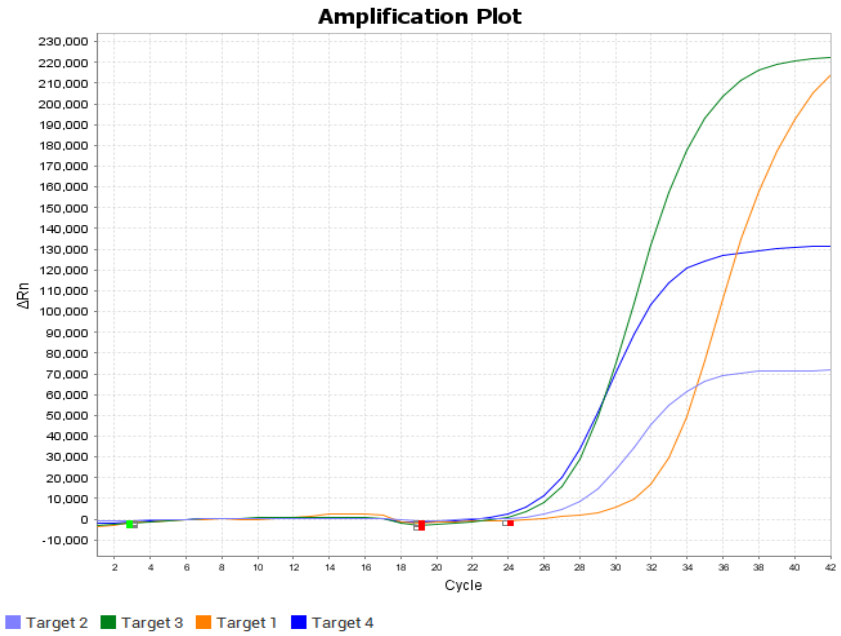

Figure 9: Amplification Curve for Quant Studio 5

9.5.6. Experiment Setup for Q Real-Time Quantitative PCR Instrument

Instrument Setup:

- Ensure that the Q Real-Time qPCR Instrument is connected to a power source.
- Press the power button to turn on the instrument and allow it to warm up and stabilize according to the manufacturer's instructions.

Reaction Setup:

- Dispense the PCR Master Mix into PCR tubes or strips, taking care to avoid any contamination.
- Add the DNA templates and positive/negative controls to the appropriate wells, ensuring proper sample identification.

Load the Strips/Tubes:

- Carefully load the strips/tubes with the prepared PCR Master Mix and samples, following the proper sample identifications defined in the software.
- Ensure that the strip/tube caps properly closed to prevent contamination and evaporation.

Thermal Cycling Conditions:

- Set the thermal cycling conditions on the Q Real-Time qPCR Instrument based on the recommendations provided by Bosphore CT/NG/MG Panel Kit v1.
- Specify the denaturation and annealing temperatures and times for each cycle.

Data Analysis and Interpretation

Threshold Determination:

- Define a threshold fluorescence value that distinguishes the background signal from the amplification signal.
- If necessary, set the "Fluorescent Cutoff Level" up to 15% to optimize the threshold determination.

C_T Calculation:

- Determine the cycle threshold (C_T) value for each sample, representing the cycle number at which the fluorescence signal crosses the threshold.
- Use the instrument's software or appropriate data analysis tools to automatically calculate the C_T values.

Data Interpretation:

- Analyze the qPCR data to draw meaningful conclusions and interpretations based on the experimental objectives.
- Compare and contrast the quantification levels between different samples or experimental conditions.

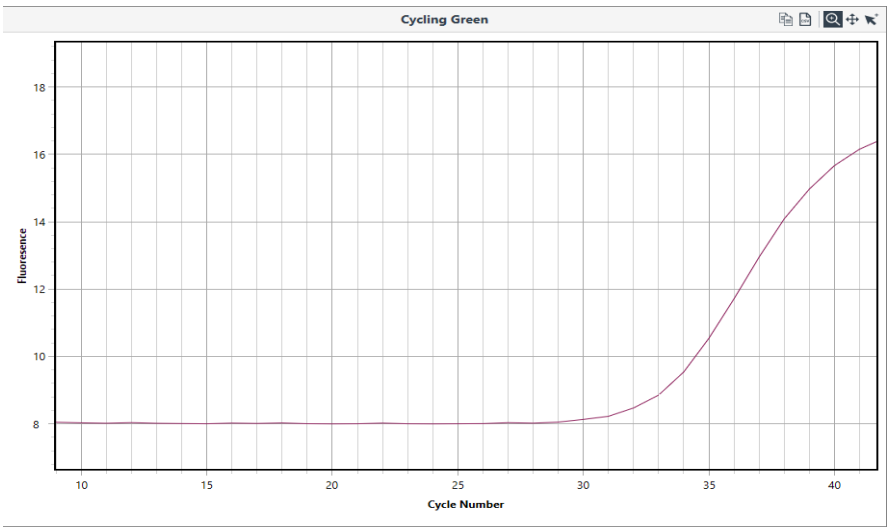

Figure 10: Amplification Curve for Q Real-Time Quantitative PCR Instrument

The amplification curve is for a single channel. Please refer to the Q Real-Time qPCR Instrument user manual for detailed instructions specific to your kit and instrument model.

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel who have received the required training from the manufacturer, consider it necessary if the system allows pulling down the threshold as much as possible to detect low amplifications, attention should be paid to keep the threshold line above the background.

The negative control is essential for accurate result analysis. Please check the negative control and ensure it shows no amplification outside the associated internal control filter. If the negative control has a signal outside the filter associated with the internal control, please do not report the results. Repeat the experiment after taking the necessary precautions against contamination. If the same result is encountered again, please contact the manufacturer.

Internal control and positive control of Bosphore CT/NG/MG Panel Kit v1 are essential for accurate result analysis. The cycle threshold acceptance criteria for the internal control and positive control are listed below:

Component / Parameter	Threshold Value (C_T)
Positive Control	≤35
Internal Control	≤35

Table 6: Bosphore CT/NG/MG Panel Kit v1 Acceptance Criteria

Test results should not be reported unless there is an amplification of the internal control in negative samples. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The samples that cross the threshold in FAM, Texas RED and HEX channels are displayed with their positive/negative results, samples that do not cut the threshold are displayed as "No C_T". These samples must be regarded as negative or having a bacterial load below the detection limit of the assay. For these undetectable samples, Cy5 data of the internal control should also be checked to avoid false negative results. The delayed amplification of the internal control may indicate a problem in nucleic acid extraction, PCR inhibition or application errors.

In this case, extraction and PCR should be repeated. The table below shows the possible results and their interpretation. Please note that this product only provides testing for CT/NG/MG and experimental results from the tube must be considered when providing a result for the patients, also in consideration of the patient's clinical findings and the guidelines of the relevant health authorities:

	***Chlamydia trachomatis* (FAM)**	***Neisseria gonorrhoeae* (HEX)**	***Mycoplasma genitalium* (Texas RED)**	**Internal Control (Cy5)**	**Result**
PCR Master Mix	+	-	-	+/-	Sample is *C. trachomatis* positive
	-	+	-	+/-	Sample is *N. gonorrhoeae* positive
	-	-	+	+/-	Sample is *M. genitalium* positive
	-	-	-	+	Sample is negative
	+	+	-	+/-	Sample is *C. trachomatis* and *N. gonorrhoeae* positive
	-	+	+	+/-	Sample is *N. gonorrhoeae* and *M. genitalium* positive
	+	-	+	+/-	Sample is *C. trachomatis* and *M. genitalium* positive
	+	+	+	+/-	Sample is *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* positive
	-	-	-	-	Test should be repeated!

Table 7: Bosphore CT/NG/MG Panel Kit v1 Results Interpretation

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend repeating the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 with dH_2O.

Montania 4896 Real-Time PCR Instrument:

- **PCR Master Mix:** Cross Talk Option should be adjusted as shown below.

	Channel 1	Channel 2	Channel 3	Channel 4
Channel 1	0,00	0,50	0,00	0,00
Channel 2	0,00	0,00	0,00	0,00
Channel 3	0,00	0,00	0,00	0,00
Channel 4	0,00	0,00	0,00	0,00

Figure 11: Montania 4896 Cross Talk Option

- **LightCycler 480 Instrument II-Roche:** Please use a white plate for Roche LightCycler 480 Instrument II. "Abs Quant/Fit Points" should be chosen as the analysis type for the appropriate threshold level selection. Analysis should be performed with the 'Cycle Range' option. For LC480, a color compensation protocol must be performed (Bosphore Color Compensation Set B or Set D).
- **CFX96-Biorad:** Use of a white plate and turning "Apply Fluorescence Drift Correction" on in "Baseline Setting" for the analysis is recommended.
- **Q qPCR MIC qPCR Cycler:** Please use "Fluorescent Cutoff Level" up to 15% if necessary.
- **Rotorgene Q-Qiagen:** Use of "Slope Correct" and "Dynamic Tube" options for the analysis is recommended. If the sigmoidal curve does not observe, try to analyze the sample without the "Slope Correct" option. It is recommended to use the "Gain" settings as 10 for all channels. Please use outlier removal options up to 5% for Green, Yellow, Orange, and Red filters.
- **Quant Studio 5:** Make sure that the passive reference dye is set to none.

11. SPECIFICATIONS

11.1. Analytical Sensitivity/Limit of Detection

Analytical sensitivity studies were performed on three different DNA Extraction systems with swab and urine matrices:

- Unio B24/B48 Extraction Systems – Unio Bacterial DNA Extraction Kit (Anatolia Geneworks) with 600 µL starting volume and 100 µL elution volume.
- Unio 96 Extraction & PCR Setup System – Unio 96 Nucleic Acid Extraction Versatile Kit (Anatolia Geneworks) with 200 µL starting volume and 60 µL elution volume.
- Bosphore STD EX-Tract Kit (Anatolia Geneworks)

Experiments were conducted in 24 replicates for each pathogen. AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) / (Vircell, Spain), AMPLIRUN TOTAL CT/NG CONTROL (URINE) / (Vircell, Spain) and AMPLIRUN TOTAL MYCOPLASMA GENITALIUM CONTROL (URINE) / (Vircell, Spain) were used as reference material and dilutions was performed according to their bacterial load/concentration.

The analytical sensitivity for nucleic acid amplification assays is expressed by the 95% positive cut-off value. This is the analyte concentration where 95% of test runs give positive results following serial dilutions of international reference material.

The LOD is the lowest analyte concentration that can be reliably detected in 95% of samples tested in a regular laboratory setting using the convenient sample matrix. Biostat Pro v 7.6.5.0 probit program was used to calculate the sensitivity levels and the LOD 95% was determined.

LOD levels of Bosphore CT/NG/MG Panel Kit v1 were determined on the Montania 4896 Real-Time PCR Instrument then LOD levels were confirmed with 5 different Real-Time PCR platforms which are Rotor-Gene Q, LightCycler 480 Instrument II, CFX96 Real-Time PCR Detection System, QuantStudio 5 Real-Time PCR Instrument and Q Real-Time PCR Cycler.

Extraction System/Kit	Sample Type		LOD Level (Obtained with Montania 4896)	Real-Time PCR Platforms Detection Rate				
				CFX96	LightCycler 480 II	RotorGene Q	Q Qpcr	Quant Studio 5
Unio B24/B48 Extraction Systems and Unio Bacterial DNA Extraction Kit	Swab Sample	CT	174 cop/mL	>95%	100%	>95%	100%	>95%
		NG	256 cop/mL	100%	>95%	>95%	>95%	100%
		MG	467 cop/mL	100%	100%	>95%	>95%	100%
	Urine Sample	CT	676 cop/mL	100%	100%	100%	100%	100%
		NG	227 cop/mL	>95%	100%	100%	100%	100%
		MG	533 cop/mL	100%	100%	100%	100%	100%
Unio 96 Extraction & PCR Setup System and Unio 96 Nucleic Acid Extraction Versatile Kit	Swab Sample	CT	291 cop/mL	>95%	100%	100%	100%	>95%
		NG	229 cop/mL	100%	100%	100%	>95%	100%
		MG	458 cop/mL	100%	100%	100%	>95%	100%
	Urine Sample	CT	382.5 cop/mL	100%	100%	>95%	100%	>95%
		NG	184 cop/mL	>100%	>95%	100%	100%	>95%
		MG	303.5 cop/mL	>95%	>95%	>95%	100%	>95%
Bosphore STD EX-Tract Kit	Swab Sample	CT	700 cop/mL	100%	>95%	100%	>95%	100%
		NG	121.4 cop/mL	>95%	>95%	100%	100%	100%
		MG	384.2 cop/mL	100%	>95%	>95%	100%	>95%
	Urine Sample	CT	531.5 cop/mL	100%	100%	100%	100%	100%
		NG	1,040 cop/mL	100%	100%	100%	100%	100%
		MG	1,625 cop/mL	100%	100%	100%	100%	100%

Table 8: LOD levels were determined on Montania 4896 Real-Time PCR Instrument and then confirmed with 5 different Real-Time PCR platforms.

11.2. Analytical Specificity

11.2.1. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were utilized. The primer and probe sequences were evaluated for possible homology to other known pathogen sequences through sequence comparison analysis using a database alignment. To mitigate the risk of cross-reactions, the kit was tested for cross-reactivity for pathogens using highly positive samples and found to be negative.

Experimental results obtained by testing these known samples at various concentrations on all systems demonstrated that the kit specifically and exclusively detects the intended pathogens it is designed for, without detecting others.

Sample	Sample Source	Concentration	Bosphore CT/NG/MG Panel Kit v1 Result
Legionella pneumophila	Vircell	800 copies/µL	**Negative**
Mycobacterium avium	Vircell	850 copies/µL	**Negative**
Mycoplasma pneumoniae	Vircell	750 copies/µL	**Negative**
Haemophilus ducreyi	Vircell	700 copies/µL	**Negative**
Haemophilus influenzae type b	Vircell	850 copies/µL	**Negative**
Streptococcus agalactiae	Vircell	750 copies/µL	**Negative**
Streptococcus pneumoniae	Vircell	900 copies/µL	**Negative**
Bordetella pertussis	Vircell	850 copies/µL	**Negative**
Neisseria meningitidis	Vircell	760 copies/µL	**Negative**
Salmonella enterica	Vircell	700 copies/µL	**Negative**
Ureaplasma urealyticum	Vircell	800 copies/µL	**Negative**
Treponema pallidum	ATCC	$5x10^3$ copies/µL	**Negative**
Gardnerella vaginalis	ATCC	$6x10^4$ copies/µL	**Negative**
HSV-1	Vircell	1,000 copies/µL	**Negative**
HSV-2	Vircell	1,000 copies/µL	**Negative**
Human Papilloma Virus	WHO	$1x10^3$ IU/mL	**Negative**
Mycoplasma hominis	Vircell	1,000 copies/µL	**Negative**
Shigella flexneri	Vircell	1,000 copies/µL	**Negative**
Trichomonas vaginalis	Vircell	1,000 copies/µL	**Negative**
Candida albicans	Vircell	1,000 copies/µL	**Negative**
Campylobacter jejuni	Vircell	1,000 copies/µL	**Negative**
Human Immunodeficiency Virus-1	WHO	10,000 IU/mL	**Negative**
Human Immunodeficiency Virus-2	ATCC	1,000 copies/µL	**Negative**

Hepatitis B virus	WHO	10,000 IU/mL	**Negative**
Giardia lamblia	*Zeptometrix	*Sample declared as High Positive	**Negative**
Entamoeba histolytica	ATCC	$4.3x10^3$ Genome copies/µL	**Negative**
Ureaplasma parvum	Vircell	1,000 copies/µL	1,000 copies/µL
Negative swab (Genital Swab)	-	-	-
Negative urine	-	-	-

Table 9: Wet lab results of cross-reactivity experiments

The in-silico analysis for this Real-Time PCR kit involves assessing the cross-reaction risk of the primers and probe sequences included in the assay. Cross-reaction risk refers to the potential formation of non-specific PCR products when both the primers and the probe sequence included in the assay exhibit 80% or more homology to non-targeted sequences that are in close proximity (within 500 base pairs) and have the correct orientation (opposite strands).

During the in-silico analysis, the data were analyzed by comparing the primer and probe sequences to various organisms through blast analyses. The main objective was to control the percentage of identical base numbers for each primer and probe. If more than 80% of identities were detected, further examination of the identities was conducted, including orientation, and base position. This detailed analysis was performed to determine the possibility of a non-specific PCR product formation.

The general criterion for cross-reaction risk assessment is based on the presence of both the primers and the probe sequences that exhibit 80% or higher homology to non-targeted sequences in close proximity (below 500 base pairs) and on opposite strands. The identified risk indicates the potential for the formation of non-specific PCR products.

Please refer to the following tables for the in silico analysis results.

Microorganism	TAXID ID	Target gene	Cross-reaction risk
Mycoplasma genitalium	2,097	*Porin*	No cross-reactivity was predicted
Metamycoplasma hominis	2,098	*Porin*	No cross-reactivity was predicted
Chlamydia trachomatis	813	*Porin*	No cross-reactivity was predicted
Ureaplasma urealyticum	2,130	*Porin*	No cross-reactivity was predicted
Treponema pallidum	160	*Porin*	No cross-reactivity was predicted
Haemophilus ducreyi	730	*Porin*	No cross-reactivity was predicted
Calymmatobacterium granulomatis	39,824	*Porin*	No cross-reactivity was predicted
Shigella	620	*Porin*	No cross-reactivity was predicted
Campylobacter	194	*Porin*	No cross-reactivity was predicted

HIV-1	11,676	*Porin*	No cross-reactivity was predicted
HIV-2	11,709	*Porin*	No cross-reactivity was predicted
Herpesviridae	10,292	*Porin*	No cross-reactivity was predicted
Papillomaviridae	151,340	*Porin*	No cross-reactivity was predicted
Hepatovirus	12,091	*Porin*	No cross-reactivity was predicted
Cytomegalovirus	10,358	*Porin*	No cross-reactivity was predicted
Molluscum contagiosum virus	10,279	*Porin*	No cross-reactivity was predicted
Deltaretrovirus	153,136	*Porin*	No cross-reactivity was predicted
Trichomonas vaginalis	5,722	*Porin*	No cross-reactivity was predicted
Entamoeba histolytica	5,759	*Porin*	No cross-reactivity was predicted
Giardia lamblia	5,741	*Porin*	No cross-reactivity was predicted
Candida albicans	5,476	*Porin*	No cross-reactivity was predicted
Phthirus pubis	121,228	*Porin*	No cross-reactivity was predicted
Sarcoptes scabiei	52,283	*Porin*	No cross-reactivity was predicted
Group B *Streptococcus*	1,319	*Porin*	No cross-reactivity was predicted
Ureaplasma parvum	134,821	*Porin*	No cross-reactivity was predicted
Gardnerella vaginalis	2,702	*Porin*	No cross-reactivity was predicted
Bacteria	2	*Porin*	No cross-reactivity was predicted
Fungi	4,751	*Porin*	No cross-reactivity was predicted
Viruses	10,239	*Porin*	No cross-reactivity was predicted
Homo sapiens	9,606	*Porin*	No cross-reactivity was predicted

Table 10: In silico analysis of *Neisseria gonorrhoeae* for exclusivity including major and essential STD pathogens

Microorganism	TAXID ID	Target gene	Cross-reaction risk
Mycoplasma genitalium	2,097	*OmpA*	No cross-reactivity was predicted
Metamycoplasma hominis	2,098	*OmpA*	No cross-reactivity was predicted
Neisseria gonorrhoeae	485	*OmpA*	No cross-reactivity was predicted
Ureaplasma urealyticum	2,130	*OmpA*	No cross-reactivity was predicted
Treponema pallidum	160	*OmpA*	No cross-reactivity was predicted
Haemophilus ducreyi	730	*OmpA*	No cross-reactivity was predicted
Calymmatobacterium granulomatis	39,824	*OmpA*	No cross-reactivity was predicted
Shigella	620	*OmpA*	No cross-reactivity was predicted
Campylobacter	194	*OmpA*	No cross-reactivity was predicted
HIV-1	11,676	*OmpA*	No cross-reactivity was predicted
HIV-2	11,709	*OmpA*	No cross-reactivity was predicted
Herpesviridae	10,292	*OmpA*	No cross-reactivity was predicted

Papillomaviridae	151,340	*OmpA*	No cross-reactivity was predicted
Hepatovirus	12,091	*OmpA*	No cross-reactivity was predicted
Cytomegalovirus	10,358	*OmpA*	No cross-reactivity was predicted
Molluscum contagiosum virus	10,279	*OmpA*	No cross-reactivity was predicted
Deltaretrovirus	153,136	*OmpA*	No cross-reactivity was predicted
Trichomonas vaginalis	5,722	*OmpA*	No cross-reactivity was predicted
Entamoeba histolytica	5,759	*OmpA*	No cross-reactivity was predicted
Giardia lamblia	5,741	*OmpA*	No cross-reactivity was predicted
Candida albicans	5,476	*OmpA*	No cross-reactivity was predicted
Phthirus pubis	121,228	*OmpA*	No cross-reactivity was predicted
Sarcoptes scabiei	52,283	*OmpA*	No cross-reactivity was predicted
Group B *Streptococcus*	1,319	*OmpA*	No cross-reactivity was predicted
Ureaplasma parvum	134,821	*OmpA*	No cross-reactivity was predicted
Gardnerella vaginalis	2,702	*OmpA*	No cross-reactivity was predicted
Bacteria	2	*OmpA*	No cross-reactivity was predicted
Fungi	4,751	*OmpA*	No cross-reactivity was predicted
Viruses	10,239	*OmpA*	No cross-reactivity was predicted
Homo sapiens	9,606	*OmpA*	No cross-reactivity was predicted

Table 11: In silico analysis of *Chlamydia trachomatis* for exclusivity including major and essential STD pathogens

Microorganism	TAXID ID	Target gene	Cross-reaction risk
Chlamydia trachomatis	813	*mgpC*	No cross-reactivity was predicted
Metamycoplasma hominis	2,098	*mgpC*	No cross-reactivity was predicted
Neisseria gonorrhoeae	485	*mgpC*	No cross-reactivity was predicted
Ureaplasma urealyticum	2,130	*mgpC*	No cross-reactivity was predicted
Treponema pallidum	160	*mgpC*	No cross-reactivity was predicted
Haemophilus ducreyi	730	*mgpC*	No cross-reactivity was predicted
Calymmatobacterium granulomatis	39,824	*mgpC*	No cross-reactivity was predicted
Shigella	620	*mgpC*	No cross-reactivity was predicted
Campylobacter	194	*mgpC*	No cross-reactivity was predicted
HIV-1	11,676	*mgpC*	No cross-reactivity was predicted
HIV-2	11,709	*mgpC*	No cross-reactivity was predicted
Herpesviridae	10,292	*mgpC*	No cross-reactivity was predicted
Papillomaviridae	151,340	*mgpC*	No cross-reactivity was predicted
Hepatovirus	12,091	*mgpC*	No cross-reactivity was predicted
Cytomegalovirus	10,358	*mgpC*	No cross-reactivity was predicted

Molluscum contagiosum virus	10,279	*mgpC*	No cross-reactivity was predicted
Deltaretrovirus	153,136	*mgpC*	No cross-reactivity was predicted
Trichomonas vaginalis	5,722	*mgpC*	No cross-reactivity was predicted
Entamoeba histolytica	5,759	*mgpC*	No cross-reactivity was predicted
Giardia lamblia	5,741	*mgpC*	No cross-reactivity was predicted
Candida albicans	5,476	*mgpC*	No cross-reactivity was predicted
Phthirus pubis	121,228	*mgpC*	No cross-reactivity was predicted
Sarcoptes scabiei	52,283	*mgpC*	No cross-reactivity was predicted
Group B *Streptococcus*	1,319	*mgpC*	No cross-reactivity was predicted
Ureaplasma parvum	134,821	*mgpC*	No cross-reactivity was predicted
Gardnerella vaginalis	2,702	*mgpC*	No cross-reactivity was predicted
Bacteria	2	*mgpC*	No cross-reactivity was predicted
Fungi	4,751	*mgpC*	No cross-reactivity was predicted
Viruses	10,239	*mgpC*	No cross-reactivity was predicted
Homo sapiens	9,606	*mgpC*	No cross-reactivity was predicted

Table 12: In silico analysis of *Mycoplasma genitalium* for exclusivity including major and essential STD pathogens

Microorganism	TAXID ID	Target gene	Cross-reaction risk
Chlamydia trachomatis	813	synthetic DNA	No cross-reactivity was predicted
Mycoplasma genitalium	2,097	synthetic DNA	No cross-reactivity was predicted
Metamycoplasma hominis	2,098	synthetic DNA	No cross-reactivity was predicted
Neisseria gonorrhoeae	485	synthetic DNA	No cross-reactivity was predicted
Ureaplasma urealyticum	2,130	synthetic DNA	No cross-reactivity was predicted
Treponema pallidum	160	synthetic DNA	No cross-reactivity was predicted
Haemophilus ducreyi	730	synthetic DNA	No cross-reactivity was predicted
Calymmatobacterium granulomatis	39,824	synthetic DNA	No cross-reactivity was predicted
Shigella	620	synthetic DNA	No cross-reactivity was predicted
Campylobacter	194	synthetic DNA	No cross-reactivity was predicted
HIV-1	11,676	synthetic DNA	No cross-reactivity was predicted
HIV-2	11,709	synthetic DNA	No cross-reactivity was predicted
Herpesviridae	10,292	synthetic DNA	No cross-reactivity was predicted
Papillomaviridae	151,340	synthetic DNA	No cross-reactivity was predicted
Hepatovirus	12,091	synthetic DNA	No cross-reactivity was predicted
Cytomegalovirus	10,358	synthetic DNA	No cross-reactivity was predicted
Molluscum contagiosum virus	10,279	synthetic DNA	No cross-reactivity was predicted
Deltaretrovirus	153,136	synthetic DNA	No cross-reactivity was predicted

Trichomonas vaginalis	5,722	synthetic DNA	No cross-reactivity was predicted
Entamoeba histolytica	5,759	synthetic DNA	No cross-reactivity was predicted
Giardia lamblia	5,741	synthetic DNA	No cross-reactivity was predicted
Candida albicans	5,476	synthetic DNA	No cross-reactivity was predicted
Phthirus pubis	121,228	synthetic DNA	No cross-reactivity was predicted
Sarcoptes scabiei	52,283	synthetic DNA	No cross-reactivity was predicted
Group B *Streptococcus*	1,319	synthetic DNA	No cross-reactivity was predicted
Ureaplasma parvum	134,821	synthetic DNA	No cross-reactivity was predicted
Gardnerella vaginalis	2,702	synthetic DNA	No cross-reactivity was predicted
Bacteria	2	synthetic DNA	No cross-reactivity was predicted
Fungi	4,751	synthetic DNA	No cross-reactivity was predicted
Viruses	10,239	synthetic DNA	No cross-reactivity was predicted
Homo sapiens	9,606	synthetic DNA	No cross-reactivity was predicted

Table 13: In silico analysis of Internal Control for exclusivity including major and essential STD pathogens

11.2.2. Interferences

Potential interferences on Bosphore CT/NG/MG Panel Kit v1 were tested with endogenous or exogenous substances that may be present in swabs and/or urine samples.

Negative and 3xLOD samples for urine and swab samples containing each interfering substance were tested in 5 replicates. DNA extraction was performed by Unio B24 Extraction System with Unio Bacterial DNA Extraction Kit with 600 µL starting volume and 100 µL elution volume. All interfering substances studies showed that no interference was detected in specified materials and concentrations, listed below:

		For Negative Samples	**For Positive Samples (3xLOD)**	**Results**
Substance	**Concentrations**	**Tested Negative**	**Tested Positive**	
Whole blood	2.5, 5, and 10 % v/v	5/5	5/5	No interference was detected
Urea	1, 2, and 4% v/v	5/5	5/5	No interference was detected
Antibiotic (Amoxicillin trihydrate and *clavulanic acid)* *	0.5, 1, and 2 mg/mL	5/5	5/5	No interference was detected
Antiviral (Oseltamivir)	1, 2, and 4 mg/mL	5/5	5/5	No interference was detected

Painkiller (Paracetamol)	0.5, 1, and 2 mg/mL	5/5	5/5	No interference was detected
Base pool for urine specimen (pH4)	-	5/5	5/5	No interference was detected
Base pool for urine specimen (pH9)	-	5/5	5/5	No interference was detected
Alcohol	1, 2, and 4 % v/v	5/5	5/5	No interference was detected

Table 14: *In vitro* analysis: potential interfering substances spiked into negative and positive CT/NG/MG clinical urine sample base pools

		For Negative Samples	For Positive Samples (3xLOD)	Results
Substance	**Concentrations**	**Tested Negative**	**Tested Positive**	
Whole blood	2.5, 5, and 10 % v/v	5/5	5/5	No interference was detected
Antibiotic (Amoxicillin trihydrate and clavulanic acid)	0.5, 1, and 2 mg/mL	5/5	5/5	No interference was detected
Antiviral (Oseltamivir)	1, 2, and 4 mg/mL	5/5	5/5	No interference was detected
Painkiller (Paracetamol)	0.5, 1, and 2 mg/mL	5/5	5/5	No interference was detected
Base pool for swab specimen (pH4)	-	5/5	5/5	No interference was detected
Base pool for swab specimen (pH9)	-	5/5	5/5	No interference was detected
Hygiene product	0.05, 0.1, and 0.2 % v/v	5/5	5/5	No interference was detected

Table 15: *In vitro* analysis: potential interfering substances spiked into negative and positive CT/NG/MG clinical swab sample base pools

However, it is important to note that this evaluation was limited to the substances listed in the tables above. Other substances or conditions not tested in this study may potentially interfere with the assay. If you suspect the presence of any such substances or conditions in your swab or urine samples, it is recommended to consult with a healthcare professional or contact our technical support team for further guidance.

11.3. Measuring Range

The measuring range of this assay has been established through experimental procedures involving plasmids, as it was not feasible to obtain a sufficient number of high positive clinical samples.

A standard curve was generated from diluted reference material (AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) (Ref.: MBTC024-R) with a known concentration and the samples containing serial dilutions of plasmids were quantified. The measurement range was determined based on the highest and lowest concentrations observed.

The measuring range of Bosphore CT/NG/MG Panel Kit v1 has been determined by evaluating the correlation coefficient and slope value of the standard curves. The acceptance criteria were met for both parameters, indicating the reliability of the results obtained from negative samples containing plasmids. The measuring range for the FAM channel, specifically for *Chlamydia trachomatis*, was found to be between $1.36x10^9$ copies/mL and $1.2x10^2$ copies/mL.

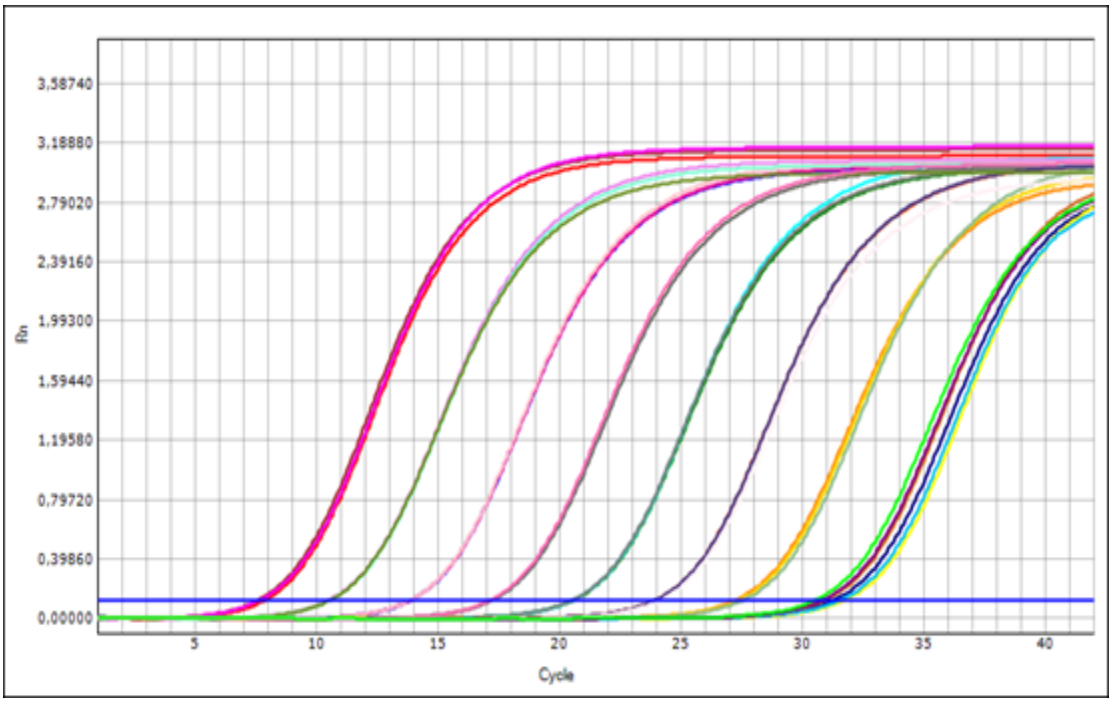

Figure 12: Measuring range amplification curves of *Chlamydia trachomatis* containing serial dilutions of the sample

The measuring range for the HEX channel, targeting *Neisseria gonorrhoeae*, was determined to be between $3.26x10^9$ copies/mL and $1.2x10^2$ copies/mL.

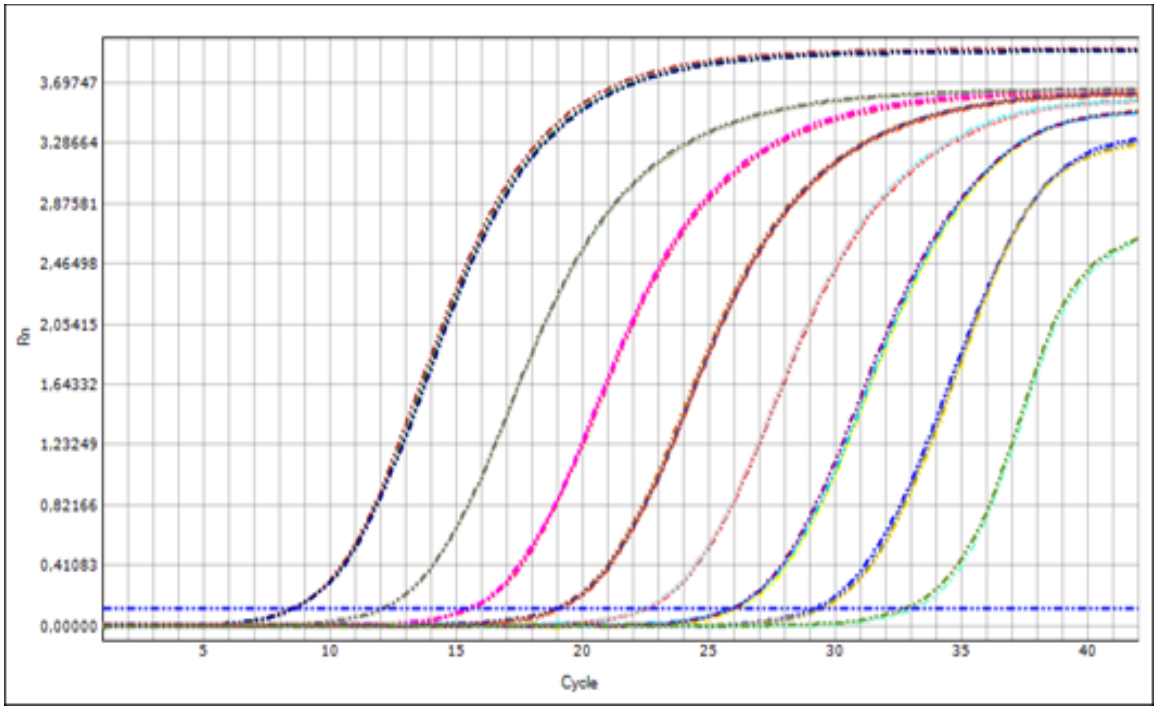

Figure 13: Measuring range of amplification curves of *Neisseria gonorrhoeae* containing serial dilutions of the sample

For the Texas RED channel, designed for *Mycoplasma genitalium* detection, the measuring range was established between $2.01x10^{10}$ copies/mL and $2.85x10^{2}$ copies/mL.

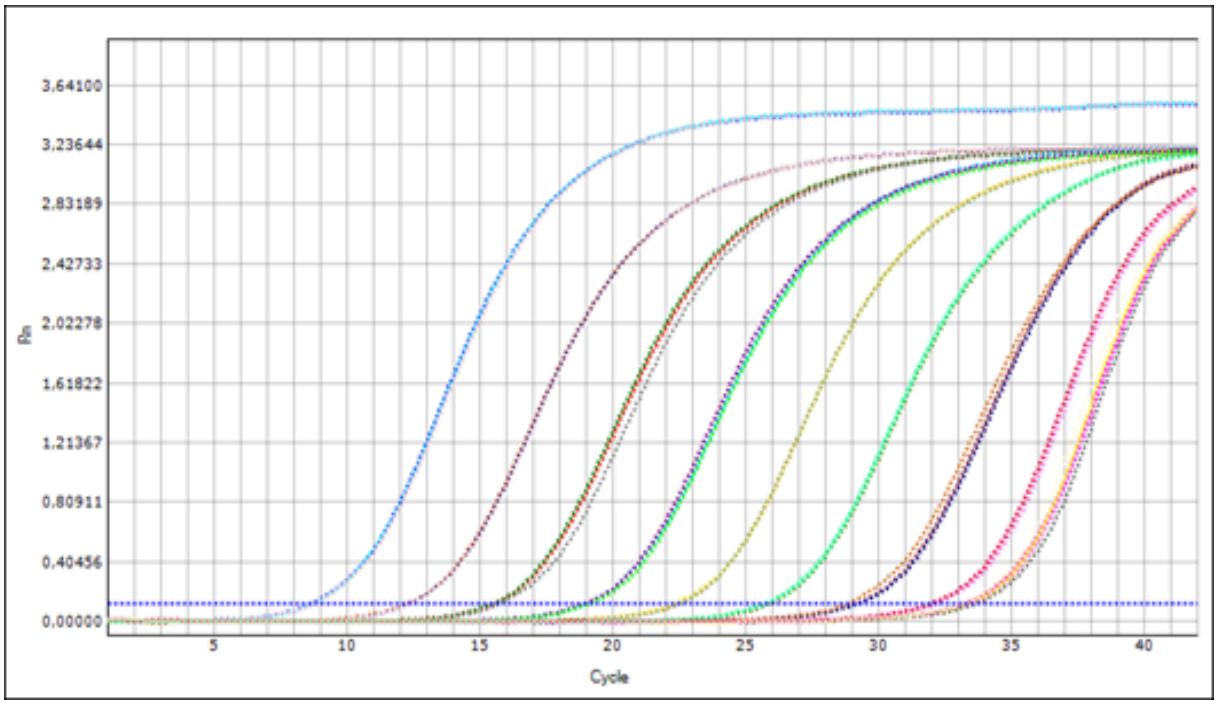

Figure 14: Measuring range of amplification curves of *Mycoplasma genitalium* containing serial dilutions of the sample

11.4. Accuracy

11.4.1. Precision Study

Reproducibility data for the precision study was obtained by analyzing diluted CT/NG/MG positive clinical samples at expected LOD levels (based on C_T values). The study was performed by two different operators, in three different settings, over five different days, with the use of two different lots and five replicates. Additionally, samples at three different concentrations (3xLOD, 100xLOD and High Positive) were used. The experimental design is presented in the table below. Unio B24 Extraction System and Unio Bacterial DNA Extraction Kit (with an initial volume of 600 and an elution volume of 100) were used for extraction, followed by the Montania 4896 Real-Time PCR Instrument.

	Site 1 (Anatolia R&D Laboratory)						**Site 2 (Anatolia Production Laboratory)**						**Site 3 (Anatolia Free Zone Branch Laboratory)**					
	Instrument 1						**Instrument 2**						**Instrument 3**					
	Lot 1/Lot 2						**Lot 1/Lot 2**						**Lot 1/Lot 2**					
	Operator 1			**Operator 2**			**Operator 1**			**Operator 2**			**Operator 1**			**Operator 2**		
Replicates	**Day 1**	**Day 3**	**Day 5**	**Day 2**	**Day 4**	**Day 6**	**Day 1**	**Day 3**	**Day 5**	**Day 2**	**Day 4**	**Day 6**	**Day 1**	**Day 3**	**Day 5**	**Day 2**	**Day 4**	**Day 6**
	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Table 16: Precision Experiments Plans

The PCR results (C_T Values) have been grouped separately for each channel:

- FAM - *Chlamydia trachomatis*
- HEX - *Neisseria gonorrhoeae*
- Texas RED - *Mycoplasma genitalium*

Within each channel, the three different concentrations (3xLOD, 100xLOD and High Positive) have been evaluated individually.

After the grouping, statistical calculations have been performed. Calculations were made for each target within the concentrations divided into sub-groups. The means, standard deviations, variances and coefficient of variations have been calculated for statistical analysis using "Analyse-it (Method Validation Edition)- version 6.15.4".

Repeatability and reproducibility results are presented on the next page:

	Specimen Type		Sample Concentration	Mean Value	N	Within Run		Between Run		Between Operator		Within Laboratory		Between Laboratory		Reproducibility	
						SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%
FAM CHANNEL	SWAB	Lot 1	3xLOD	30,252	90	0,462	1,5	0,268	0,9	0,0	0,0	0,534	1,8	0,13	0,4	0,55	1,8
			100XLOD	25,373	90	0,132	0,5	0,097	0,4	0,0	0,0	0,164	0,6	0,0	0,0	0,164	0,6
			High Positive	19,098	90	0,099	0,5	0,103	0,5	0,087	0,5	0168	0,9	0,046	0,2	0,174	0,9
		Lot 2	3xLOD	30,225	90	0,389	1,3	0,225	0,7	0,074	0,2	0,456	1,5	0,0	0,0	0,456	1,5
			100XLOD	25,311	90	0,096	0,4	0,036	0,1	0,023	0,1	0,105	0,4	0,042	0,2	0,113	0,4
			High Positive	19,069	90	0,062	0,3	0,167	0,9	0,0	0,0	0,179	0,9	0,093	0,5	0,201	1,1
	URINE	Lot 1	3xLOD	31,82	90	0,69	2,2	0,24	0,8	0,05	0,3	0,73	2,3	0,19	0,6	0,76	2,4
			100XLOD	26,83	90	0,29	1,1	0,18	0,7	0,0	0,0	0,34	1,3	0,0	0,0	0,34	1,3
			High Positive	19,29	90	0,10	0,5	0,15	0,8	0,0	0,0	0,18	0,9	0,03	0,2	0,18	0,9
		Lot 2	3xLOD	31,804	90	0,752	2,4	0,39	1,2	0,0	0,0	0,847	2,7	0,098	0,3	0,853	2,7
			100XLOD	26,815	90	0,157	0,6	0,077	0,3	0,0	0,0	0,175	0,7	0,0	0,0	0,175	0,7
			High Positive	19,259	90	0,078	0,4	0,075	0,4	0,0	0,0	0,109	0,6	0,0	0,0	0,109	0,6

Table 17: Calculations and comparisons of C_T values obtained for different matrices and different concentrations in the FAM - *Chlamydia trachomatis* Channel for Lot 1 and Lot 2

	Specimen Type		Sample Concentration	Mean Value	N	Within Run		Between Run		Between Operator		Within Laboratory		Between Laboratory		Reproducibility	
						SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%
HEX CHANNEL	SWAB	Lot 1	3xLOD	31,259	90	0,458	1,5	0,342	1,1	0,0	0,0	0,572	1,8	0,162	0,5	0,594	1,9
			100XLOD	26,386	90	0,202	0,8	0,156	0,6	0,0	0,0	0,255	1,0	0,135	0,5	0,289	1,1
			High Positive	20,663	90	0,171	0,8	0,218	1,1	0,0	0,0	0,277	1,3	0,069	0,3	0,286	0,3
		Lot 2	3xLOD	31,199	90	0,418	1,3	0,053	0,2	0,158	0,5	0,450	1,4	1,137	0,4	0,470	1,5
			100XLOD	26,294	90	0,172	0,7	0,027	0,1	0,0	0,0	0,174	0,7	0,116	0,4	0,209	0,8
			High Positive	20,620	90	0,141	0,7	0,178	0,9	0,080	0,4	0,241	1,2	0,0	0,0	0,241	1,2
	URINE	Lot 1	3xLOD	32,58	90	0,78	2,4	0,0	0,0	0,18	0,5	0,80	2,5	0,38	1,2	0,89	2,7
			100XLOD	27,37	90	0,48	1,8	0,22	0,8	0,0	0,0	0,53	1,9	0,07	0,3	0,53	1,9
			High Positive	20,82	90	0,25	1,2	0,24	1,1	0,0	0,0	0,35	1,7	0,11	0,5	0,36	1,7
		Lot 2	3xLOD	32,464	90	0,537	1,7	0,27	0,8	0,0	0,0	0,601	1,9	0,161	0,5	0,622	1,9
			100XLOD	27,319	90	0,143	0,5	0,059	0,2	0,0	0,0	0,155	0,6	0,088	0,3	0,178	0,7
			High Positive	20,765	90	0,153	0,7	0,205	1,0	0,0	0,0	0,256	1,2	0,051	0,2	0,261	1,3

Table 18: Calculations and comparisons of C_T values obtained for different matrices and different concentrations in the HEX – *Neisseria gonorrhoeae* channel for Lot 1 and Lot 2

	Specimen Type		Sample Concentration	Mean Value	N	Within Run		Between Run		Between Operator		Within Laboratory		Between Laboratory		Reproducibility	
						SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%
Texas RED CHANNEL	SWAB	Lot 1	3xLOD	32,973	90	0,778	2,4	0,322	1,0	0,0	0,0	0,842	2,6	0,197	0,6	0,865	2,6
			100XLOD	27,812	90	0,178	0,6	0,194	0,7	0,097	0,3	0,280	1,0	0,125	0,5	0,307	1,1
			High Positive	20,327	90	0,094	0,5	0,197	1,0	0,198	1,0	0,294	1,4	0,0	0,0	0,294	1,4
		Lot 2	3xLOD	32,591	90	0,716	2,2	0,337	1,0	0,149	0,5	0,805	2,5	0,0	0,0	0,805	2,5
			100XLOD	27,726	90	0,155	0,6	0,141	0,5	0,0	0,0	0,210	0,8	0,187	0,7	0,281	1,0
			High Positive	20,300	90	0,171	0,8	0,196	1,0	0,0	0,0	0,260	1,3	0,073	0,4	0,27	1,3
	URINE	Lot 1	3xLOD	31,79	90	0,51	1,6	0,30	1,0	0,0	0,0	0,59	1,9	0,20	0,6	0,63	2,0
			100XLOD	26,80	90	0,13	0,5	0,20	0,7	0,0	0,0	0,24	0,9	0,15	0,6	0,28	1,1
			High Positive	20,44	90	0,12	0,6	0,17	0,8	0,13	0,7	0,25	1,2	0,0	0,0	0,25	1,2
		Lot 2	3xLOD	31,672	90	0,623	2,0	0,323	1,0	0,0	0,0	0,701	2,2	0,181	0,6	0,724	2,3
			100XLOD	26,733	90	0,120	0,5	0,152	0,6	0,0	0,0	0,194	0,7	0,032	0,1	0,197	0,7
			High Positive	20,479	90	0,088	0,4	0,172	0,8	0,052	0,3	0,2	1,0	0,0	0,0	0,2	1,0

Table 19: Calculations and comparisons of C_T values obtained for different matrices and different concentrations in the Texas RED - *Mycoplasma genitalium* channel for Lot 1 and Lot 2

			Repeatability		Between Lot		Reproducibility	
			SD	CV%	SD	CV%	SD	CV%
SWAB	FAM (CT)	3xLOD	0.358	1.2	0.112	0.4	0.375	1.3
		100xLOD	0.086	0.3	0.061	0.2	0.105	0.4
		High Positive	0.072	0.4	0.1	0.5	0.123	0.6
	HEX (NG)	3xLOD	0.355	1.2	0.0	0.0	0.355	1.2
		100xLOD	0.14	0.5	0.0	0.0	0.14	0.5
		High Positive	0.135	0.7	0.0	0.0	0.135	0.7
	T.RED (MG)	3xLOD	0.842	2.5	1.113	3.4	1.396	4.2
		100xLOD	0.135	0.5	0.0	0.0	0.133	0.5
		High Positive	0.084	0.4	0.129	0.7	0.154	0.8
			Repeatability		Between Lot		Reproducibility	
			SD	CV%	SD	CV%	SD	CV%
URINE	FAM (CT)	3xLOD	0.733	2.3	0.265	0.8	0.779	2.4
		100xLOD	0.150	0.6	0.069	0.3	0.165	0.6
		High Positive	0.095	0.5	0.025	0.1	0.099	0.5
	HEX (NG)	3xLOD	0.48	1.5	0.0	0.0	0.48	1.5
		100xLOD	0.085	0.3	0.087	0.3	0.121	0.4
		High Positive	0.075	0.4	0.045	0.2	0.088	0.4
	T.RED (MG)	3xLOD	0.538	1.7	0.0	0.0	0.538	1.7
		100xLOD	0.121	0.5	0.0	0.0	0.121	0.5
		High Positive	0.058	0.3	0.008	0.0	0.058	0.3

Table 20: Comparison between Lot 1 and Lot 2 for different matrices and different concentration

In experiments, a precision study performed by 2 different operators, on 3 different sites, on 5 different days, using 2 different lots, in 5 replicates, it was observed that there was no significant difference (≤5% differences) among those using the same concentration of samples.

The "0.0" results given in the tables above are the results that the "Analyse-it (Method Validation Edition)" application used when performing the statistical calculations found "not significant.

11.4.2. Trueness

In the trueness study, a reference sample named AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) (Ref.: MBTC024-R) was prepared in three different concentrations. Linear analysis was performed on these dilutions and testing was carried out on two separate days, with each day consisting of 20 replicates.

For the extraction process, Unio B24 Extraction System and Unio Bacterial DNA Extraction Kit were employed. The starting volume was 600 µL and elution was performed using 100 µL.

Subsequently, the Montania 4896 Real-Time PCR Instrument was used for the PCR analysis. The obtained results were evaluated in relation to one another.

During the trueness study, it is crucial to compare the results only within the concentration range to which they belong. Additionally, in the 20-repeat experiment, it is important for all replicates to yield positive results. The different testing days and concentrations were considered independent parameters to ensure accurate assessment.

			Mean	Standard Deviation	CV%
Day 1	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:2 Dilution	**FAM (CT)**	26.026	0.060033	0.230667
		HEX (NG)	27.452	0.113428	0.413188
		T.RED (MG)	29.765	0.182058	0.61165
	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:8 Dilution	**FAM (CT)**	27.865	0.110295	0.395819
		HEX (NG)	29.352	0.220082	0.749802
		T.RED (MG)	31.548	0.305411	0.968084
	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:32 Dilution	**FAM (CT)**	29.918	0.189278	0.632655
		HEX (NG)	31.4965	0.308728	0.980197
		T.RED (MG)	33.6365	0.711184	2.114322

Table 21: Day 1: Statistical analysis of C_T values obtained from different concentrations of AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R)

			Mean	Standard Deviation	CV%
Day 2	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:2 Dilution	**FAM (CT)**	25.998	0.07724	0.297099
		HEX (NG)	27.451	0.120744	0.439851
		T.RED (MG)	29.6995	0.177129	0.596405
	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:8 Dilution	**FAM (CT)**	27.9635	0.14914	0.533338
		HEX (NG)	29.382	0.190226	0.647424
		T.RED (MG)	31.549	0.378456	1.199582
	AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R) 1:32 Dilution	**FAM (CT)**	29.969	0.244313	0.81522
		HEX (NG)	31.2815	0.327082	1.045609
		T.RED (MG)	33.602	0.78347	2.331619

Table 22: Day 2: Statistical analysis of C_T values obtained from different concentrations of AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R)

The trueness study, conducted over two days and involving 20 replicates for each of the three different concentrations of AMPLIRUN TOTAL CT/NG/TV/MGE CONTROL (SWAB) for CT/NG/MG bacterial DNA (Ref.: MBTC024-R), yielded positive results for all samples, as expected.

11.5. Carry-Over

Experimental studies were employed to control the carry-over effect of the device. In order to test this parameter, one highly positive clinical sample was used initially, followed by one negative clinical sample. Unio B24 Extraction System and Unio Bacterial DNA Extraction Kit, with a starting volume of 600 µL and an elution volume of 100 µL, were utilized for the extraction process. Subsequently, the Montania 4896 Real-Time PCR Instrument was employed for analysis.

In each run, 5 highly positive samples were tested, with 5 negative clinical samples interspersed between them. This approach ensured that the performance criteria were met. Specifically, the expectation was that all 5 negative samples would yield negative results in every run, without any contamination from the preceding high positive specimen. The acceptance criteria were defined as the absence of a C_T value in the negative samples. The results obtained from the experimental studies assessing the carry-over effects of Bosphore CT/NG/MG Panel Kit v1 are provided below:

Sample	Run 1	Run 2	Run 3	Run 4	Run 5
High positive CT/NG/MG sample (Replicate 1)	Positive	Positive	Positive	Positive	Positive
Negative sample (Replicate 1)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)
High positive CT/NG/MG sample (Replicate 2)	Positive	Positive	Positive	Positive	Positive
Negative sample (Replicate 2)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)
High positive CT/NG/MG sample (Replicate 3)	Positive	Positive	Positive	Positive	Positive
Negative sample (Replicate 3)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)
High positive CT/NG/MG sample (Replicate 4)	Positive	Positive	Positive	Positive	Positive
Negative sample (Replicate 4)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)
High positive CT/NG/MG sample (Replicate 5)	Positive	Positive	Positive	Positive	Positive
Negative sample (Replicate 5)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)	Negative (No C_T)

Table 23: Carry-over effects of Bosphore CT/NG/MG Panel Kit v1

Based on the data obtained, all the negative samples showed "No C_T," as expected. And it was determined that there was not any carry-over effect for all negative samples in all runs.

11.6. Real-Time Stability

According to extrapolated data and accelerated stability data, as well as real-time stability experiments, indicate that the kit's shelf life is 18 months for optimal performance.

11.7. In-Use Stability

Bosphore CT/NG/MG Panel Kit v1 has undergone comprehensive in-use stability testing to ensure reliable performance in various conditions. The stability studies followed guidelines from CLSI25A, and EN ISO 23640:2015.

To evaluate the kit's stability, experimental setups were designed to simulate real-world scenarios. Multiple freeze/thaw cycles were performed on two different concentrations (100xLOD and 3xLOD) of urine and swab samples, along with positive and negative controls. The kit was then compared to the 0th-day results. For statistical analysis, the t-test (two-sample assuming unequal variances, two-tailed) was used.

The findings indicated that Bosphore CT/NG/MG Panel Kit v1 remains stable up to three freeze/thaw cycles. So, it is recommended to limit the kit to three freeze/thaw cycles for optimal performance.

Additionally, the kit's stability during a 90-minute incubation in the cold rack of Unio 96 Extraction & PCR Setup System was evaluated. The analysis involved testing two concentrations (100xLOD and 3xLOD) of urine and swab samples, comparing them to a reference kit. Statistical analysis demonstrated that the kit remained stable during the cold rack incubation ($p>0.05$).

These in-use stability studies assure users that Bosphore CT/NG/MG Panel Kit v1 maintains its performance, ensuring accurate and reliable results for Real-Time PCR applications.

11.8. Shipping Stability

This section provides instructions for evaluating the shipping stability (transport stability) of Bosphore CT/NG/MG Panel Kit v1 through experimental studies conducted on the Montania 4896 Real-Time PCR Instrument (Anatolia Geneworks). The purpose of this evaluation is to determine if the kit's performance is affected by transportation conditions.

To assess the shipping chain, the kit was shipped via air freight under routine shipping conditions and returned to the headquarters. This process allowed us to test the stability of the device during transportation.

According to the statistical data obtained, it was determined that there was no statistically significant difference between all matrices and all concentrations in the tested device before and after shipping. As a result, the Bosphore CT/NG/MG Panel Kit v1 Real-Time PCR Kit is stable, according to statistical data ($p > 0.05$), despite being transported, spending time on dry ice while being transported, and even time lost when returning.

Transport stability has been validated with 25 kg of dry ice, 2 cold chain gel packs and 1 thermal indicator for up to 6 days. The transportation box has to be made of styrofoam with the specifications; outer part measurement of 675x520x300 mm, inside measurement of 575x420x200 mm, wall thickness 50 mm, density 30 kg/m^3. The cold chain gel packs properties must have a weight of 500±50 grams and must be filled with cold chain gel. Thermal indicator circle must be white after transportation. Finally, the styrofoam box and the lid indentations have to be neatly aligned and tightly closed with pressing from the top and sealed with a duct tape with a size of 45 mm x 100 m, sealing around the lid with at least 3 rounds. The styrofoam box containing the Real Time PCR kit, of the specified dimensions, must not be opened at any stage of transportation and must contain dry ice when it received. Also, if any of the stated conditions are altered, the transport stability study must be repeated. Based on these findings, the validated transportation temperature range must be between -20°C and -90°C.

11.9. Metrological Traceability

Metrological traceability of the positive control of Bosphore CT/NG/MG Panel Kit v1 is calibrated in terms of concentration/C_T value against the Amplirun Total CT/NG/TV/MGE Control (SWAB) DNA.

Experimental results proved that the concentration of the positive control for *C. trachomatis* is 3.598x10^6 copies/mL and this corresponds to a mean C_T value of 20.6 (According to the Montania 4896 Real-Time PCR Instrument).

Experimental results proved that the concentration of the positive control for *N. gonorrhoeae* is 1.134x10^{6}copies/mL and this corresponds to a mean C_T value of 21.96 (According to the Montania 4896 Real-Time PCR Instrument).

Experimental results proved that the concentration of the positive control for *M. genitalium* is 2.467x10^6 copies/mL and this corresponds to a mean C_T value of 20.63 (According to the Montania 4896 Real-Time PCR Instrument).

12. TECHNICAL ASSISTANCE

For any technical assistance or inquiries regarding your Real-Time PCR kit, we are here to help you. We understand that there might be situations where you require expert guidance and support. Whether you have questions about kit usage, troubleshooting, or data interpretation, our dedicated team of technical experts is available to assist you.

To reach our technical support team, please use the contact information in Section 16.

When contacting us, please provide detailed information about your specific query or concern. This will enable us to provide you with the most accurate and efficient assistance. We kindly request that you have the following information ready:

Experimental Details: Describe your experimental setup, including the sample type, target gene, reaction conditions and any specific issues you are encountering. The more information you can provide, the better we can understand your need and offer the correct support.

Instrumentation and Software: If applicable, please mention the Real-Time PCR instrument and software you are using. This information will allow us to provide guidance specific to your instrument's capabilities and features.

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14. SYMBOLS

	Manufacturer		This product is in compliance with relevant EU requirements
	*In vitro* Diagnostic Device		Unique Device Identifier
	Catalog Number		Batch Number
	Serial Number		Consult (Electronic) Instructions for Use http://www.anatoliageneworks.com/support
	Expiry Date		Temperature Limit
	Caution		Note

15. ORDERING INFORMATION

Catalog Number:

ABMNC3 (100 rxn/box)
ABMNC2 (50 rxn/box)
ABMNC1 (25 rxn/box)

16. CONTACT INFORMATION

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27th April 2023	V1	Published Document	Ömer Alperen Arslantaş
28th May 2023	V2	Formal review and minor edits	Merve Ölçen Erdem
31th October 2023	V3	Online Link for Summary of Safety and Performance Added	Merve Ölçen Erdem
03rd November 2023	V4	Correction of Information Regarding Endocervical Sample Use	Ömer Alperen Arslantaş
22nd March 2024	V5	Transport/Shipping stability, Real-time stability, Specimen stability information update and minor edits	Ömer Alperen Arslantaş
08th May 2024	V6	Section 5.1.1, 11.5, and 14 have revised.	Ömer Alperen Arslantaş