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EliGene® STD NEI/TRI RT

REF 90090-RT **(for 50 samples)**

Kit components:

5 x 150 µl **STD 2 Mix**
2 x 50 µl **PC DNA STD 2**
5 x 200 µl **IAC DNA**
Instruction for Use

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® STD NEI/TRI RT kit is intended for DNA diagnostic of pathogenic species *Neisseria gonorrhoeae* and *Trichomonas vaginalis* from isolated DNA sample.

Principle of the method

This diagnostic kit is based on RealTime PCR method. In this kit primers and dual labeled fluorescent probes for the detection of DNA are used.

Channel	HEX	Texas Red	Cy5
DNA target	IAC	Trichomonas	Neisseria

Introduction

Neisseria gonorrhoeae *(gonococci)* is gram-negative, non-motile and non-sporulated diplococcic. Of the eleven species of *Neisseria* colonizing humans, only two are pathogens, *N. gonorrhoeae* and *N. meningitidis. N. gonorrhoeae* is the causative agent of sexual transmitted disease gonorrhea which is the third most common sexual disease, worldwide is registered about 62.2 million cases of gonorrhea. In males and females the gonorrhea is clinically manifested as urethritis with a purulent (or pus-like) discharge from the genitals, in females also as cervicitis. Complication can be infection in pelvic region leading up to infertility in females. The classical penicillin family of antibiotics is relatively well effective in therapy, but the number of resistance strains is increasing. Also the simultaneous infection by *Chlamydia* infection is common. For DNA diagnostic purposes the urine, urethral swab or vaginal swab are recommended.

Trichomonas vaginalis is an anaerobic parasitic protozoan, that causes trichomoniasis, a sexually transmitted disease. There are about 30 million infections in sub-Saharan Africa each year. Based on a systematic review of articles published in recent years, estimated prevalence of Trichomonas vaginalis in Europe is 0.2 % in men and 1.6 % in women. The infection is asymptomatic in about 50 % of women. However, when symptoms appear, women suffer for vaginal discharge, pruritus or dysuria, vaginitis and cervicitis. In men, symptoms include urethritis, prostatitis and decreased fertility. For DNA diagnostic purposes the urine, urethral swab or vaginal swab are recommended.

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Primary sample collection, handling and storage

Clinical material:	Recommended DNA isolation procedure:
Urine, swabs	Manual: EliGene® Urine Isolation Kit
	Automatic: **ZEPHYRUS Magneto** (ELISABETH PHARMACON)

WARNING: To keep the sensitivity of the test we recommend to strictly follow the pre-analytical procedures mentioned in this instruction. Specially do not change the recommended procedures of isolation as for stated amount of sample, centrifugation force, etc. For the DNA isolation other isolation kits can be used, but the pre-analytical procedures of storage, transport and centrifugation must be held.

Cervical swabs, urethral swabs:

These samples should be collected according to standard protocol in collection tubes with transport medium (remel MicroTest M4RT Transport or remel MicroTest M4 Transport). Samples in this transport medium should be transported to the laboratory at 4 °C. Samples can be stored at 4 °C up to seven days.

Recommended procedure for swab specimens processing:

1. Vortex well the collection tubes for at least 10 seconds before the DNA isolation. Open the tube and by pressing the swab along the tubes wall press out all solution from the swab. Discard the swab.
2. Centrifuge the collection tubes for 15 minutes at 6000 x g. If there is not centrifuge for collection tubes, split the medium into two 1.5 ml microtubes and centrifuge. Microtubes can be centrifuged 10 minutes at 10000 x g.

Manual isolation:

1. Aspirate supernatant and add 200 µl of MI3 solution from EliGene® Urine Isolation Kit and 180 µl of molecular grade water to the pellet and re-suspend the pellet by pipetting (if you used for the centrifugation two microtubes firstly re-suspend the pellet in one tube with total volume of buffer and consequently in other microtubes with the buffer mixture from the first one). Add 10 µl of Proteinase K and 20 µl of Internal Control (IAC DNA) and continue according to instructions in standard protocol of EliGene® Urine Isolation Kit.
2. Use 50 µl of Elution buffer. Use isolated DNA for the detection immediately after DNA isolation or store DNA hours to one week at 4 °C. For longer period than one week freeze DNA at -20 °C.

Automatic isolation:

1. Open the tubes and carefully aspirate all supernatant. Then re-suspend pellets in 180 µl of PBS by vortexing and add 20 µl of Internal Control (IAC DNA).
2. Isolate DNA from the sample by using MAGNETO BodyFluid DNA/RNA isolation kit according to protocol for Plasma samples with Elution to 50 µl of Elution buffer.

Urine:

According to standard protocol, take the samples of the urine into sterile tube. Use sterile tubes without conservation additives. Urine samples must be stored and transported at 4 °C. It is possible to store the samples at 4 °C up to 7 days.

The sample of urine must be centrifuged at 6000 x g for 15 minutes before the DNA isolation. Use 8–15 ml of urine sample. **Do not use lower centrifugation force or shorter time of centrifugation!** In the case that laboratory do not have centrifuge for large volumes, aliquot urine sample to lower volumes but total sample volume must be at least 8 ml.

Manual isolation:

1. Aspirate supernatant. Take care; all urine must be aspirated before the re-suspending! Resuspend pellet in 180 µl of molecular grade water with 200 µl of MI3 solution from EliGene® Urine Isolation Kit, add 10 µl of Proteinase K and 20 µl of Internal Control (IAC DNA). Vortex for 15 seconds. Continue according to instructions in standard protocol in EliGene® Urine Isolation Kit.
2. Use 100 µl of Elution buffer. Isolated DNA use for the detection immediately after DNA isolation or store DNA hours to one week at 4 °C. For longer period than one week freeze DNA at -20 °C.

Automatic isolation:

1. Open the tubes and carefully aspirate all supernatant. Re-suspend pellets in 180 µl of PBS buffer by vortexing and add 20 µl of Internal Control (IAC DNA).

   WARNING: The rest of urine in the tube can influence the purity of isolated DNA.
2. Isolate DNA from the sample by using MAGNETO BodyFluid DNA/RNA isolation kit according to protocol for Plasma samples with elution to 50 µl of Elution buffer.

Additional required equipment

- Automatic pipette 5–20 µl and sterile tips with filter DNA-, RNA- free, DNase-, RNase- free (we recommend plastic with CE certificate for diagnostic purposes).
- Sterile stand DNA-, RNA- free, DNase-, RNase- free.
- Equipment for RealTime PCR – the kit is designed for Quant Studio 5 (Thermofisher Scientific), CFX96 Touch Real-Time PCR Detection System (Bio-Rad), MIC qPCR Cycler (Bio Molecular Systems).
- Sterile plastic (strips, plates, tubes) DNase-, RNase- free compatible with given RealTime PCR system.
- Lab safety gloves

Configuration of Real Time instrument

Follow the cycler manufacturer's manual when using the kit. Below is a list of cyclers that have been used in testing EliGene® STD NEI/TRI RT kit.

- For *Trichomonas vaginalis* detection the probe labeled with TexasRed is used (exc.589 nm – em. 615 nm)
- For *Neisseria gonorrhoeae* detection the probe labeled with Cy5 is used (exc. 650 nm – em. 670 nm)
- For Internal Control detection the probe labeled with HEX is used (exc. 520 nm – em. 548 nm)

QuantStudio 5 (ThermoFisher Scientific):

Select the options Experiment type, “Presence/Absence”, Chemistry “TaqMan Probes” and Run Mode “Standard”. As reporter dyes use VIC/HEX (IAC DNA), ROX (Trichomonas), Cy5 (Neisseria) and select NONE as the passive reference dye.

Set up the following temperature profile:

Holding stage

95°C	2 min	Ramp rate (1.6°C/s)

Cycling stage- 45 Cycles

95°C	10 s	Ramp rate (1.6°C/s)

55°C	20 s	Ramp rate (1.6°C/s)	Data collection ON
67°C	30 s	Ramp rate (1.6°C/s)	

Post-Read Stage

40°C	1 min	Ramp rate (1.6°C/s)

Collect emission signal at the cycling stage at 55 °C.

The complete temperature profile can be up-loaded from Run Template "EliGene _STD_QS5_v00.edt". The Run Template can be imported to the software from the CD included in the kit.

CFX96 Touch (Bio-Rad):

Select the option "Startup Wizard", set up "New Experiment" and create new protocol by selecting "Create New Protocol".

Set up the following temperature profile:

Step 1	95°C	2 min
Step 2	95°C	10 s
Step 3	55°C	20 s + Plate Read
Step 4	67°C	30 s
Step 5	*GOTO Step 2*	*44x*
Step 6	40°C	60 s

Set "Sample volume" at 20 µl.

Collect emission signal at the Step 3 at 55° C.

For filter settings use the "Scan Mode" All Channels but in Plate Manager select for the samples only fluorophores HEX, TxRed, Cy5. Then assign the samples with positions as an "Unknown" sample or "Standard". The complete temperature profile can be up-loaded from Run Template "EliGene_STD_CFX96_v00.pcrl". The Run Template can be imported from the CD included in the kit.

MIC qPCR (Bio Molecular Systems Pty Ltd):

In Run Setup Create a new Run Profile for the MIC instrument. Enter the Sample Volume of 20 µl and Temperature Control "Fast TAQ (v3)".

Set up the following temperature profile:

Holding stage

95°C	2 min

Cycling stage – 45 cycles

95°C	10 s	
55°C	20 s	Acquire on "Yellow", "Orange", "Red"
67°C	30 s	

Holding stage

40°C	60 s

The complete temperature profile can be up-loaded from Run Template "EliGene_STD_MIC_v00.mictemplate". The Run Template can be copied from the CD included in the kit.

Reagent preparation

- To avoid the contamination keep all tubes closed and follow the instructions.
- Before the usage, all reagents must be completely thawed, briefly mixed on vortex and shortly spun.
- In the step of Proteinase K addition of Isolation protocol add 20 µl of Internal Control (IAC DNA) to isolated sample. In no case add the internal control to isolated DNA just before the analysis.
- **If you do not use all the volume of MasterMix, store the tube at dark at temperature 4°C up to 14 days. For long-term storage use the freezer (-20 °C, dark). MasterMix should not go through more than five freeze- thaw cycles.**

Preparation of Reaction Mix for the instruments Quant Studio 5, CFX96 and MIC qPCR

1. Detection: Take one microtube with STD 2 Mix and after the thawing pipette 15 µl of the mix to amplification microtube or plate and add 5 µl of isolated DNA. During the pipetting of samples be careful to avoid cross-contamination of samples.
2. Positive control: Take one microtube with STD 2 Mix and after the thawing pipette 15 µl of mix to amplification microtube or plate and add 5 µl of PC DNA STD 2. During the pipetting of positive control be careful to avoid contamination of other samples. Use separate pipette for positive controls!

Insert the microtubes or plate with samples to the RealTime PCR instrument and run the program according to chapter “Configuration of Real Time instrument” above.

Result reading

QuantStudio 5 (ThermoFisher Scientific):

In “**Analyse Settings**” edit the original parameters for Ct for individual channels (VIC, ROX, Cy5). Enter the Threshold level for each channel to a value equal to **1/3 of the maximal intensity of positive control**, select “Automatic Baseline” option and analyze results by selecting “**Apply**”.

Positive result for *Trichomonas vaginalis*: The positive result is characterized by the growth of fluorescence signal in ROX channel. In a case of negative results, the amplification will not occur.

Positive result for *Neisseria gonorrhoeae*: The positive result is characterized by the growth of fluorescence signal in Cy5 channel. In a case of negative results, the amplification will not occur.

The Internal Control (IAC DNA) must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in VIC (HEX) channel.

CFX96 Touch Real-Time PCR Detection System (Bio-Rad):

In Data Analysis window choose “Quantification”. In “Settings” menu choose option “Baseline Setting” and select “Baseline Subtracted Curve Fit” option and “Apply Fluorescence Drift Correction” option.

In Data Analysis window select a single fluorophore (HEX, TxRed, Cy5) by the clicking the box next to the fluorophore name located under the amplification chart and read the results for individual samples.

Positive result for *Trichomonas vaginalis*: The positive result is characterized by the growth of fluorescence signal in TexasRed channel (em. 615 nm). In a case of negative results, the amplification will not occur.

Positive result for *Neisseria gonorrhoeae*: The positive result is characterized by the growth of fluorescence signal in Cy5 channel (em. 670 nm). In a case of negative results, the amplification will not occur.

The Internal Control (IAC DNA) must be amplified in each sample. The Internal Control amplification is

characterized by the growth of fluorescence signal in HEX channel (em. 548 nm).

MIC qPCR (Bio Molecular Systems):

To view and analyze acquired data, click the small cross next to Cycling under Analysis module and then select the target – Non-Assay Yellow (HEX), Non-Assay Orange (TxRed) and Non-Assay Red (Cy5). In the down right corner **increase** "**Fluorescence Cut-off Level**" **to 20 %**. A quantification cycle (Cq) value is displayed for each positive well. This is the cycle number at which the sample fluorescence rises above the threshold (the red horizontal line near the bottom of the amplification curve chart). In the Samples panel on the right, click sample names to hide or display the associated amplification curves and result data.

Positive result for *Trichomonas vaginalis*: The positive result is characterized by the growth of fluorescence signal in Orange channel. In a case of negative results, the amplification will not occur.

Positive result for *Neisseria gonorrhoeae*: The positive result is characterized by the growth of fluorescence signal in Red channel. In a case of negative results, the amplification will not occur.

The Internal Control (IAC DNA) must be amplified in each sample. The Internal Control amplification is characterized by the growth of fluorescence signal in Yellow channel.

Interpretation of results

Negative result:

If the increasing of amplification signal in channel TxRed or Cy5 does not appear before cycle number 40, the result of test should be interpreted as probably *Neisseria gonorrhoeae* and *Trichomonas vaginalis* DNA negative or with concentration of DNA below the detection limit of this kit (10 genomic DNA/reaction). The signal for Internal Control must be positive. This result does not exclude the occurrence of the above-mentioned pathogens and possible infection because results of this test are dependent on proper sample collection and elaboration. Results are also dependent on enough quantity of analyzed DNA.

Positive result:

Amplification signal in TexasRed channel (589-615 nm) for STD 2 Mix appears before cycle number 40. *Trichomonas vaginalis* DNA was detected in the sample. The sample is *Trichomonas vaginalis* DNA positive.

Amplification signal in Cy5 channel (650-670 nm) for STD 2 Mix appears before cycle number 40. *Neisseria gonorrhoeae* DNA was detected in the sample. The sample is *Neisseria gonorrhoeae* DNA positive.

WARNING: The contamination in laboratory space is also possible. Use separate pipette for MasterMixes, separate pipette for positive controls and separate pipette for samples. Follow all recommendations for laboratories of DNA analyses.

Inhibited sample:

In the case that increasing of amplification signal specific for DNA of the above-mentioned pathogens and also increasing of amplification signal specific for internal control in HEX channel is not observed, it is necessary to repeat the analysis. The best, there is to use DNA samples prepared by new DNA isolation.

Control procedure

EliGene® STD NEI/TRI RT involves Internal Control (IAC DNA) and Positive Control (PC DNA STD 2). Internal control isolation follows the quality of DNA isolation and detects mistakes in the isolation process. It detects the occurrence of an inhibition of amplification process. In the case that the sample is negative, the Cp of internal

control must be Cp < 35.

Positive control follows the proper function of MasterMix. Minimal Cp of positive control must be 35 or less. The Cp higher than 35 for positive control can't be accepted and DNA detection must be repeated with new sample. In the case of repeatedly higher Cp contact manufacturer ELISABETH PHARMACON.

Use negative control for each run. As negative control use the water for molecular biology used in your laboratory. For negative control use the pipette for DNA samples.

Reference material:

To monitor all the examination processes covering DNA isolation and RealTime PCR detection is possible to use sample positive for *Neisseria gonorrhoeae* and *Trichomonas vaginalis* DNA. The commercial positive material is not available.

Troubleshooting:

1. If there is no amplification of Internal Control, there is some problem in the isolation of DNA or the kit is after the expiration date or there is RealTime instrument breakdown.
2. If there is no amplification of Positive Control, the kit is after the expiration date or there is RealTime instrument breakdown.

Performance characteristics

Analytical performance characteristics:

Kit EliGene® STD NEI/TRI RT has a very high sensitivity – detects 10 genomic or plasmid DNA added to the amplification mix. Analytical sensitivity of the test was verified as follows. The insert with specific DNA concentration was prepared by cloning and subsequently diluted to desired concentration of target sequence. The addition of human DNA to sample in common concentration has no effect to sensitivity of the method.

Analytical sensitivity is 10 copies of *Neisseria gonorrhoeae* and *Trichomonas vaginalis* DNA in reaction mix.

Analytical specificity of method was verified by searching the DNA database NCBI (GenBank, http://www.ncbi.nlm.nih.gov/) for the sequences of primers and probes and by addition of human DNA to MasterMix. 50 different samples of human DNA did not give false positive result. False positive result was not observed after the addition of DNA from these organisms *B. burgdorferi sensu lato*, *M. tuberculosis*, *M. bovis*, *M. cansasii*, *M. xenopii*, *M. avium*, *M. marinum*, *Lactobacillus sp.*, *Enterococcus faecalis*, *Pseudomonas sp.*, *E. coli*, *A. niger*, *C. albicans*, *S. aureus*, *S. agalactiae*, *Ch. trachomatis*, *U. urealyticum, U. parvum*, HBV, EBV, CMV, HSV1, HSV2, VZV.

Clinical specificity was tested on clinical urine samples and swab samples from men and women urogenital tract. A total of 50 clinical samples (urine samples and swabs from men and women urogenital tract) were collected in the Laboratory Diagnostic Center ELISABETH PHARMACON, spol. s r.o., Rokycanova 4437/5, Brno. Of these, 8 were positive for *Neisseria gonorrhoeae* and 3 were positive for *Trichomonas vaginalis.* Compared to the reference methods, 100 % agreement was achieved in results.

Diagnostic performance characteristics:

Clinical sensitivity and specificity of the EliGene® STD NEI/TRI RT kit is 100 %.

Measuring interval

The kit enables the detection of 10^1–10^8 of *Neisseria gonorrhoeae* and *Trichomonas vaginalis* DNA molecules in reaction mix.

Internal control of quality

As an internal control of quality the Internal Control (IAC DNA) for checking the process of DNA isolation and amplification together with Positive Control for functional control of MasterMix is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with sample (isolation of DNA). It is strictly recommended to use isolation kits and procedures mentioned above.

The sensitivity of DNA detection depends on sample collection, storage (store at 4 °C) and processing methods (DNA isolation, sample collection date, detection immediately after isolation).

Biological reference intervals

Not applicable information for this kit.

Warning

After mixing, MasterMix is stable at -20 °C. Do not freeze tubes with MasterMix more than 5 times! Do not mix components of the kits of different lots.

Warnings and general precautions

- Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.
- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturer´s kit.

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar safety box. Tubes containing different samples must be never opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled under PCR box (not in laminar flow box). The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Amplification products must be handled in such way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

- The tubes containing mix (STD 2 Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- The tubes containing IAC DNA and PC DNA STD 2 are disposable and therefore must be used once only in the preparation of the reaction mixture.
- These mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.
P281 Use personal protective equipment as required.

Literature

Barbara A. Bannister, Norman T. Begg and Stephen H. Gillespie: Infectious Disease. Blackwell Science, 2th Ed., 2000

Whiley DM, Buda PP, Freeman K, Pattle NI, Bates J, Sloots TP. 2005. A real-time PCR assay for the detection of *Neisseria gonorrhoeae*in genital and extragenital specimens. Diagn Microbiol Infect Dis. 52(1):1-5

Whiley DM, Garland SM, Harnett G, Lum G, Smith DW, Tabrizi SN, Sloots TP, Tapsall JW. 2008. Exploring 'best practice' for nucleic acid detection of *Neisseria gonorrhoea*. Sex Health. 5(1):17-23.

Symbols

Catalog number


Upper limit of temperature

Batch code


Use by (last day of month)

in vitro diagnostic medical device


Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.

Contains sufficient for "N" tests


Attention, consult instructions for use


Manufacturer

Manufacturer

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