EURx® MOLECULAR BIOLOGY PRODUCTS

Duplex Mycoplasma qPCR Kit

Kit Components

Component	Cat. No. E0449-01 50 reactions of 25 µl	Cat. No. E0449-02 100 reactions of 25 µl
Duplex Mycoplasma Master Mix (2x)	2 x 325 µl	4 x 325 µl
IC-internal control	1 x 500 µl	2 x 500 µl
PC—positive control	1 x 100 µl	1 x 200 µl
Water, nuclease free	1 x 1000 µl	2 x 1000 µl

Storage

Store the components at -20°C in the dark.
Repeated thawing and freezing (> 2x) should be avoided, as this may reduce the sensitivity.

Description

- Duplex Mycoplasma qPCR Kit is a ready-to-use system for the quantitative detection of Mollicutes (Mycoplasma, Acholeplasma, Spiroplasma, Ureaplasma) DNA in cell cultures, animal tissue samples and other biological matrices.
- The kit enables the direct detection of all Mollicutes species known as contaminants of cell cultures.
- Duplex Mycoplasma qPCR Kit contains reagents and primers for the amplification of the 16S rRNA coding region in the mycoplasma genome. The detection of the mycoplasma-specific amplicon is based on using the specific FAM-labelled probe.
- In addition, the kit contains a second heterologous amplification system to monitor the DNA isolation procedure and identify possible PCR inhibition simultaneously. The internal control system consists of: primers and the HEX-labelled probe present in Duplex Mycoplasma Master Mix (2x) and exogenous DNA fragment (IC-internal control). The amplification of the inner control does not interfere with the amplification of the specific mycoplasma DNA and confirms DNA isolation and/or PCR reaction correctness.
- IC can be added at the DNA isolation stage (point a) - then it is both a control of the DNA isolation and PCR inhibition, or only at the PCR stage (point b) - to assess the quality of the purified DNA and possible PCR inhibition.

a. Add IC to the lysis buffer or to the mixture of lysis buffer and sample material (do not add to the sample material directly) at a ratio 0,1 µl per 1 µl elution volume. For example, 5 µl of IC should be added initially using 50 µl elution volume. It is recommended to use carrier RNA (cat. no. E0307) when isolating DNA from from cell free body fluids and material low in DNA/RNA content. Carrier RNA increases the efficiency of DNA binding to silica membranes in the presence of small amounts of genetic material in the sample.

b. Add 0.5 µL of IC for every 25 µL of PCR reaction.

- The specificity of Duplex Mycoplasma qPCR Kit has been confirmed by PCR using purified DNA (source: DSMZ) from: *Acholeplasma laidlawii*, *Mycoplasma pneumoniae*, *Mycoplasma arginini*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, *Mycoplasma fermentans*, *Spiroplasma citri*.
- The kit meets EP 2.6.7 critreria and allows detection over 100 Mollicute species.
- Duplex Mycoplasma qPCR Kit provides the efficient detection of Mollicutes with high sensitivity (LOD ≤ 10 CFU/ml or LOD ≤ 5 Mollicutes DNA copies per reaction).
- Duplex Mycoplasma Master Mix contains onTaq DNA Polymerase, optimized reaction buffer and dNTPs.
- onTaq DNA Polymerase is a modified “hot start” enzyme that is blocked at moderate temperatures and allows room temperature reaction setup.
- The polymerase activity is restored during the initial denaturation step when amplification reactions are heated at 95°C for 15 minutes.
- ROX passive reference dye included in the master mix allows fluorescence normalization on certain cyclers . The use of ROX passive reference dye is necessary for all real-time PCR cyclers from Applied Biosystems and optional for cyclers from Stratagene. ROX compensates for variations of fluorescent signal between wells due to slight differences in reaction volume and fluorescence fluctuations. ROX is not involved in PCR reaction and does not interfere with real-time PCR on any instrument.

EURx Ltd. 80-297 Gdańsk Poland ul. Przyrodników 3, NIP 957-07-05-191, KRS 0000202039
www.eurx.com.pl, orders@eurx.com.pl, *tel.* +48 58 524 06 97, *fax* +48 58 341 74 23

List of Mollicute strains detected with Duplex Mycoplasma qPCR Kit

Mycoplasma

M. agalactiae, *M. agassizii*, *M. alkalescens*, *M. alligatoris*, *M. alvi*, *M. amphoriforme*, *M. anserisalpingitidis*, *M. arginini*, *M. arthritidis*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovis*, *M. buccale*, *M. californicum*, *M. canadense*, *M. canis*, *M. capricolum*, *M. citelli*, *M. cloacale*, *M. collis*, *M. columbinum*, *M. columborale*, *M. conjunctivae*, *M. corogypsi*, *M. cottewii*, *M. cricetuli*, *M. crocodyli*, *M. cynos*, *M. dispar*, *M. edwardii*, *M. enhydrae*, *M. equirhinis*, *M. falconis*, *M. faucium*, *M. feliminutum*, *M. felis*, *M. fermentans*, *M. flocculare*, *M. gallisepticum*, *M. gateae*, *M. genitalium*, *M. gypis*, *M. hominis*, *M. hyopneumoniae*, *M. hyorhinis*, *M. hyosynoviae*, *M. imitans*, *M. iowae*, *M. leachii*, *M. lipofaciens*, *M. lipophilum*, *M. maculosa*, *M. microti*, *M. moatsii*, *M. molare*, *M. mucosicanis*, *M. muris*, *M. mustelae*, *M. mycoides*, *M. opalescens*, *M. orale*, *M. ovipneumoniae*, *M. oxoniensis*, *M. penetrans*, *M. phocae*, *M. phocicerebrale*, *M. phocidae*, *M. phocirhinis*, *M. phocoenae*, *M. phocoeninasale*, *M. pirum*, *M. pneumoniae*, *M. primatum*, *M. procyoni*, *M. pullorum*, *M. pulmonis*, *M. putrefaciens*, *M. salivarium*, *M. sp. ovine/caprine*, *M. spermatophilum*, *M. spumans*, *M. sualvi*, *M. synoviae*, *M. testudineum*, *M. testudinis*, *M. timone*, *M. tullyi*, *M. venyonii*, *M. verecundum*, *M. volis*, *M. vulturii*, *M. yeatsii*, *M. zalophi*

Ureaplasma

U. canigenitalium, *U. diversum*, *U. gallorale*, *U. parvum*, *U. urealyticum*,

Spiroplasma

S. apis, *S. citri*, *S.ixodetis*, *S*, *kunkelli*, *S. poulsonii*

Acholeplasma

DNA sample preparation

Boiling method for cell culture screening

Transfer 100 to 500 µl of the supernatant from the cell culture to a 1.5 ml reaction tube. Incubate the sample at 95°C for 10 min. Centrifuge the sample for 30 sec at max. speed. Use 2 µl of the supernatant directly for qPCR. Use of the higher volume (>2 µl) is not recommended due the probability of PCR inhibition. Extracted DNA may be stored at +4°C (up to 1 week) or at -20°C (long term storage).

DNA purification kits

To achieve the highest level of sensitivity it is recommended to use dedicated, commercially available DNA purification kits based on silica membrane or magnetic beads technologies. The high purity of nucleic acids obtained by the kits allows to use of 2-12.5 µl of DNA sample per 25 µl reaction volume.

Sample concentration step

To further enhance the sensitivity of a Mycoplasma DNA purification kit perform a sample concentration step. Transfer 1 ml of the supernatant from the cell culture to a 1.5 ml reaction tube. Centrifuge the sample at ≥ 12000 × g for 10 min to pellet mycoplasma particles. Discard the supernatant and resuspend the pellet in 200 µl Tris buffer (10 mM Tris-HCl, pH 8.0). Vortex the sample and proceed immediately with DNA extraction.

Sample stabilization

Sample stabilization is necessary if DNA purification is not performed immediately after sample collection or sample concentration step. Stabilize samples by heating (see Boiling method above). Please note that sample stabilization by heat inactivation cannot be performed before the sample concentration step.

EURx Ltd. 80-297 Gdańsk Poland ul. Przyrodników 3, NIP 957-07-05-191, KRS 0000202039
www.eurx.com.pl, orders@eurx.com.pl, *tel.* +48 58 524 06 97, *fax* +48 58 341 74 23

Protocol

Preparation of PCR Reaction:

Component	Negative Amplification Control -NAC	Negative Extraction Control-NEC	Positive Extraction Control-PEC	Sample DNA	Positive Control Mycoplasma-TPC
Duplex Mycoplasma Master Mix (2x)	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl
IC-internal control (optional)	-	-	-	0.5 µl	0.5 µl
PC-positive control	-	-	-	-	2 µl
Sample of: extracted H_2O/ extracted IC extracted DNA	- - -	2-12.5 µl - -	- 2-12.5 µl -	- - 2-12.5 µl	- - -
Water, nuclease free	12.5 µl	To 25 µl	To 25 µl	To 25 µl	To 25 µl
Total volume	25 µl	25 µl	25 µl	25-25.5 µl	25-25.5 µl

Notes:

1. A reaction volume of 25 µl should be used with most real-time cyclers. Other reaction volumes may be used if recommended for a specific instrument.
2. Thaw, gently vortex and briefly centrifuge all solutions. Keep Duplex Mycoplasma Master Mix on ice.
3. Set up PCR reactions at room temperature according to the table above.
4. Add:
   - 12.5 µl of water (negative amplification control-NAC),
   - 2-12.5 µl of water extracted with DNA purification kit (negative extraction control-NEC),
   - 2-12.5 µl of IC extracted with DNA purification kit (positive extraction control-PEC),
   - 2 µl of DNA extracted with the boiling method or 2-12.5 µl of DNA extracted with DNA purification kit (sample DNA),
   - 2 µl of PC (positive control, Mycoplasma-TPC).
5. Mycoplasma-TPC gives a signal in the FAM channel only, when no IC-internal control was added to the reaction. To obtain a signal in both the FAM and HEX channels, add 0.5 µl of IC to the reaction. Increasing the PCR reaction volume for the Mycoplasma-TPC by 0.5 µL to 25.5 µL (when preparing one reaction mix for DNA samples and controls) does not adversely affect the efficiency of the PCR reaction.
6. Centrifuge briefly to settle down the reaction components and remove bubbles. Bubbles interfere with fluorescent detection.
7. Place the samples in the cycler and start the program.
8. Perform data analysis.

Thermal Cycling Conditions:

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	15 min	1
Denaturation	95°C	10 s	40
Annealing/Extension	60°C	60 s	
Cooling	4°C	Indefinite	1

Notes:

1. Run PCR reactions using standard ramp rate (standard mode).
2. During the initial denaturation step onTaq DNA Polymerase is activated. onTaq DNA polymerase requires 15 min incubation at 95°C to restore activity.
3. Fluorescence detection should be set at the end of the extension step and executed using FAM and HEX channels. FAM channel detects Mycoplasma DNA and HEX channel detects the inner control.

EURx Ltd. 80-297 Gdańsk Poland ul. Przyrodników 3, NIP 957-07-05-191, KRS 0000202039
www.eurx.com.pl, orders@eurx.com.pl, *tel.* +48 58 524 06 97, *fax* +48 58 341 74 23

Results Interpretation:

Sample type	FAM channel	HEX channel	Result
Negative Amplification Control -NAC	-	-	correct
Negative Extraction Control-NEC	-	-	correct
Positive Extraction Control-PEC	-	+	correct
Positive Control, Mycoplasma-TPC (- IC) (+ IC)	+ +	- +	correct
1	-	+	negative Mycoplasma$^-$
2	+	+	positive Mycoplasma$^+$
3	+	-	positive Mycoplasma$^+$
4	-	-	incorrect

Notes:

1. A test can be considered valid/correct if:
- negative amplification control-NAC does not give a signal in both the FAM and HEX channels,
- negative extraction control-NEC does not give a signal in both the FAM and HEX channels,
- positive extraction control (when IC was purified according to the DNA isolation procedure) gives a signal only in the HEX channel,
- positive control, Mycoplasma-TPC gives a signal only in the FAM channel when no IC was added to the reaction or in both the FAM and HEX channels when IC was added to the reaction.

2. The sample is positive (Mycoplasma$^+$) and the test is valid if:
- the conditions of point 1 are met,
- the sample gives a signal in the FAM channel regardless of any signal in the HEX channel.

   In some circumstances, when the reaction is strongly positive (Mycoplasma$^+$), the sample may give a weak or no signal in the HEX channel. The reason for this is competition between Mycoplasma and IC-internal control DNA amplification reactions.

3. The sample is negative (Mycoplasma$^-$) and the test is valid if:
- the conditions of point 1 are met,
- the sample gives a signal only in the HEX channel.

4. The presence of a fluorescence signal in the HEX channel for a given sample indicates that the DNA isolation process and PCR reaction were correct (in the case of adding IC to the DNA isolation process), or only that the PCR reaction was not inhibited (in the case of adding IC to the PCR reaction mixture). Very high C_T values (> 34) in the HEX channel may indicate a partial inhibition of the PCR reaction. In turn, the complete absence of a signal in the HEX channel with the simultaneous lack of a signal in the FAM channel may indicate complete inhibition of the PCR reaction or failure to add DNA to the PCR reaction with the simultaneous addition of IC in the DNA isolation process. In this case, it is recommended to dilute the sample 10 times or repeat the DNA isolation and rerun the PCR reaction.
5. Lack of signal for the Mycoplasma-TPC indicates an error in PCR preparation.