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Technical Datasheet

EZdetect™ PCR Kit for *Mycoplasma* Detection

Based on 16s-23s rRNA spacer region

Product Code: CCK009

1. Introduction:

*Mycoplasma*s are the smallest known, simple prokaryotes. They constitute one of the most widely spread contaminants found in cell culture. They are virtually undetectable because they do not change physical parameters such as pH or turbidity. They also cannot be detected by microscopy due to their small size. There are over a hundred *Mycoplasma* species and their genome size varies from 0.5 - 1.3Mbp. Mycoplasma contamination has been shown to alter the growth rate of cells in culture, induce chromosomal aberrations, influence amino acid and nucleic acid metabolism and cause membrane aberrations. *Mycoplasma* detection methods include PCR, biochemical detection, microbiological cultivation on broth and agar, DNA staining using fluorophores such as DAPI or Hoechst derivatives, ELISA, immonofluorescence staining. PCR based detection of *Mycoplasma* is one of the most convenient methods as it is rapid, sensitive and can be completed within a few hours.

2. About the kit:

The EZdetect™ PCR kit has been designed for detection of *Mycoplasma* based on amplification of spacer region between 16S and 23S rRNA genomic DNA sequence. This sequence is highly conserved between various mycoplasma species. This allows for detection of commonly encountered cell culture contaminants. The detection spectrum includes *M. fermentans, M. hominis, M. neurolyticum, M. orale, M. pirum, M. pulmonis, M. hyorhinis and M. salivarium.* Eukaryotic DNA is not amplified. A successful PCR reaction is indicated by a distinct band of 350-400bp derived from the internal control DNA on the agarose gel.

3. Kit Contents:

Volumes of components provided are sufficient for 25 reactions.

Code	Description	Quantity	Storage
CCK009(A)	PCR buffer (10X)	1500µl	-30°C to -10°C
CCK009(B)	Taq polymerase (1U/µl)	3 x 10µl	-30°C to -10°C
CCK009(C)	dNTP mix (2mM)	3 x 25µl	-30°C to -10°C
CCK009(D)	Forward primer mix (10X)	100µl	-30°C to -10°C
CCK009(E)	Reverse primer mix (10X)	100µl	-30°C to -10°C
CCK009(F)	MgCl2 solution (50mM)	100µl	-30°C to -10°C
CCK009(G)	Positive DNA control	25µl	-30°C to -10°C
CCK009(H)	Water (DNAse, RNAse free)	1500µl	-30°C to -10°C

4. Materials required but not provided in the kit:

- Centrifuge
- Thermocycler
- Heating Block
- Agarose gel electrophoresis set-up
- PCR tubes
- Micropipettes and tips
- Buffers for DNA electrophoresis
- Gel loading dye and DNA staining reagents.

5. Directions for use:

Ensure that all the vials of the kit are frozen on receipt
Users are advised to review entire procedure before starting the assay

5.1 Sample collection and preparation of template DNA:

1. Prior to collecting the samples, the cell line to be tested for mycoplasma contamination should be in continuous culture for several days and without any antibiotics (even penicillin and streptomycin) or after thawing for at least 2 weeks.
2. Samples should be derived from cultures that are at least 90-100% confluent.

3. Aseptically withdraw 1ml of supernatant of adherently growing cells or of cultures with settled cell suspension.
4. Centrifuge the microfuge tube(s) at 13000rpm for 30 minutes.
5. Pipette and discard the supernatant without disturbing any pellet that may be visible at the bottom of the tube.
6. Add 50µl of the 1 X TE buffer to the pellet and vortex briefly.
7. Incubate the tubes on a heating block maintained at 95°C for 10 minutes.
8. Centrifuge the tubes briefly to pellet cell debris.
9. Use 2.5µl for the PCR reaction.
10. Store the remaining sample at -80°C for future use.

5.2 Protocol for setting up the PCR reactions:

1. The place where the DNA is extracted, the PCR reactions is set up, and the gel is run after the PCR should be separated from each other.
2. It is recommended to reserve pipettes, tips and tubes for PCR work and irradiate the pipettes frequently by ultraviolet (UV) light.
   Note: Use of sterile filter tips is preferred for addition of PCR reagents
3. Succession of PCR setup should be followed strictly.
4. Wear gloves during the entire sample preparation and PCR setup.

5.2.1 Preparation of the PCR Mix:

1. Thaw out the components of the kit.
2. Pipette out the components as per the Table 1 given below into a 1.5 - 2.0ml micro centrifuge tube.
3. Mix gently by pipetting up and down few times.

Table 1: Recommended volumes for PCR Mix

Component	Volume (in µl) for			
	1 reaction	5 reactions	10 reactions	25 reactions
Water	9	45	90	225
PCR buffer	2.5	12.5	25	62.5
$MgCl_2$ solution	2.5	12.5	25	62.5
dNTP mix	2.5	12.5	25	62.5
Forward primer mix	2.5	12.5	25	62.5
Reverse primer mix	2.5	12.5	25	62.5
Taq polymerase	1	5	10	25
Total Volume	22.5	112.5	225	562.5

**Adjust the total number of reactions needed to include the positive control and a negative control.*

5.2.2 Preparation of the final PCR reactions

1. Pipette out the components into PCR tubes as per the Table 2 given below:

Table 2: Recommended volumes for final PCR

Component	Test samples (µl)	Positive DNA sample (µl)	Negative Control (µl)
PCR Mix (from above table)	22.5	22.5	22.5
Template DNA	2.5	--	--
Positive DNA sample	--	2.5	--
Negative control (Water)	--	--	2.5
Total Volume	25.0	25.0	25.0

2. Place the tubes in the thermocycler and start the program as per the recommendations given below.

6. PCR amplification procedure:

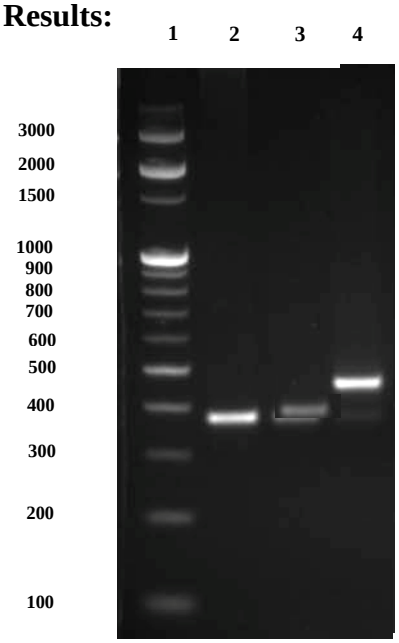
Program the thermocycler as per the PCR program given below:

Cycles	Temperature	Duration
For activation of Taq polymerase	95°C	5 minutes
For next 40 cycles	95°C	30 seconds
	55°C	30 seconds
	72°C	30 seconds
Extended elongation	72°C	8 minutes
	10°C	Until taken out from thermocycler

7. Gel electrophoresis protocol:

1. Prepare 2% agarose gel containing ethidium bromide or any other DNA stain, with the appropriate number of wells.
2. Prepare samples by adding the loading dye to the sample. Mix thoroughly.
3. Load appropriate volume of DNA marker in the first well.
4. Load the samples into the wells.
5. Electrophorese at 120V – 150V until the tracking dye migrates up to 3/4th length of the gel. Do not allow dye to run off the gel.
6. Observe the gel under the UV transilluminator.

8. Results:



1. Positive control
2. Positive sample infected with 1 species
3. Positive sample infected with 2 species
4. Negative control

Figure 1: *Gel run in 1X TAE buffer showing amplification of positive DNA (M. Hominis gDNA) and mycoplasma from infected cultures.*

9. Interpretation of the results:

1. Presence of a band of size 370-500bp in test samples along with presence of a band in positive control and absent band in negative control suggests the presence of a Mycoplasma contaminant in the culture.
2. Presence of more than one band of sizes 370-500bp in test samples along with appropriate positive and negative controls suggests the presence of more than a single Mycoplasma contaminant in the culture.
3. Presence of a positive band of suggested size does not lead to the identification of species of the contaminant. When species determination is needed, use of specific products or other methodologies is recommended.
4. In general, intensity of the band is a qualitative measure of the amount of mycoplasma contamination. The greater the intensity of the band, greater the template DNA concentration in the reaction and greater the amount of mycoplasma contaminants. This kit is however not designed to quantify the amount of mycoplasma present in the culture.
5. Positive bands should not be confused with un-incorporated primer-dimer bands which may occur below the level of the tracking dye i.e. < 100bp in size.

10. Storage and shelf life:

Store at -30ºC to -10ºC .

If stored properly, the kit is stable until the expiry period mentioned on the product label.

11. Related products:

EZKill™ Mycoplasma Elimination kit

Code No: CCK006-1KT

EZdetect™ DAPI Stain Kit for *Mycoplasma* Detection

Code No: CCK007-100NO

EZdetect™ Hoechst Stain Kit for *Mycoplasma* Detection

Code No: CCK008-100NO

EZdetect™ PCR Kit for *Mycoplasma* Detection

Based on 16s rRNA sequence

Code No: CCK022-25R

12. Troubleshooting:

Observation	Interpretation	Suggestions for resolving
No band of size 370-500bp in lane of positive control	This suggests that the PCR did not work. This may happen due to following reasons- a. Degradation of template DNA	Store the extracted DNA at -30°C to -10°C.
	b. Inadequate mixing of reaction mixture	Ensure that all components were pipetted in correct volumes for the assays and are mixed well after addition.
	c. Inappropriate storage of reagents	Ensure that the reagents supplied in the kit are stored at the correct temperature. Avoid repeated freeze-thaw cycles of reagents.
	d. Absence of $MgCl_2$ in the reaction mixture	Ensure that all the reagents are added in the reaction mixture.
Band of size 370-500bp in lane of negative control	Possible cross contamination of positive test sample or positive controls	Repeat the procedure. If the band re-appears in the negative control lane, it is possible that one or more of the reagents of the PCR reaction is cross contaminated. In such cases, it is recommended to use completely fresh reagents.
Band of size other than 370-500bp in test-sample lanes	Just an artifact of the PCR reaction which occurs sometimes	Repeat the procedure