Photometric application note
Multiskan EX

Mycoplasma PCR ELISA
Summary

Mycoplasmas are common and serious infections of animal cell cultures. A sensitive and specific detection method of Mycoplasma compromise PCR amplification of a mycoplasma-specific DNA sequence, hybridization with a biotinylated capture probe and binding of the hybrid to a streptavidin coated microplate. Detection is done in an immunoreaction using peroxidase enzyme and TMB as substrate. The hybridization and detection is a one day test, easy to perform and is well suited to be used with Thermo Labsystems microplate instrumentation.

Thermo Labsystems
products used:

- Multiskan EX, Cat.no. 51118 170
  Instead of Multiskan EX any other microplate photometer from Thermo Labsystems may be used.
- iEMS Incubator/Shaker HT
- Multiwash microplate washer.
  This washer is not anymore available, instead any 96-well microplate washer from Thermo Labsystems may be used, such as:
  - Wellwash 4 (Cat.no. 5160740)
  - Wellwash 4 Mk2 (Cat.no. 5160770)
  - Wellwash AC (Cat.no. 5161020)
- Finnpipettes + Finntips
- Finnpipette® PDP (positive displacement pipette) and Finntip® PDP
Introduction

Mycoplasma are common and serious infections of animal cell cultures (1) originating often from serum, laboratory personnel and contaminated cultures. An array of physiological and biochemical parameters are affected by the presence of mycoplasma in the cells. The infection causes changes in metabolism, growth, viability, macromolecule synthesis, morphology etc. and therefore a sensitive routine testing for contamination of cell cultures is essential.

The Mycoplasma PCR kit from Roche is based on the amplification of a mycoplasma-specific DNA sequence by PCR* and subsequent detection of the amplicon by ELISA (2-3).

The assay is performed as follows: Mycoplasmas are enriched by centrifugation and lysed. Neutralized lysate is subjected for PCR. PCR is carried out in the presence of mycoplasmal group-specific primers and digoxigenin-11-dUTP, leading to the incorporation of this label into amplified DNA. After rendering amplified DNA single-stranded by denaturation, a biotinylated capture probe is hybridized with the amplicon. The hybrid formed is immobilized on a streptavidin coated microplate. Detection is done in an immunoreaction using antidigoxigenin-antibodies conjugated to peroxidase (POD) and 3,3´,5,5´- tetramethyl benzidine (TMB) as a substrate. After substrate incubation the absorbances are measured at 450 nm and 690 nm. 690 nm is used as reference wavelength.

The specificity of the Mycoplasma PCR ELISA is very good (3). The assay detects all mycoplasma-, acholeplasma and ureaplasma species tested and DNA from other bacteria (except Clostridium spec.) yeast and eukaryotic cells is not detected. The sensitivity is excellent but varies, ranging from 1 pg. to <1 fg, depending on the mycoplasma species (3).

Reagents

- Mycoplasma PCR ELISA kit, Roche, Cat.No. 1 663 925.
- Sterile autoclaved deionized water.

Instrumentation and equipment

- PCR thermal cycler
- PCR tubes or microplates
- Positive displacement pipette, Finnpipette® PDP and Finntip® PDP or Finnpipette® and Finntip Filters
- Multiskan EX microplate photometer. Instead of Multiskan EX any other microplate photometer from Thermo Labsystems may be used.
- 96-well microplate washer from Thermo Labsystems such as: Wellwash 4 (Cat.no. 5160740) Wellwash 4 Mk2 (Cat.no. 5160770) Wellwash AC (Cat.no. 5161020).
- iEMS Incubator/Shaker HT

Preparation of samples and controls

Culture medium from animal cell lines are centrifuged at room temperature for 10 minutes at 200 x g to remove the cell debris. 1 ml of the supernatants are centrifuged for an additional 10 minutes at 4°C using 13000 x g. Thereafter the supernatants are removed carefully and 10 µl of water and 10 µl of lysis reagent are added to the “invisible” pellet. For a positive control 10 µl of control DNA and for

* PCR is a patented technology owned by Hoffmann-La Roche Inc.
negative control, 10 µl of water are transferred into tubes and 10 µl of lysis reagent is added.

Controls and samples are incubated for 1 h at +37°C, thereafter 30 µl of neutralization reagent is added.

Amplification

10 µl of the pretreated sample or control is used in a 50 µl PCR reaction containing 25 µl of the PCR ready-to-go mix and 15 µl water. Following PCR program is recommended: First 5 min. at 95°C, then 40 cycles: 30 s at 94°C, 30 s at 62°C and 1 min. at 72°C, and final extension, 10 min. at 72°C. Amplification can be carried out in various thermal cyclers but minor modifications of the program might be required.

Hybridization and detection

10 µl of the amplification product is transferred into a tube containing 40 µl of denaturation reagent and the tubes are incubated at room temperature for 10 minutes. 450 µl of hybridization reagent is added and after mixing, 200 µl of the mixture is transferred into a microplate well. The wells are covered and incubated at +37°C with shaking at 400 rpm in an iEMS Incubator/shaker for 3 hours.

After washings using a microplate washer the anti-Dig-POD conjugate is added. The plate is incubated as above for 30 min. Thereafter the wells are washed five times and 100 µl of the TMB substrate is added. After incubation at room temperature for 20 minutes with shaking at 400 rpm, the reaction is stopped using 100 µl of stop reagent. Absorbances are measured at 450 nm using 690 nm as a reference wavelength.

Interpretation of the results

The delta absorbance, ∆A, is calculated: 

\[ ∆A = A_{450} - A_{690} \]

for all controls and samples. The mean value of the controls is subtracted from all sample delta absorbances. The samples are regarded as mycoplasm-contaminated if the difference in absorbances is higher than 0.2.

For the assay to be valid, the \[ ∆A = A_{450} - A_{690} \] absorbance of the negative control should be lower than 0.25 units and ∆A of the positive control higher than 1.25.

Results from a test experiment

The tests were performed according to the instructions of the kit. 1 ml aliquots of supernatants from various animal (e.g. mice, monkey and human) cell cultures, grown without antibiotics, were pretreated as described above. 2-10 µl of the positive control DNA was added to some of the tubes prior to the lysis buffer.

PCR was performed using 0.5 ml PCR tubes with an oil layer. After PCR 5 µl of the mixture was subjected to 1.8 % Methaphor agarose gel electrophoresis. Gel electrophoresis with ethidium bromide label revealed that samples with positive control DNA contained one amplified PCR fragment.

Hybridizations and detection were performed in triplicates using 10 µl or 2.5 µl of the amplified PCR product as a target. Results were obtained by measuring the absorbances at 450 nm and 690 nm using Multiskan. Mean absorbances are shown in Table 1.
Table 1. Mean absorbances obtained using the Mycoplasma PCR ELISA kit for various cell lines and Multiskan microplate reader

<table>
<thead>
<tr>
<th></th>
<th>A 450 nm</th>
<th>A 690 nm</th>
<th>∆A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.080</td>
<td>0.035</td>
<td>0.045</td>
</tr>
<tr>
<td>Positive control:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in PCR: 10 µl</td>
<td>3.06*</td>
<td>0.040</td>
<td>&gt;3</td>
</tr>
<tr>
<td>in PCR: 2 µl</td>
<td>3.18*</td>
<td>0.038</td>
<td>&gt;3</td>
</tr>
<tr>
<td>+ and only 2.5 µl</td>
<td>2.82*</td>
<td>0.036</td>
<td>&gt;2.8</td>
</tr>
<tr>
<td>in hybridization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 10 µl positive control</td>
<td>0.084</td>
<td>0.036</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>3.42*</td>
<td>0.037</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Mice cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 2 µl positive control</td>
<td>0.080</td>
<td>0.036</td>
<td>0.044</td>
</tr>
<tr>
<td>+ and only 2.5µl</td>
<td>3.12*</td>
<td>0.037</td>
<td>&gt;3</td>
</tr>
<tr>
<td>in hybridization</td>
<td>2.71*</td>
<td>0.035</td>
<td>&gt;2.6</td>
</tr>
<tr>
<td>Human cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 2 µl positive control</td>
<td>0.130</td>
<td>0.037</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>2.98*</td>
<td>0.041</td>
<td>&gt;2.9</td>
</tr>
</tbody>
</table>

* Sample is positive but the absorbance is too high to be accurate.
Conclusion

The absorbances obtained using positive control DNA of the kit are very high. Using only one forth of recommended amount of the amplified product in the hybridization reaction gave still high signals. The absorbances from positive control were not affected by possible cell components in the sample and different cell lines gave similar results. The hybridization and detection is a one day test, easy to perform and suits well to be used together with Thermo Labsystems microplate instruments.

References


Contact Information

Thermo Labsystems Oy
Sorvaajankatu 15,
P.O. Box 208
FIN-00811 Helsinki, Finland

Tel. +358-9-329 100
Fax. +358-9-3291 0415
www.labsystems.fi