Protocol for mycoplasma detection in JCRB Cell Bank

Translation and modification by Hideyuki Tanabe

1. Vero-Hoechst method: using indicator cells detected by staining with fluorescent dye
   (Indirect method: refer to slide-show)

1. Preparations
   The indicator cells,\(^1\) Vero (JCRB0111; African green monkey, kidney epithelial-like cells), are inoculated into the tissue culture chamber slide (Lab-Tek Chamber Slide; NUNC; Catalogue No. 177437; 4 separate chamber) one day before starting the detection experiment. We recommend to put 1ml of cell suspension with \(10^4\) Vero cells/ml to each chamber. If this type of chamber slide can not be available promptly, instead you can use the normal glass slides that are placed on the bottom of culture dish when you start the culture. However, in this case you should use 3 or 4 slides per sample including the positive and/or negative controls.

2. Culture
   The next day you check that Vero cells were attached on the chamber slide, and then remove the supernatant of 100-200 ul from each chamber. After that samples of cell suspension\(^2\) are applied to each chamber with the same amount of removed medium (100-200 ul; we apply 2 or 3 volumes from one sample, e.g. 100, 150, and 200 ul). Culture the samples on the chamber slide for 5 to 7 days in 5% CO\(_2\) incubator at 37C.

3. Fixation and Staining
   1. After the culture, discard the supernatant from each chamber.
   2. Wash 2 times with PBS (-).
   3. Fixative: ethanol : acetic acid = 3 : 1 (should be freshly prepared and chilled) is diluted in 1/3 with PBS (-) and cells are fixed for 15 min at room temperature.
   4. Discard the diluted fixative and apply undiluted fixative to each chamber for 5 min.
   5. Discard the fixative and wash 3 times with PBS (-).
   6. Stain with Hoechst33258 (or DAPI) (0.5 ug/ml in PBS (-)\(^3\)) in 1 ml for 10 min.
   7. Discard the staining solution and wash 3 times with PBS (-).
   8. Remove the frame of the chamber and dry thoroughly.
   9. Drop the mounting medium\(^4\) and cover with the coverglass.
   10. Observe the samples under the fluorescent microscope throughby the equipment of
UV filter cube. Take photographs at several magnifications.

11. Example of the result. This case showed LoVo cells were contaminated by a kind of mycoplasma species. The negative control sample cells showed non-contaminated LoVo cells derived from the different lot (Comparison between both of them).

Notes

*1) Vero, CKT-1, or 3T6-swiss albino cells have been used for the indicator cells. After thawing the indicator cells, the available passage number is up to about 50 passages and the viability should be more than 80% when the cells are inoculated into the chamber slide. It is necessary to freeze several stock ampules of indicator cells in order to occasionally test mycoplasma contamination.

*2) Cell suspension: Sample cells were cultured in the medium without antibiotics at least for 3 passages, i) Floating cells are collected with medium after cultured for 7-10 days and adjust the cell density about $10^6$ cells/ml, ii) Attached cells are collected with medium by the cell-scraper without using trypsin. The supernatant without cells can be used as test samples, but this is less sensitive.

*3) Staining solution: Prepare 100-1000 times concentrated solution into brown bottle, distribute them to the small tubes, and freeze for the stock solution. The working (diluted) solution should be freshly prepared and once diluted, discard the remained of the solution.

*4) Mounting medium: Prepare the mixture with 0.1M citric acid (22.2ml) + 0.2M Na$_2$HPO$_4$ (27.8ml) + glycerol (50ml), and adjust pH5.5. Store at –20C.

Supplements

1. By this method 100-200ul of sample medium was examined, thus you should note that in principle the positive detection is possible only in case there are more than one individual mycoplasma in 100-200 ul sample. It is seldom that the living mycoplasma can grow with 100% rate, so the limit of detection will be lower than the above. Besides, if the contained number of mycoplasma is less than those of them, it is better to centrifuge the sample to enrich the mycoplasma individuals and then it can be applied to the indicator cells. Recently developed method by means of PCR has already become a practical use, so far use together in JCRB Cell Bank.

2. It is relatively difficult to eliminate mycoplasma completely from the culture by applying the general antibiotics, but MC210 provided quite effective results. However, no matter how you have the negative result once by the method above, it does not always show the complete elimination and repeated tests are needed.
2. Mycoplasma detection by PCR method
   (Direct method)

**General matter**

To confirm the detection sensitively, the supernatant of the chamber slide by Vero-Hoechst method should be started as the first material of test samples which are suspected to contain mycoplasmas that were amplified by co-culturing with Vero. The DNA was extracted from the supernatant of each sample. After two step PCR-amplification of mycoplasma DNA, detection was done by the gel electrophoresis. The PCR primers were designed to cover the consensus sequences of which are detectable every kind of mycoplasma species. It is possible to detect the mycoplasma species by the length of amplified DNA or by its restriction enzyme digested pattern. In case of highly contamination with mycoplasma, PCR method is in vain and the result of positive detection can be easily obtained by merely Hoechst33258 staining. However, to confirm the judgement of results precisely, both methods of Vero-Hoechst staining and PCR have been carried out routinely in the JCRB Cell Bank.

In this test we use the products of Perkin Elmer company for the PCR experiment.

**Experiment**

1. **Preparation of the template DNA**

   **A. From cultured supernatant (in case out of cells)**

   1. The sample aliquots (600ul / sample) were obtained from the supernatant of which the sample cells were co-cultured on Vero cells in the chamber. Practically, we use a 1.5ml eppendorf tube per one sample.
   2. Add same amount of TE-saturated phenol (600ul) to each eppendorf tube and mix vigorously by vortex mixer for several seconds.
   3. Centrifuge at 15000rpm for 5 min at room temperature.
   4. Transfer the supernatant of 400ul to the new eppendorf tube and add 10ul of 3M sodium acetate.
   5. Mix well and spin down the aliquots in the tubes.
   6. Add 2.5 times volume of absolute ethanol (1ml), mix well, and stay at –80C for 15 min.
   7. Centrifuge at 15000rpm for 10 min at 4C.
   8. Discard the supernatant by micropipet and rinse with 80% ethanol.
   9. Centrifuge at 15000rpm for 10 min at 4C.
   10. Discard the supernatant by micropipet completely and air-dried.
11. Dissolve in DDW 40ul by vortex vigourously and use this as a template DNA for
1st PCR.

**Note:** If you know that the sample was highly contaminated by mycoplasma, you can
use the supernatant from cultured sample cells directly to PCR reaction mixture.
In this case 3-5ul of the supernatant will be applicable to the reaction mixture of
1st PCR without all the above procedures and also without phenol treatment.

**B. From frozen ampule of sample cells**
1. Thaw the frozen ampule at room temperature.
2. Open the ampule and take 600ul of cell suspension to the eppendorf tube.
3. Add same amount of TE-saturated phenol (600ul) to each eppendorf tube and
   mix vigourously by vortex mixer for several seconds. From this step, perform all
   the same procedures described in A. 2-11.

   In this protocol the final 40ul dissolved solution becomes little bit viscously
because of containing much amount of genomic DNA of the sample cells. Therefore,
vortex mixing is needed for longer time.

**2. 1st PCR**

<table>
<thead>
<tr>
<th>Reaction mixture per sample (per one tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
</tr>
<tr>
<td>Stock solution A</td>
</tr>
<tr>
<td>Template DNA</td>
</tr>
<tr>
<td>Ampli. Taq Gold [5U/ul]</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

1. Beforehand we make “Stock solution A’ and “Master mixture A’ for 10 tubes as
follows. Distribute **45ul** of “Master mixture A” to each tube.

<table>
<thead>
<tr>
<th>Stock solution A (15 ul for 1 sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xPCR Buffer II *1)</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
</tr>
<tr>
<td>dNTPs (each 2.5mM)</td>
</tr>
<tr>
<td>Primer F1 (10pmol/ul) *2)</td>
</tr>
<tr>
<td>Primer R1 (10pmol/ul) *2)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
Master mixture A (45ul per one tube)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>312.4 ul</td>
</tr>
<tr>
<td>Stock solution A</td>
<td>157.5 ul</td>
</tr>
<tr>
<td>Ampli. Taq Gold</td>
<td>2.6 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>472.5 ul for 10 samples</strong></td>
</tr>
</tbody>
</table>

2. Add **5.0ul** of template DNA, mix well, and spin down.

3. Perform PCR cycling by the thermal cycler machine as following schedule:

- **95C 9min**
- **94C 30 sec | ***
- **55C 2 min | * ← 30 cycles repeated**
- **72C 2 min | ***
- **72C 5 min**

4. Store the samples at 4°C

3. 2nd PCR

**Reaction mixture per sample** (per one tube)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>32.75 ul</td>
</tr>
<tr>
<td>Stock solution B</td>
<td>15.0 ul</td>
</tr>
<tr>
<td>1st PCR product</td>
<td>2.0 ul</td>
</tr>
<tr>
<td>Ampli. Taq Gold [5U/ul]</td>
<td>0.25 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50.0 ul</strong></td>
</tr>
</tbody>
</table>

1. In advance same as 1st PCR we make “Stock solution B” and “Master mixture B” for 10 tubes as follows. Distribute **48ul** of “Master mixture B” to each tube.

**Stock solution B** (15 ul for 1 sample)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xPCR Buffer II</td>
<td>5 ul</td>
<td>for 95 samples; 475 ul</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>4 ul</td>
<td>(1 eppendorf tube) 380 ul</td>
</tr>
<tr>
<td>dNTPs (each 2.5mM)</td>
<td>4 ul</td>
<td></td>
</tr>
<tr>
<td>Primer F2 (10pmol/ul)</td>
<td>1 ul</td>
<td>95 ul</td>
</tr>
<tr>
<td>Primer R2 (10pmol/ul)</td>
<td>1 ul</td>
<td>95 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15 ul</strong></td>
<td><strong>1425 ul</strong></td>
</tr>
</tbody>
</table>

**Master mixture B** (48ul per one tube)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDW</td>
<td>343.9 ul</td>
</tr>
<tr>
<td>Stock solution B</td>
<td>157.5 ul</td>
</tr>
<tr>
<td>Ampli. Taq Gold</td>
<td>2.6 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>504.0 ul for 10 samples</strong></td>
</tr>
</tbody>
</table>
2. Add **2.0ul** of 1st PCR product, mix well, and spin down.

3. Perform PCR cycling by the thermal cycler machine as following schedule:
   
   \[
   \begin{align*}
   95C & \quad 9\text{min} \\
   94C & \quad 30 \text{ sec} \ | \ * \\
   55C & \quad 2 \text{ min} \ | \ * \ \leftarrow 30 \text{ cycles repeated} \\
   72C & \quad 2 \text{ min} \ | \ * \\
   72C & \quad 5 \text{ min}
   \end{align*}
   \]

4. Store the samples at 4C

**Notes:** Stock solutions A and B are stored in –20C. Master mixtures A and B can also be stored in –20C but in this case it is better without Ampli. Taq GOLD enzyme.

*1) 10 x PCR BufferII (Perkin Elmer)
   100mM Tris-HCl (pH8.3)
   500mM KCl

*2) PCR primers were designed as follows:

   Primer F1 : 20 mer; (5') ACACCATGGGAG(C/T)TGGTAAT (3')
   Primer R1 : 27 mer; (5') CTTC(A/T)TCGACTT(C/T)CAGACCCAAGGCAT(3')
   Primer F2 : 20 mer; (5') GTG(G/C)GG(A/C)TGGATCACCTCCT (3')
   Primer R2 : 19 mer; (5') GCATCCACCA(A/T)A(A/T)AC(C/T)CTT (3')

4. **Agarose gel electrophoresis**

1. 10 ul of 2nd PCR products were loaded onto 2% agarose gel.

2. Stain the gel with ethidium bromide (0.1ug/ml) for 10 min and take photograph under UV light.

**An example of the result: Control experiment**

   The purified *Mycoplasma orale* DNA was used as a positive controle standard with the condition that the sample aliquots contain enriched genomic DNA derived from Vero cells. The number under the lanes indicates the DNA amount of *Mycoplasma orale* in the 1st PCR reaction mixture.

**References**