Detection of Mycoplasma in cell cultures

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Mycoplasma is a prokaryotic organism that is a frequent and occult contaminant of cell cultures. This organism can modify many aspects of cell physiology, rendering experiments that are conducted with contaminated cells worthless. Because of their small size, Mycoplasmas can pass through filters used to prevent bacterial and fungal contamination and potentially spread to all the cultures in a laboratory. It is essential that all new cell cultures entering a laboratory and all cell banks are tested for the presence of Mycoplasma. It is recommended that two techniques be used, selected from a PCR-based method, indirect staining and an agar and broth culture. This protocol describes these three tests for detecting Mycoplasma, which take from 1 d to 3–4 weeks, and such tests should be an obligatory component of quality control in every tissue culture laboratory.

INTRODUCTION

Mycoplasma contamination of cell cultures is widespread, ranging from 5 to 35% in published reports[1]. The use of contaminated cells compromises almost all aspects of cell physiology, and consequently the results and conclusions from any experiment[2,3]. It is essential that all cell stocks and all new cultures entering a laboratory are tested for the presence of Mycoplasma and a regime of routine testing be put in place. This protocol describes tests for detecting Mycoplasma.

Introduction to Mycoplasma

Mycoplasmas are small, round or filamentous prokaryotic organisms. They are members of the order Mollicutes and there are over 100 species, which is one reason why single tests can fail to detect contamination. *Mycoplasma pneumoniae* is pathogenic, but most Mycoplasma species are not. The species most frequently found in tissue culture are *Mycoplasma hyorhinis*, *Mycoplasma orale*, *Mycoplasma arginini* and *Acholeplasma laidlawii*[1,2,4].

Owing to their small size and deformability, Mycoplasmas can pass or be forced through filters (220-nm pores) used to sterilize tissue culture media. The lack of a rigid cell wall makes Mycoplasmas unresponsive to antibiotics that target cell-wall synthesis.

The concentration of Mycoplasma can reach 10^8 cells per ml of tissue culture medium without causing obvious cloudiness and have no apparent effect on cell growth. Consequently, Mycoplasmas can be difficult to detect in routine cell lines culture work[1]. Many cells support low levels of contamination that can only be detected using highly sensitive tests. Consequently, laboratories that do not test for Mycoplasma or use an insensitive method have a high probability of being infected.

The source of contamination is generally other infected cultures, although contaminated reagents such as serum or infection by personnel can be responsible[1]. In addition, feeder cells used for cultures such as human embryonic stem cell lines can contaminate the supported cells.

Detection of Mycoplasma

Many methods are available for the detection of Mycoplasma (see **Table 1**), including isolation on selective microbiological growth media, direct or indirect fluorescent staining, ELISA, autoradiography, immunostaining and direct or nested PCR[3]. Culture in agar is usually regarded as the 'gold standard' assay, but even using this assay, some 'fastidious' species of Mycoplasma, which require specialized culture conditions, can be missed. Consequently, the recommendation is to use two assays from isolation in broth/agar culture, indirect staining and a PCR-based technique[5].

There are some basic principles for Mycoplasma detection. Protocols and precautions for cell culture are available in the relevant *Nature Protocol*[6]. Cells for testing should be prepared fresh whenever possible, as storage or snap freezing may reduce the number of viable organisms. The cultures must have been grown without antibiotics for at least three subcultures or for 2 weeks (whichever is greater). Appropriate positive and negative controls must always be included in every assay.

If Mycoplasma contamination is found, how should it be managed? If the contamination is found in a new cell line retained in quarantine, the cells can either be discarded or the Mycoplasma treated with antibiotics. Antibiotic treatment of cells for Mycoplasma is a long process (3 months or more) but can be successful. If the contamination is found within the routine tissue culture laboratory, cell culture work should be closed down immediately and all living cultures discarded. All surfaces (particularly hoods and incubators) must be thoroughly cleaned and disinfected and all partly used medium discarded before taking fresh cultures from the cell bank and retesting all the cells.

One of the expectations of a *Nature Protocol* is that a competent graduate student could successfully implement the protocol with minimum guidance. Given the importance of Mycoplasma contamination in the tissue culture laboratory, and the considerable experience needed in optimization and interpretation of tests, a laboratory will wish to provide thorough training to its inexperienced personnel and confirm any positive or negative results obtained by them.

Overview of procedure

In this protocol, we describe three methods for the detection of Mycoplasma. We recommend the use of two of the three options.

(**A**) *Agar and broth culture*: the inclusion of a positive control, where possible, is mandatory in every experiment. However, for Mycoplasma testing, the provision of a positive control entails the growth of live organisms and/or contaminated cells in the environment, in which stringent efforts are being made to exclude them. Consequently, cases in which the use of a positive control might compromise the sterility of a laboratory, it may be preferable to outsource testing using this method. It should be noted that minor modifications will be needed to conform to the European Pharmacopoeia

TABLE 1 | Mycoplasma detection methods, their sensitivity, and advantages and disadvantages.

Method	Sensitivity	Advantages	Disadvantages
Direct DNA stain (e.g., Hoechst 33258)	Low	Rapid, cheap	Can be difficult to interpret
Indirect DNA stain (e.g., Hoechst 33258) with indicator cells (e.g., 3T3)	High	Easy to interpret because contamination amplified	Indirect and thus more time-consuming
Broth and agar culture	High	Sensitive	Slow and may require expert interpretation
PCR	High	Rapid	Requires optimization
Nested PCR	High	Rapid	More sensitive than direct PCR, but more likely to give false positives
ELISA	Moderate	Rapid	Limited range of species detected
Autoradiography	Moderate	Rapid	Can be difficult to interpret if contamination is at low level
Immunostaining	Moderate	Rapid	Can be difficult to interpret if contamination is at low level

(6th edition, 2007, section 2.6.7) and the guidelines of CBER/FDA, which relate to vaccine production rather than cell culture (Test for Mycoplasmas, 21 CFR 610.30, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=610.30).

(**B**) *Indirect staining*: Mycoplasmas can be detected in cell cultures stained with a fluorescent dye such as Hoechst 33258 or DAPI (4′,6-diamidino-2-phenylindole), which binds to DNA[7]. As Mycoplasmas contain DNA, small dots or flecks of fluorescence can be seen, concentrated on the cell surface and in the surrounding medium and on the culture dish. In contrast, cell nuclei will appear as large fluorescent oval areas.

Direct staining of cultures is not recommended, as it often gives equivocal results and will only reliably detect heavily contaminated cultures. Interpretation is difficult in cases in which few microorganisms are present and DNA from bacterial contamination or degraded DNA from cells may give rise to small points of fluorescence that mimic Mycoplasma. Therefore, it is essential to infect a reporter line that is known to support high levels of Mycoplasma proliferation using culture medium from the cells under test. Vero are the reporter cells most frequently used, but 3T6, MDCK and A549 cell lines are also suitable[3].

(**C**) *Direct PCR*: PCR can give false positives or false negatives. The reliability of detection by PCR will depend on the sensitivity of the method, the quality of the test sample and the specificity of primers. The method needs to be optimized under local conditions.

A large number of different sets of primers—single, multiplexed and nested—are available, giving a range of sensitivity and specificity[9,10]. Most primers use highly conserved sequences, attempting to detect a broad range of Mycoplasma species.

The following direct PCR protocol allows detection of a range of mollicutes. These include *Acholeplasma laidlawii*, *M. arginini*, *Mycoplasma fermentans*, *M. hyorhinis*, *M. orale*, *M. pneumoniae*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Spiroplasma citri*, species representing an optimal selection in terms of frequency of occurrence as contaminants in cell cultures and phylogenetic relationships, based on the guidelines provided in the European Pharmacopoeia. In addition, this protocol also detects *Mycoplasma arthritidis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Mycoplasma penetrans*, *Mycoplasma salivarium*, *Ureaplasma urealyticum*, *Mycoplasma bovis*, *Mycoplasma hyopneumoniae* and *Mycoplasma pirum*. The primers used are derived from a conserved region within the *16S rRNA* gene[11] and do not detect eukaryotic DNA or bacterial genera with close phylogenetic relation to mycoplasmas such as *Clostridium*, *Lactobacillus* and *Streptococcus*.

The following controls should be used for the PCR method:

- Negative control sample—a sample of sterile water or growth medium, which must produce a negative result, i.e., no band visible on the PCR gel.
- Positive control sample—a sample from a Mycoplasma-contaminated cell line or a standard Mycoplasma organism, e.g., Acholeplasma laidlawii, which must produce a positive result, i.e., a clear band visible on the PCR gel.
- Standard Mycoplasma organism—a standard preparation of a known Mycoplasma species.

MATERIALS

REAGENTS

For all methods: cells to be tested are grown as described in reference 6.

For agar and broth culture method

- Mycoplasma agar plates and broths (Mycoplasma Experience or Difco). Alternatively, agar plates and broths can be produced in the laboratory[8]
- Positive control—authenticated Mycoplasma reference strains can be obtained from a *bona fide* reference collection (see http://www.wfcc.info) such as the European Directorate for the Quality of Medicine and Health Care (http://crs.edqm.eu) or specialist companies such as Mycoplasma Experience. Alternatively, a known Mycoplasma-contaminated cell line may be used.

For indirect staining

- Hoechst 33258 (Sigma, cat. no. 861405)
- Reporter culture growth medium
- Reporter cell line (e.g., Vero)
- Vectashield mounting solution (Vector laboratories, cat. no. H-1000)

For PCR

- 10× CoralLoad PCR buffer (supplied in HotStar *Taq* Plus DNA polymerase kit; Qiagen, cat. no. 203605) ▲ **CRITICAL** Store at −20 °C until manufacturer's expiration date.
- HotStar *Taq* Plus DNA polymerase (supplied in HotStar *Taq* Plus DNA polymerase kit; Qiagen, cat. no. 203605) ▲ **CRITICAL** Store at −20 °C until manufacturer's expiration date.
- Deoxynucleotide triphosphate (dNTP) mix (10 mM) (Promega, cat. no. U1511) ▲ **CRITICAL** Store at −20 °C until manufacturer's expiration date.

- Antisense primer MGSO (5′-TGCACCATCTGTCACTCTGTTAACCTC-3′), (10 μM) (Integrated DNA Technologies) ▲ CRITICAL Store at −20 °C in small aliquots. Primers are stable for several years at −20 °C. Avoid multiple freeze–thaw cycles.
- Sense primer GPO-3 (5′- GGGAGCAAACAGGATTAGATACCCT-3′), (10 μM) (Integrated DNA Technologies) ▲ CRITICAL Store at −20 °C in small aliquots. Primers are stable for several years at −20 °C. Avoid multiple freeze–thaw cycles.
- DNase-free water (Qiagen, cat. no. 129114)
- DNeasy blood and tissue kit (Qiagen, cat. no. 69504)
- Standard Mycoplasma organism, e.g., *Acholeplasma laidlawii* (Mycoplasma Experience)

For agarose gel electrophoresis (part of direct PCR)

- UltraPure agarose—electrophoresis grade (Invitrogen, cat. no. 15510–027)
- 10× TBE (162 g Tris + 27.5 g boric acid + 9.3 g EDTA in 1 liter UltraPure water, adjusted to pH 8.3 with glacial acetic acid) (Media Services, NIBSC)
- UltraPure sterile water (Media Services, NIBSC)
- SYBR Safe DNA gel stain 10,000× concentrate in DMSO (Invitrogen, cat. no. S33102)
- 100-bp DNA ladder (Promega, cat. no. G2101)

EQUIPMENT

For agar and broth culture method

- 37 °C/5% CO_2 incubator dedicated for Mycoplasma tests
- Sterile plastic pipettes, 1 or 2 ml
- Microbiological safety cabinet, class II
- Petri dishes
- Inverted microscope with ×10 phase objectives or a stereomicroscope

For indirect staining

- Epifluorescence microscope with high-power objective (e.g., ×63)
- Incubation culture vessels, e.g., 24-well culture plates
- Sterile microscope coverslips to fit test incubation cultures (typically 13 mm-diameter round glass coverslips can be used that can be autoclaved in aluminum foil packages)
- Class II laminar flow cabinet

For PCR

- 1.5-ml Eppendorf tubes
- 0.2-ml PCR tubes or 96-well PCR plate
- Thermocycler
- Microcentrifuge capable of 22,000*g*

For agarose gel electrophoresis (part of PCR)

- Weighing balance
- Conical flask
- Microwave
- Gel preparation tank
- Electrophoresis tank
- UV transilluminator
- Camera

PROCEDURE

1| Carry out two of the following three options.

(A) Agar and broth culture method ● TIMING results are obtained within 3–4 weeks

(i) Grow cells for at least three subcultures or for 2 weeks (whichever is greater) in antibiotic-free medium.

(ii) Inoculate 0.1–0.2 ml of antibiotic-free cell suspension (fresh sample where possible, otherwise store at 4 °C for up to 2 weeks) into a vial containing 1.5-ml Mycoplasma broth, and onto the surface of a Mycoplasma agar plate. Incubate at 37 °C for 28 d in an atmosphere enriched with 5% CO_2.
▲ CRITICAL STEP Incorporate Mycoplasma-positive controls in each test to ensure that Mycoplasma grows on the batch of broths/agar plates in use.

(iii) Subculture from broths to agar plates at 3–5 d, and at 14 d. Incubate as above.

(iv) Observe the agar plates under a microscope each week, noting the presence of any Mycoplasma colonies (some species have a typical 'fried-egg' appearance as in **Fig. 1**, but this is not always the case). Record the results after 28 d of incubation, then discard the broths and agar plates.

(B) Indirect staining ● TIMING results are obtained within 10 d

(i) Grow test cells for at least three passages or for 2 weeks (whichever is greater) in antibiotic-free medium.

(ii) Culture test cells for 7 d or until peak concentration/density is achieved in an antibiotic-free medium. Simultaneously, seed enough reporter cells in antibiotic-free medium with a sterile glass coverslip in the base of the culture vessel (e.g., '24-well plate') to produce an exponentially growing culture on top of the coverslip after 7 d (e.g., 10–20,000 Vero cells per 2.5 cm^2).
▲ CRITICAL STEP Clearer results are obtained if the reporter cells are healthy and subconfluent, i.e., in the exponential phase of growth at the time of testing; hence, it may be more convenient to plate the reporter cells at a later time point.

(iii) Add 1-ml medium from the test cells to the medium of the reporter cells.

(iv) Incubate in a 5% CO_2 atmosphere for 3 d.

(v) Remove the medium with a pipette (if a vacuum aspirator is used, cross-contamination through the tubing should then be prevented by careful disinfection by aspirating bleach through the tubing after use).
▲ CRITICAL STEP It is important to be as gentle as possible with all rinsing steps as the majority of Mycoplasmas will be on the cell surface or on the surface of the plastic. Most will be discarded in the medium, which can be tested if desired by spinning down in a cytocentrifuge and staining (not recommended as the equipment is difficult to sterilize and the process can disseminate infection).

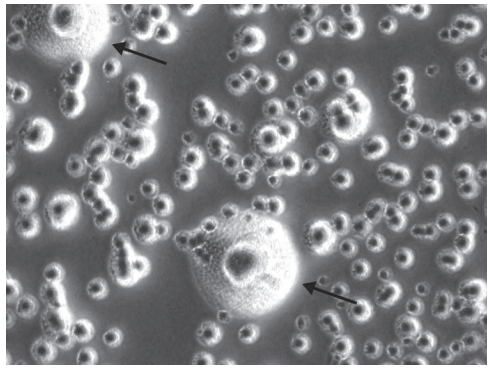

Figure 1 | Typical Mycoplasma colony, having the classic 'fried-egg' morphology (courtesy of NIBSC-HPA).

(vi) Rinse the cells with medium or PBS.
(vii) Fix the cells in an ice-cold mixture of acetic acid and methanol (freshly prepared 1:3 ratio) for at least 5 min.
(viii) Discard the fixative, wash gently with distilled water and dry each coverslip, taking care to keep control of sample identity represented by each coverslip.
■ **PAUSE POINT** Coverslips can be stored at room temperature at this point if necessary.
(ix) Add 50 ng ml^{-1} of Hoechst 33258 in distilled water for 10 min at room temperature.
(x) Remove stain and rinse gently with distilled water three times.
(xi) Add a drop of mountant, place one edge of a coverslip close to the drop and, using a needle, gently lower onto the drop (being careful not to create bubbles).
(xii) Remove excess fluid.
(xiii) Examine cultures using UV epifluorescence with a 330/380-nm excitation filter and a 440-nm barrier filter.
! **CAUTION** UV light is hazardous, and can cause burning of the eyes and may permanently damage sight. Consequently, if a microscope lacking safety features to prevent eye exposure is used, full training and explanation of the importance of the barrier filter must be given to trainees and persons using UV microscopy on an occasional basis.

(C) PCR ● TIMING results are obtained within 4–5 h

(i) Collect 1 ml of supernatant from adherent cells or cells growing in suspension in an Eppendorf tube.
▲ **CRITICAL STEP** Wear gloves throughout the protocol.
(ii) Add 8.25 µl of premix solution (see below) to each tube or well.

PCR master mix	For one reaction (µl)	For ten reactions (µl)
10× CoralLoad PCR buffer	5	50
MGSO primer (10 µM)	1	10
GPO-3 primer (10 µM)	1	10
dNTP mix (10 mM each)	1	10
HotStar*Taq* plus DNA polymerase	0.25	2.5
Total premix solution	8.25	82.5

? TROUBLESHOOTING

(iii) Centrifuge test samples at 200*g* for 1 min at room temperature to pellet cellular debris. Transfer the supernatant to a fresh tube, avoiding the pelleted cells. This supernatant is used as the template in PCR.
(iv) Add 1 µl of supernatant to the appropriate tube or well. To adjust the final reaction volume to 50 µl, add 40.75 µl of nuclease-free water to each reaction.
(v) For the inhibition-control reaction, add 1 µl of supernatant and 1 µl of Mycoplasma-positive control to each tube or well. Add 39.75 µl of nuclease-free water to each reaction to adjust the final reaction volume to 50 µl.
? TROUBLESHOOTING
(vi) Mix the contents of the tubes and tap or centrifuge to remove air bubbles.
(vii) Place the tubes or plate in a thermal cycler.
(viii) Run the samples at 95 °C for 5 min; 40 cycles at 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 1 min; 72 °C for 10 min and hold at 4 °C until the plate is removed.
? TROUBLESHOOTING
(ix) Agarose gel electrophoresis: prepare 500 ml of 1× TBE by adding 50 ml of 10× TBE to 450-ml UltraPure sterile water.
(x) Weigh out 2 g of agarose and add to a conical flask containing 100 ml of 1× TBE.
(xi) Place conical flask into a microwave to dissolve the agarose. Microwave on full power for 1 min, and then check to determine whether the agarose mix is dissolved. If not, repeat the microwave procedure until the agarose is completely dissolved.
(xii) Add 10 µl SYBR Safe DNA gel stain to the agarose mix and pour the gel mix into the gel preparation tank.
(xiii) Place a 15-, 20- or 30-well comb into the gel mix, depending on the number of samples for testing.
(xiv) Allow the gel to set. This takes about 30 min.
(xv) Once the gel has set, carefully remove the comb and place the gel in the electrophoresis tank.
(xvi) Pour enough 1× TBE into the tank to cover the gel.
(xvii) Run 18 µl of all PCR-amplified samples on 2% agarose gel at 100 V for 1 h. Include a 100-bp DNA ladder.
? TROUBLESHOOTING

(xviii) Run the gel at 100 V for 60 min.
(xix) Take and store an image of the gel exposed to UV light (using a UV transilluminator).

● TIMING

Step 1(A), Agar and broth culture method: results are obtained within 3–4 weeks (5–10 min to set up each plate or broth; 5–10 min to check plates and subculture at 3–5 d and at the 14-d time points; and finally another 5–10 min to check at the end of incubation after 28 d. Colonies are visible after 3–5 d unless the contamination is at a low level).
Step 1(B), Indirect staining: results are obtained within 10 d (15 min to plate cells, 5 min to transfer medium to reporter line and 30 min to stain and inspect cells).
Step 1(C), PCR: results are obtained within 4–5 h (~30–60 min for the PCR setup, 2.5 h for PCR and 1 h for agarose gel electrophoresis).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table for PCR.

Step	Problem	Possible reason	Solution
C(viii)	Absence of a 270 bp band in positive control lane	Incorrect annealing temperature	Use recommended annealing temperature
C(ii) and C(v)		Error in set up	Repeat the experiment. Ensure that all reagents are added in correct volumes
C(viii)		Error in cycling	Ensure that the temperature on display in the thermal cycler is the actual block temperature. Check that the program used is correct
C(xvii)		Error in gel analysis/loading	Ensure that samples are loaded in the correct wells in the gel
C(viii)	Presence of non-specific multiple bands in the gel	Annealing temperature is too low	Use recommended annealing temperature. Run a temperature gradient in 2 °C increments
C(ii)		Priming starting during set up	Set up reaction on ice or use a hot-start *Taq* DNA polymerase
		Contamination	Check negative control reaction for bands
	Negative control shows a 270 bp band	Contaminated reagents, water or culture medium used in the negative control reactions	Prepare fresh premix solution, water and culture medium used as templates in negative controls
		Pipettes contaminated	Use filter tips and clean pipettes. Ensure that a fresh pipette tip is used for each reaction
		Aerosol contamination	Pipette and expel reagents and template carefully. Use filter tips
Box 1	Absence of amplification in the inhibition control reaction	Presence of inhibitory subs tances in the supernatant	Remove the inhibitory substances from test samples by DNA extraction
C(v)		Error in set up	Ensure that the positive control is added to each inhibition control reaction

ANTICIPATED RESULTS

An example of a Mycoplasma colony that grows by the agar and broth culture method from a contaminated culture can be seen in **Figure 1**.

In cells examined by indirect staining, the nuclei of cells will stain blue. If there is Mycoplasma contamination in these samples, spots or flecks of bright blue stain will be seen at high magnification scattered over the cells and on the plastic surrounding the cells. If the results are equivocal, the test should be repeated.

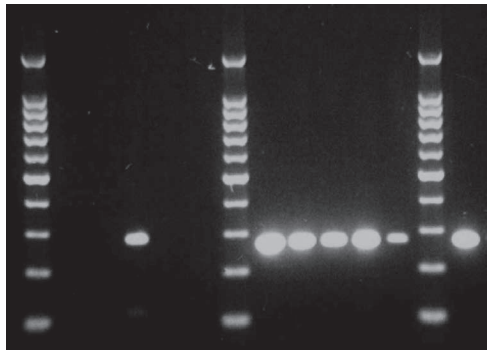

Figure 2 | Typical gel photo from direct PCR. Numbering from left to right, lanes 1, 7 and 13, 100-bp DNA ladder; lane 2, negative control; lane 3, test sample A; lane 4, test sample B; lane 5, test sample C; lane 6, test sample D; lane 8, inhibition control for negative control; lane 9, inhibition control for test sample A; lane 10, inhibition control for test sample B; lane 11, inhibition control for test sample C; lane 12, inhibition control for test sample D; and lane 14, positive control.

The results using PCR are interpreted by comparing the presence and size of the PCR product from test samples with those of the positive control reaction. The positive control and the inhibition control should both show a 270-bp band, whereas the negative controls should show no bands in this region (**Fig. 2**). A test is only accepted as valid if the negative controls give negative results and both the positive control and the inhibition control give positive results. If the results obtained using PCR are equivocal (i.e., the band of 270 bp seen by gel electrophoresis is faint) the suspected contamination can be amplified by inoculation of test material onto a reporter cell line (such as Vero cell monolayer) known to be negative for the presence of Mycoplasma. The inoculated cell line is then incubated for a few days to cultivate and support high levels of Mycoplasma proliferation, prior to harvesting of culture medium for PCR testing.

For test samples that are inhibitory to the PCR reaction (indicated by the absence of the 270-bp band in the inhibition control reaction), repeat the PCR as described in **Box 1**.

BOX 1 | FURTHER PROCEDURE TO BE USED WHERE PCR TEST SAMPLES ARE INHIBITORY TO THE PCR REACTION

(1) Centrifuge the supernatant from adherent or suspension cells in an Eppendorf tube at 200*g* for 1 min at room temperature to pellet cell debris.
(2) Transfer the supernatant to a fresh Eppendorf tube, avoiding any cell pellet.
(3) Centrifuge supernatant at 22,000*g* for 15 min.
(4) Carefully decant the supernatant. Resuspend the pellet (which may not be visible) in 100 µl of PBS.
(5) Extract DNA using a commercially available DNA extraction kit such as the DNeasy blood and tissue kit (Qiagen), or equivalent. The extracted DNA is used as the template in the PCR.
(6) Proceed to PROCEDURE Step 1C(ii).

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