Standard Operating Procedure SOP200

Title: Internal PCR Mycoplasma Testing

Location: All CBE labs

1. PURPOSE

The intent of this SOP is to describe the process for Internal Mycoplasma screening using PCR.

2. SCOPE

This SOP applies to containment level 2 (CL2) CBE laboratories, including the CBE Laboratory Unit (located in the Holywell Park) and the CBE Tissue Engineering Laboratory T208B (located in the Wolfson School). This is to prevent the entrance and spread of mycoplasma in CBE laboratories. Any new cells lines being brought into the CBE will be screened along with cell lines already being used within the CBE. This testing will be done periodically using the PCR mycoplasma test as described below.

3. RESPONSIBILITES

CBE Laboratory Users

Shall provide spent medium from cell lines that are in culture periodically for mycoplasma screening, or if they believe their cells in culture could be at risk of having Mycoplasma present. If the latter is the case, they should raise their suspicions with the responsible person or the Lab manager and an internal PCR mycoplasma test can be done immediately.

Responsible Person (RP)/Laboratory Manager (LM)

Shall periodically perform or when required perform Internal PCR Mycoplasma Testing and provide the interested parties with the results and also provide a copy of the results to be filed in the CBE office as a record of internal PCR mycoplasma testing.

4. EQUIPMENT AND MATERIALS

Reagents	Sourced from	Order number
My Taq HS DNA Polymerase	Bioline	BIO-21112
5X My Taq Reaction buffer	Bioline	BIO-21112
Oligos	IDT	
Mycoplasma DNA (Positive Control)		
Molecular Biology Grade Water	Thermofisher	10505854
Agarose	Bioline	BIO-41025
Midori Advance Green DNA Stain	Geneflow	S6-0022-X
Tris Base	SLS	T1503
Boric Acid		
Hyperladder 25bp	Bioline	BIO-33031
Crushed ice (available from GV Highbay area)		

0.5M EDTA	Lonza	LZ5134

1- Life technologies PCR system
2- PCR plates/ Strips and adhesive film or caps.
3- Gel tank – H29
4- Gel comb-H29
5- Gel tray-H29
6- Electrophoresis Gel tank-H29
7- Power pack-H29

5. Background Information

PCR Primer
The primers were taken from Wong-Lee JG, Lovett M (1993): Rapid and sensitive PCR method for identification of Mycoplasma species in tissue culture.

Primer length-18 to 20 base pairs
Amplicon length – approx. 460 base pairs

Mycoplasma Primers
Forward: 5'-GGCGAATGGGTGAGTAACACG-3'
Reverse: 5-CGGATAACGCTTGCGACCTATG-3'

Primer annealing temperature 58°C

In addition, a control can be added to the experimental set-up if amplifying from DNA. In this case, GAPDH is amplified along with the Mycoplasma primer either mixed together or separately.

Name	Sequence	Range
Myco-F	GGCGAATGGGTGAGTAACACG	435-470
Myco-R	CGGATAACGCTTGCGACCTATG	435-470
GAPDH_Myco_F	GCATTCGCCCTCTTAATGGG	336
GAPDH_Myco_R	TGACGGTGCCATGGAATTTG	336

Table 1: Primer Information

6. PROCEDURE

Step 1: Culturing Cells for Mycoplasma Detection

- Thaw cells according to your specific lab protocols.

- Ideally, culture cells without antibodies for at least 2 weeks after thawing.
- Allow cells to grow in unchanged medium for at least 48 hours before sampling.
- For sampling from adherent cells; remove 1ml of medium from the flask and put into a sterile micro-centrifuge tube or 15ml falcon tube.
- Ideally the sample should be used for the PCR straight away but it can be stored at 2-8°C for up to two hours or in the -20°C freezer for longer term storage. If frozen, the sample needs to be thawed first.

Step 2: PCR Set up

- Centrifuge the medium sample at 200g for 2 minutes and sample a minimum volume of 100µL of supernatant to ensure removal of debris before boiling.
- Incubate the samples at 95°C for a minimum of 5 mins in a heat block. **WARNING – Do not touch the heat block with bare hands; beware of the potential for Eppendorf tubes to pop open if left in the heat block for too long. This is both a safety and sample contamination concern. Samples that are rendered non-sterile before PCR amplification could result in a false positive result**.
- The sample(s) can then be placed on ice until used.
- Collect all of the required reagents; these are stored in the fridge or freezer in H23. Thaw the frozen reagents. Vortex all reagents gently, and spin in a micro centrifuge for a short time to remove any bubbles and to bring all the liquid to the bottom of the tube. These should be stored on ice until required.
- Make up the mastermix as below. (Note: When making up the master mix account for the two controls (+/-) and a two sample excess so that you do not run out of mix. E.g. If there are six samples plus two controls, make the master mix up for ten samples. Vortex the mastermix briefly to make sure it is thoroughly mixed. Centrifuge briefly.

Reagents	Volume (µL)	Master mix (x10 samples) (µL)
5X MyTaq Reaction Buffer	3	30
Forward Primer (10uM)	0.75	7.5
Reverse Primer (10uM)	0.75	7.5
MyTaq HS DNA Polymerase	0.5	5
DNase/RNase free water	8.5	85

Total Volume	13.5	135
Template (Your sample)	1.5	0

Table 2: Master mix

- Pre-plan a sample order template in a lab book to keep track of where each sample and control will be placed. Circle the sample wells on the plate to easily visualise the correct wells.
- Add 13.5µL of master mix to each sample well followed by 1.5µL of sample. There should be a total volume of 15uL in each well. A positive control and a negative control need to be added to the plate. For the positive control add 1.5µL of Mycoplasma DNA, for the negative control add 1.5uL of DNase / RNase free water to the well.
- Use a new pipette tip for each sample well and fill the positive control well last to avoid any potential contamination of sample wells with Mycoplasma DNA, this would result in a false positive result.
- Once all the medium samples and controls have been added to the plate the plate needs to be sealed using an adhesive sheet or caps. This needs to be firmly pressed on and needs to be checked to make sure that a seal has been formed over the top of each well or the sample could evaporate.

Step 3. Put plate onto the thermal cycler.

The thermocycler program is shown below (Named Ade_Myco1).

Number of Cycles	Temperature (°C)	Time
1	95°C	1 min
35	95°C	15 sec
	58°C	15 sec

	72°C	10 sec
1	72°C	5 min
1	10°C	Infinity

Table 3: Thermal Cycler program set up

The thermal cycler should be set to hold the temperature at 10°C until the PCR plate is taken off.

Once the PCR cycles have finished, the samples can be ran on a 1.5% agarose gel immediately or can be stored in the fridge.

Step 4. Prepare and run an agarose gel.

- Prepare stock 5x TBE buffer in a clean glass duran bottle.

Reagent	Amount
Tris-Base	54g
Boric Acid	27.5g
0.5M EDTA	20mL
Purified water	980mL
Total Volume	1000mL

Table 4: Stock 5x TBE buffer

- Prepare 1X TBE buffer
    - To 200mL of 5x TBE buffer add 800ml of purified water to make 1000mL of 1x TBE buffer (5:1 dilution).
- Make the Gel
    - Take the gel tray and use the rubber end caps to seal the ends to form a mold. Insert the comb into the slot above the red line in the tray. Additional samples can be run on the same gel by placing a second comb over the second red line halfway down the tray. Place the tray on a flat surface ready to pour the gel.
    - Add 1.5g of agarose powder per 100mL of the 1x TBE running buffer into a small duran bottle with the lid on very loosely.
    - Place into the microwave in H29 (Beware of the lab coat procedure in the microbiology laboratory) and heat for approximately 5 minutes or until the agarose has fully dissolved, leaving a clear, colourless liquid without any lumps. Do not allow the agarose solution to over boil. **WARNING – Loosen the top of the duran bottle when placing it into the**

microwave. This will allow for hot air to escape and prevent the bottle from exploding. The duran bottle and agarose solution will be very hot!

- Allow to cool slightly (to around 60 °C) and remove carefully from the microwave using the heat proof gloves.
- Add 8µL of Midori Green advance DNA stain to the agarose solution.
- Mix gently by swirling the bottle to make sure the DNA stain is mixed evenly through the agarose solution.
- Gently pour the solution into the mold. Try to avoid causing bubbles as this can affect the PCR products from running properly. Move any bubbles by using a pipette tip to drag them quickly to the side of the tray.
- Allow the gel to set for approximately 30 minutes or until it is solidified.
- If the gel is not going to be used straight away, remove the comb(s) and the rubber caps from gel tray, wrap the gel in Clingfilm and store it at 2-8°C (short term storage only).

- Running the gel
  - Carefully remove the comb from the gel to create the sample wells, avoid tearing the gel.
  - Carefully remove the rubber caps from both ends of the tray.
  - Transfer the gel (still in its tray) into the electrophoresis gel tank, with the wells at the back (black cathode). As the PCR products are negatively charged, they will run towards the red anode.
  - Add 1x TBE running buffer so that the gel is completely covered. There is a max fill line on the side of the tank.
- Prepare PCR product for loading
  - Add 5µL of loading dye to each sample and gently mix with the pipette.
  - Decide on the loading order of the samples, it is best to leave a few wells either end if possible and leave room at either end of the sample wells to load DNA ladder. This is a DNA size standard and is used to see the size of the DNA fragments and to check that the gel has ran properly.
  - Using a pipette take up 10µL of the sample, place the end of the pipette tip into the well and slowly release the sample. Avoid putting the tip into the gel or releasing the sample into the

well too fast as this can lead to the sample spilling out of the well and potentially contaminating other wells.

- Put the lid of the gel tank on (checking it is in the correct orientation) – the end with the black power lead goes on the black end of the tank.

- Connect the leads from the tank to the power pack. The red lead connects to the red slot, and the black lead connects to the black slot.

- Switch on the power pack and set to a constant voltage. If it is working correctly then small bubbles should start to appear in the gel tank. Begin with 150 watts, the higher the voltage the quicker the samples will move towards the anode. Before leaving check that the samples are travelling in the right direction. You can see this as the blue dye should be starting to move down the gel.

- Whereas a higher voltage will allow the gel to run quicker, a lower voltage will be slower but provide better clarity between DNA fragment bands, especially if the difference in b.p. is small.

- Allow the gel to run until it has migrated to an appropriate distance down the gel.

  NOTE: Do not leave the gel running for too long, or the samples will run off the end of the gel.

- Stop the power pack and view the gel under the UV illuminator.

Step 5: Viewing the gel

- Turn the H29 PC on and log in using your university profile.
- Open the UVP program.
- Put the gel onto the viewing surface. Close the door and turn on the UV light to trans.
- Check that the gel is situated correctly and that the samples are illuminating using the front viewing port. **WARNING – Do not open the viewer while the UV is on, only use the front viewing port.**
- Once the program has loaded, click the Acquisition Tab.
- Click on the “Preview” button. This step may take a while. A window will come up which shows the gel, if you cannot view the gel clearly you may need to alter the exposure level. Click on the arrows in the window for exposure time, until you are able to visualize the gel clearly. The gel background should show dark and the positive control and DNA ladders should be bright.
- The negative control should be blank and if the samples being tested are Mycoplasma free they should be blank also.

- To save an image of the result press Print Screen “PrtScn” on the keyboard.
- Open MS Paint and paste “Ctrl + V”.
- The image can then be cropped down to size and the gel labelled.
- Save the image as a JPEG.
- Turn the UV light on the gel viewer off.
- Take the gel out and if finished with dispose of in the autoclavable waste (orange route).
- Wipe clean the UV box and close the door.
- Close all the programs and turn off the computer.

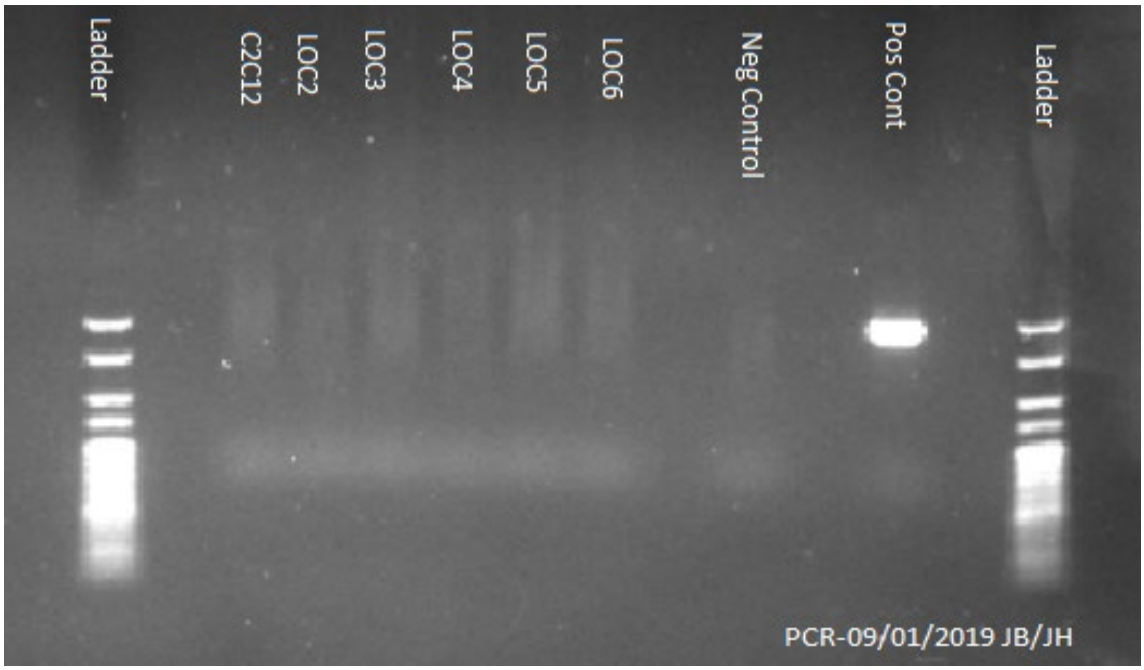

Image 1: Example of a labelled PCR electrophoresis gel.

This shows that the samples are all negative for Mycoplasma, along with the negative Control. The Positive control shows a positive result and the size ladders are shown on either end.

- This gel picture then needs to be sent to the Responsible Person or a Lab Manager so that it can be put in the internal Mycoplasma testing record. There is a folder on the workspace in the Bioengineering-lab-leaders called “Internal Mycoplasma PCR results” where a copy will be located and a hard copy will be stored in the office.

DOCUMENTATION

There are no documents for this SOP.

Standard Operating Procedure **SOP200**

Title: Internal PCR Mycoplasma Testing

Location: All CBE labs

SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
		[Insert specific changes from previous SOP] < e.g. changes in accountabilities, process steps, deviation actions, or records>.	