Chapter 14: Conventional Polymerase Chain Reaction (PCR)

Chapter Overview and Introduction to Conventional PCR

PCR is frequently used in the diagnosis of bacterial pathogens because of its improved sensitivity and its high throughput. A range of PCR assay types exist, each with its own advantages and limitations. Conventional PCR is the most basic type of PCR assay that requires a post-PCR step for detection and visualization of the DNA product (Gal et al. 2005).

Several conventional PCR assays have been developed for the detection and sequencing of bacterial pathogens, including *B. pseudomallei*. Most diagnostic laboratories have now replaced routine conventional PCR testing for real-time PCR methods. However, conventional assays may still be particularly useful in resource-limited settings since the cost is reduced compared to real-time PCR machines and reagents (Garibyan & Avashia 2013). Additionally, conventional PCR still plays an essential role in bacterial sequencing and strain typing. Sequencing and strain typing of *B. pseudomallei* are discussed further in Chapter 16.

Conventional PCR Limitations

With conventional PCR, DNA sequences are amplified and analysis of the product is performed at the end of the PCR reaction. Amplified products need to be visualized with a secondary procedure after the PCR has finished, such as agarose gel electrophoresis or polyacrylamide gel electrophoresis (PAGE). Electrophoresis requires that reaction tubes are opened and handled, increasing the risk of contamination. Conventional PCR is also more time consuming and less sensitive and specific than real-time PCR (ThermoFisher 2012; Smith & Osborn 2009).

Additionally, as with other types of PCR assays, the components and procedures needed to effectively perform conventional PCR testing are very specific and cannot be altered. **If a lab is going to conduct conventional PCR testing, they must follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed below.**

B. pseudomallei Conventional PCR Detection

A number of conventional PCR assays have been developed for the identification and detection of *B. pseudomallei* (Table 17). Specific PCR targets have included 23S ribosomal RNA (rRNA) (Lew & Desmarchelier 1994), 16S rRNA (Dharakul et al. 1996), lipopolysaccharide (LPS) (Rattanathongkom et al. 1997), flagellin C (*fliC*) (Sonthayanon et al. 2002), metalloprotease gene (*mprA*) (Neubauer et al. 2007), and the type three secretion (TTS1) system gene cluster of *B. pseudomallei* (Gal et al. 2005; Winstanley & Hart 2000).

Table 17- Conventional PCR targets and primers used for the detection of *B. pseudomallei*.

Target	Primers	Reference
mprA	Bpm-f: 5'-ACTGCTTCGTTCAAGGCGACCGTC-3' Bpm-r: 5'-TGACGGCCTGAACGTCCGCGC-3'	Neubauer et al. 2007
16-23s rDNA spacer (Semi-Nested PCR)	Bp1- 5'-CGATGATCGTTGGCGCTT-3' Bp2- 5'-ACTTACGGGCATCTCA-3' Bp3- 5'-ATTAGAGTCGAACAAT-3' Bp4- 5'-CGTTGTGCCGTATTCCAAT-3'	Inglis et al. 2005
TTS1 (*orf2)*	BPTTSF- 5'-CTTCAATCTGCTCTTTCCGTT-3' BPTTSR- 5'-CAGGACGGTTTCGGACGAA-3'	Winstanley & Hart 2000
fliC	PMA-1- 5'CTGTCGTCGACGGCCGT-3' PMA-2- 5'GGTTCGAGACCGTTTGCG-3'	Sonthayanon et al. 2002
pKKU-S23 LPS	LPS1- 5'-CTCTCAGATTGCTGACAAACCC-3' LPS2- 5'-CGGATGAACTCGAAATCCACCG-3'	Rattanathongkom et al. 1997

16s rDNA	PPM3- 5' AATCATTCTGGCTAATACCCG-3' PPM4- 5' CGGTTCTCTTTCGAGCTCG-3'	Brook et al. 1997
16s rDNA (Nested PCR)	U33- 5'-AAGTCGAACGGCAGCACGG-3' OL731- 5'-TTTGCTCCCCACGCTTTCG-3' BS3L- 5'-ACGGGCTTCGGCTGGTG-3' BS4R- 5'-CACTCCGGGTATTAGCCAG-3'	Dharakul et al. 1996
23s rRNA	PPMA- 5'-GGTAGCCTGCGAAAAGCTACGGGGGAG-3' PPMB2- 5'-CCTGCGCGGAACATGTAACGGGGCT-3' PPMC- 5'-CCACCTGCGTCGGTTTGCGGTACGG-3' PPM2- 5'-CTCTCCTACCATCGAGAC-3'	Lew & Desmarchelier 1994

The current "gold standard" species-specific PCR assay for *B. pseudomallei* is based on the amplification of *orf2* in the type three secretion system 1 (TTS1) cluster. Type three secretion (TTS) systems have been identified in several gram-negative pathogens such as *Shigella* and *Salmonella spp.* as important virulence determinants that allow for bacterial invasion and escape from host cells (Stevens et al. 2003; Winstanley et al. 1999). Several studies have shown that a 548-base pair (bp)-long TTS segment that is part of *orf2* is invariably present in *B. pseudomallei* but not closely related species such as *Burkholderia thailandensis* or *Burkholderia mallei* (Novak et al. 2006; Rainbow et al. 2002; Smith-Vaughan et al. 2003).

Conventional PCR targeting the TTS1 gene cluster is performed using oligonucleotide primers BPTTSF and BPTTSR (see Table 17 above), which exhibit high sensitivity and specificity for *B. pseudomallei* (Gal et al. 2005; Winstanley & Hart 2000). The assay has been shown to be particularly robust for sputum, wound and culture specimens, reflecting the high bacterial load. However, the sensitivity of the assay from blood samples is reduced compared to other clinical specimen types, most likely due to the low concentration of *B. pseudomallei* circulating in whole blood (Wuthiekanun

et al. 2007). Improved sensitivity from blood has been shown when sample DNA is increased from 1 µL to 5 µL (Gal et al. 2005; Tellapragada et al. 2017).

B. pseudomallei TTS1 Conventional PCR Procedures

General Comments Before Starting

PCR is a very sensitive method that that is also very prone to contamination. Precautions should be taken to minimize cross-contamination between samples:

- It is recommended that separate spaces or areas are used where pre- and post-PCR steps are performed (see Chapter 12 for further detail).
- If individual rooms are not possible, separate bench spaces should be used. Separate pipettes, laboratory coats and gloves should be used in each of these areas.
- Working in a PCR laminar flow hood or PCR box is also recommended to minimize cross-contamination.
- Decontamination of surfaces and equipment with 10% bleach solution or commercial DNA decontaminant followed by 70% ethanol should be performed after any handling of DNA.
- Always use filter-barrier pipette tips and change and discard gloves frequently.
- Remove DNA templates, controls and other reagents from the freezer and allow them to thaw completely before use. Briefly vortex and centrifuge tubes for 30 seconds at 1000 rpm before using.
- It is good practice to keep reagents on ice while setting up the reaction plate and PCR master mix to prevent reagent degradation.
- Prior to beginning the PCR, plan the experiment by filling out and printing a plate template worksheet. Ensure PCR reagents are available and have been correctly calculated.

- Primer working stock solutions should be made up to 20 µM before starting. This will help to prevent primer contamination and limit repeat freeze-thaw cycles every time the reaction is performed.

Quality Controls (CLIA 2010; U.S. EPA 2004)

One of the most important aspects of any PCR method is the use of the quality controls to ensure that the procedure has worked correctly, and the results are reliable. Laboratories should analyze positive and negative controls on a routine basis to demonstrate adequate performance of the PCR. Routine PCR controls should include:

1. Negative control- PCR negative controls are used to demonstrate that contamination has not occurred during sample processing. No amplification of DNA should be detected in these controls. Two types of PCR negative controls that should be used include:
    a. Non-template control- PCR grade water should be used as a non-template control (NTC). NTCs should be included in each PCR run. It is advisable to include one NTC for work performed in the clean PCR set-up room/area and one for work performed in the dirty PCR set-up area/room.
    b. DNA extraction blank- This is used to examine if contamination has occurred during sample processing or PCR analysis. DNA extraction is done on sterile water and this is processed alongside the test samples using the same preparation and extraction procedures as the test samples.
2. Positive control- PCR positive controls are used to check that the reagents have been prepared appropriately. They should be run concurrently with each PCR batch. Positive controls are prepared by the addition of an external control to the master mix. An exogenous control can be:
    a. Purified DNA from a known isolate of *B. pseudomallei* containing the sequence of interest. Care must be taken to ensure no cross-contamination occurs between the

positive controls and the samples or negative controls. *B. pseudomallei* control DNA should be diluted in sterile PCR-grade water to minimize the risk of cross-contamination.

b. A specific template that contains the gene sequence of interest.

c. A related template sequence that is amplified with an efficiency that is comparable to the target sequence.

3. Internal positive control (IPC)- The presence of PCR inhibitors or errors during DNA extraction is a common cause of false-negative PCR results and can be easily controlled for by including an Internal Positive Control (IPC). This is used to confirm DNA amplification, detect false negatives, and examine the presence of amplification inhibitors. See Chapters 13 and 15 for further details and protocols.

Equipment and Reagents

- Primers (see below)
- Appropriate positive and negative controls

- 10x PCR Buffer
- 10mM dNTPs Mix
- HotStarTaq DNA Polymerase (QIAGEN)
- PCR-grade water
- 10% bleach or commercial DNA decontaminant
- 70% ethanol
- PPE (gloves, lab coats and safety glasses)
- Laboratory pens/markers
- Racks for 0.2 mL, 1.5mL microcentrifuge tubes and 96-well plate
- Pipettes and filter/barrier pipette tips
- Sterile PCR microcentrifuge tubes
- Benchtop microcentrifuge
- 96-well polypropylene PCR plates (e.g. Thermo Scientific™ AB0600) or PCR strips (e.g. Axygen™ 0.2 mL Polypropylene PCR Tube Strips #16272591)
- PCR strip caps to cover wells
- Vortex
- Thermocycler (such as Eppendorf Mastercycler® or T100™ Bio-Rad)

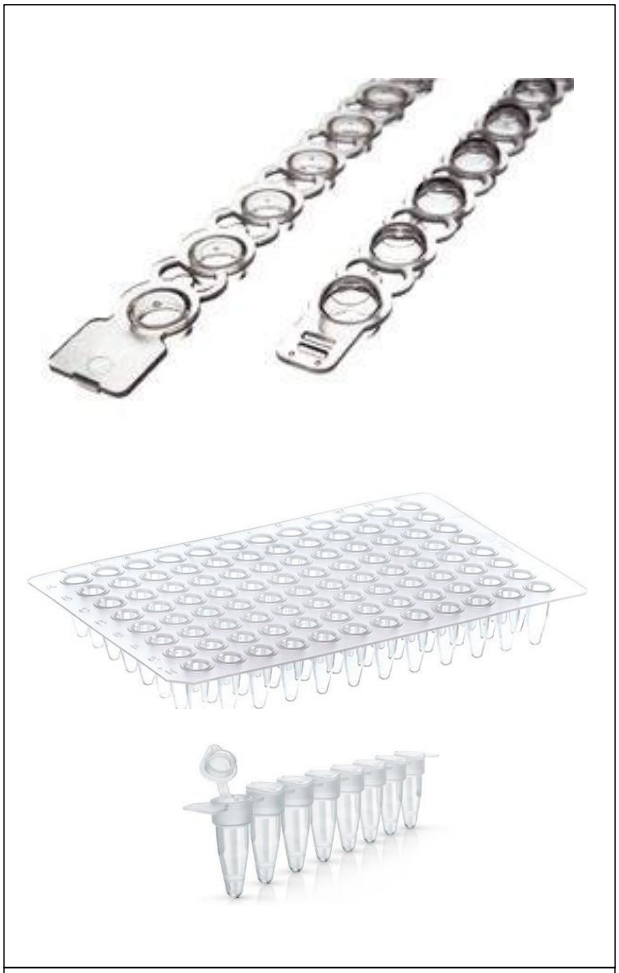


Figure 78- 96-well PCR strip caps, plate and PCR strips (Thermo-Scientific ™).

Primer Sequences-

TTS1 (*orf2*)- 548 base pair length (GenBank AF074878):

1. Forward- BPTTSF- 5'-CTTCAAT CTGCTCTTTCCGTT-3'
2. Reverse- BPTTSR- 5' -CAGGACG GTTTCGGACGAA-3'

Protocol for 25 µL Reaction

Each PCR reaction should contain:

- Deoxynucleoside triphosphates (dNTPs)
- PCR buffer containing $MgCl_2$
- HotStarTaq DNA polymerase (QIAGEN)
- Forward primer
- Reverse primer
- Extracted DNA template from specimen
- PCR-grade water

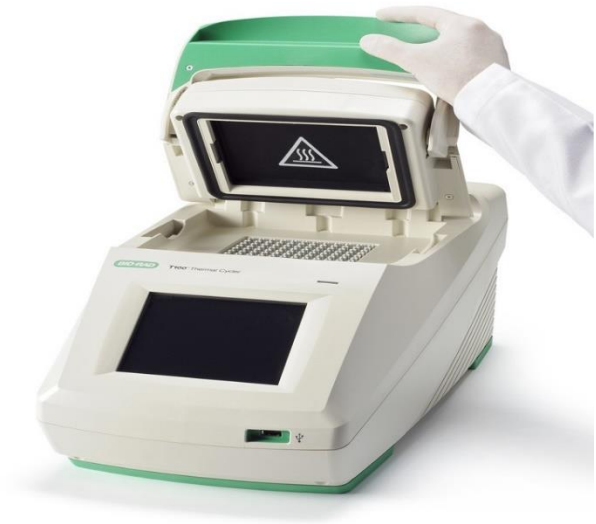


Figure 79- A conventional PCR machine and editable template for a PCR protocol available on the Bio-Rad T100™. The protocol editor displays the protocol in a graphical format using editable boxes (Bio-Rad).

A master mix should be prepared that includes all components listed above **except the DNA template. Prepare two more reactions than the number of specimens to be tested to ensure that adequate master mix is available**, as some will be lost during pipetting.

1. Combine the reagents for the master mix as shown below. Each reagent volume should be multiplied by the number of reactions being run (+2 extra reactions to account for pipetting loss) (QIAGEN HotStarTaq PCR Handbook).

Component	For 25 µL Reaction	Final Concentration
10X PCR Buffer	2.5 µL	1X
10 mM dNTP Mix	0.5 µL	200 µM of each dNTP
20 µM Forward Primer	0.625 µL	0.5 µM
20 µM Reverse Primer	0.625 µL	0.5 µM
HotStarTaq DNA Polymerase	0.125 µL	2.5 Units/reaction
PCR-grade Water	15.625µL	
DNA Template	5 µL	

2. Gently mix the master mix then briefly spin in a centrifuge.
3. Pipette 20 µL of the master mix into each well of a 96-well plate or PCR strips based on the template worksheet.
4. Cover the wells using cap strips or a plate seal. Spray down the clean workspace with 10% bleach or DNA decontaminant and repeat with 70% ethanol. Remove lab coat and gloves and put on a new pair of gloves. Take the 96-well plate or PCR strips to the DNA template addition area.
5. Put on new laboratory coat. Very carefully remove the cover from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
6. Add 5 µL of template DNA and extraction controls to the appropriate well of the 96-well plate or PCR strip based on the template worksheet.
7. Add 5 µL water to the “NTC” well.
8. Add 5 µL of the positive control template and IPC to the correct well.
9. Cover the wells of each column of the PCR plate or each PCR strip using strip caps as you go and secure caps tightly.

10. Wipe down the work area with bleach or DNAerase, followed by 70% ethanol. Where possible, quickly spin the plate at 1000 rpm to remove droplets that may have formed. Transport the plate/strips to the PCR thermocycler area and place directly in the PCR thermocycler.

PCR Thermocycler Set-Up

Set the following PCR cycle on the thermocycler:

Stage	Temperature	Time	# of Cycles
Initial Denaturation	95°C	15 minutes	1 cycle
Amplification	95°C	30 seconds	35 cycles
	55°C	30 seconds	
	72°C	30 seconds	
Final Extension	72°C	5 minutes	1 cycle
Storage	4°C	hold	

1. Double check all temperature and times are correct and press start.
2. Once the reaction has finished, store the PCR products at 4°C until running on a gel.

Gel Electrophoresis

With conventional PCR, PCR products must be further examined after the reaction has run to ensure the correct product has been amplified after completing the reaction cycle. This is usually done by analyzing the size of the DNA product by means of gel electrophoresis. Gel electrophoresis is used to separate DNA fragments based on size and charge allowing for their visualization and purification. Electrophoresis uses an electrical current to move the

DNA through a gel toward a positive electrode (Aaij & Borst 1972). The DNA molecules travel through the gel at different speeds based on size, allowing them to be separated from one another. This means it is possible to see how many different DNA fragments are present in a sample and their relative sizes. Since the gel is composed of small pores, smaller fragments of DNA will move faster through the gel than the larger ones. After the electrophoresis is complete, a dye is applied to the gel which binds to the DNA. Under UV light, this dye will fluoresce, allowing for visualization of the PCR product. By running a DNA ladder (a standard containing DNA fragments of known sizes) alongside the PCR products, the size can be determined and compared to the expected result (Brody & Kern 2004; Lee et al. 2012).

Gel electrophoresis consists of three main steps (Lee et al. 2012):

1. Pouring the Gel- Electrophoresis gels are normally composed of agarose, a polysaccharide that is typically purchased in a dry powder form. Gels can be made at different percentages but 1 or 2% are used most frequently. The mixture is heated until dissolved and a DNA intercalating dye, typically ethidium bromide (EtBr) is added. The solution is poured into a gel cast with combs (Figure 80). After cooling, this hardens into a solid gel, with small indentations at one end. These are termed “wells”, which are where the DNA samples are loaded.

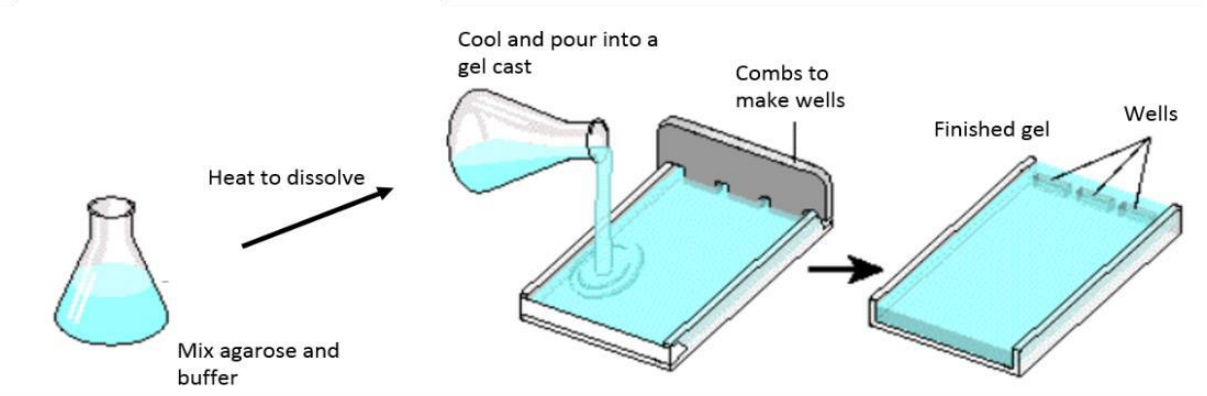


Figure 80- Pouring a gel for electrophoresis. After boiling and dissolving the solution of agarose, the solution is poured and allowed to cool, forming a solid gel. The combs are removed, leaving indentations where the samples are loaded (WUAS 2007).

2. Running the Gel- After the gel has cooled and is solid, it is placed into an electrophoresis gel box/tank. One side of the tank is connected to a positive electrode, while the other is connected to a negative electrode. The end of the gel that contains the wells should be placed at the end with the negative electrode. This is the end the DNA will migrate towards. The box is then filled with buffer which will help to conduct current through the gel. A loading dye is added to each DNA sample before being loaded into the wells. This is done to help track the DNA samples through the gel (to prevent DNA from running off the gel) and to help the samples sink into the well and not float out into the buffer (Armstrong & Schulz 2015). Alongside the samples, a DNA ladder is loaded to help analyze the DNA sample sizes. Commercial DNA ladders come in different size ranges, but one that is reflective of the size range of the expected DNA fragments should be used. Once the current is turned on, the samples migrate down the gel toward the positive end (Figure 81).

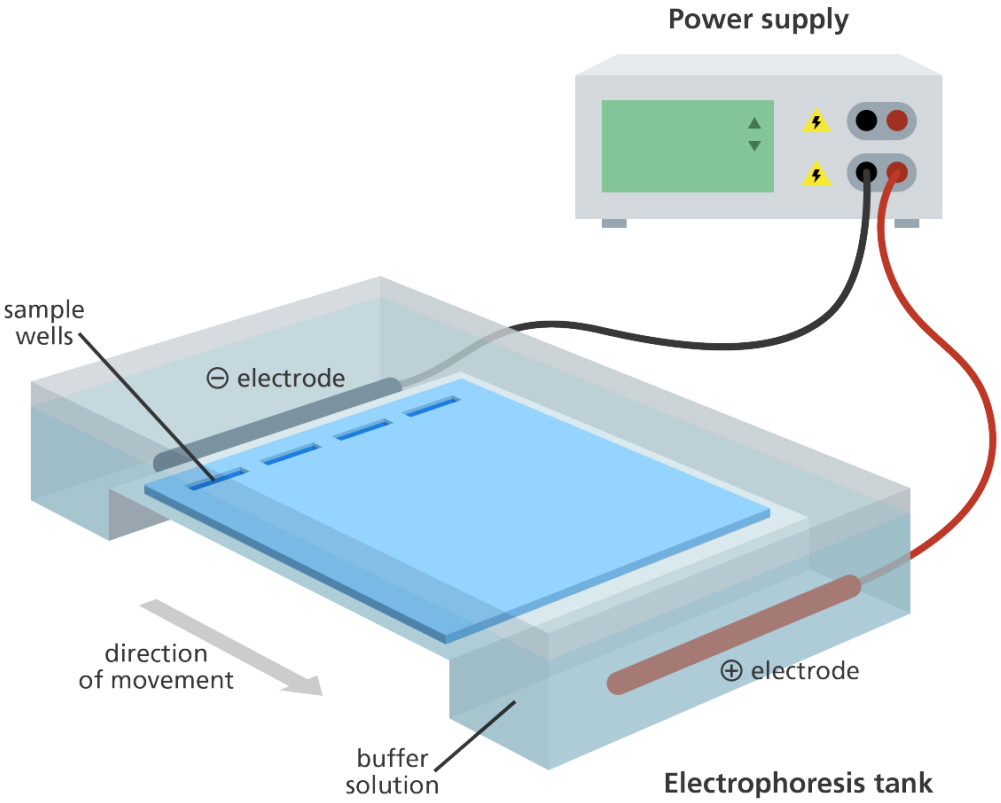


Figure 81- DNA electrophoresis equipment. A gel is placed inside an electrophoresis tank containing running buffer. The DNA samples are then loaded into the sample wells at one end of the gel and an electrical current is passed across the gel. The negatively-charged DNA moves towards the positive end of the gel (Genome Research Limited- yourgenome CC BY 4.0 2017).

3. Visualizing the DNA fragments- After the gel has finished running, the DNA bands are visualized by exposing the gel to UV light as shown in Figure 82. A distinct line that contains DNA fragments of the same size is called a band. Their approximate sizes can then be determined using the DNA ladder, which is used to indicate how many base pairs (bp) long

the DNA fragment is. If the band size is correct, the target sequence can be confirmed in the sample.

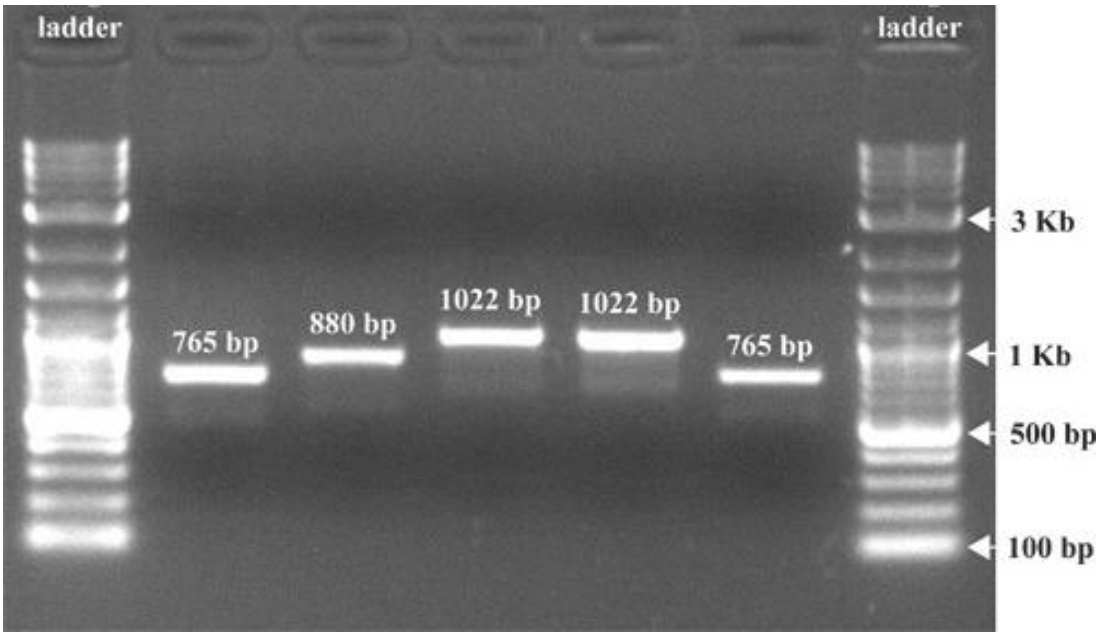


Figure 82- An image of a gel post electrophoresis using an EtBr stain. The gel was exposed to UV light and visualized with a gel documentation system (Lee et al. 2012).

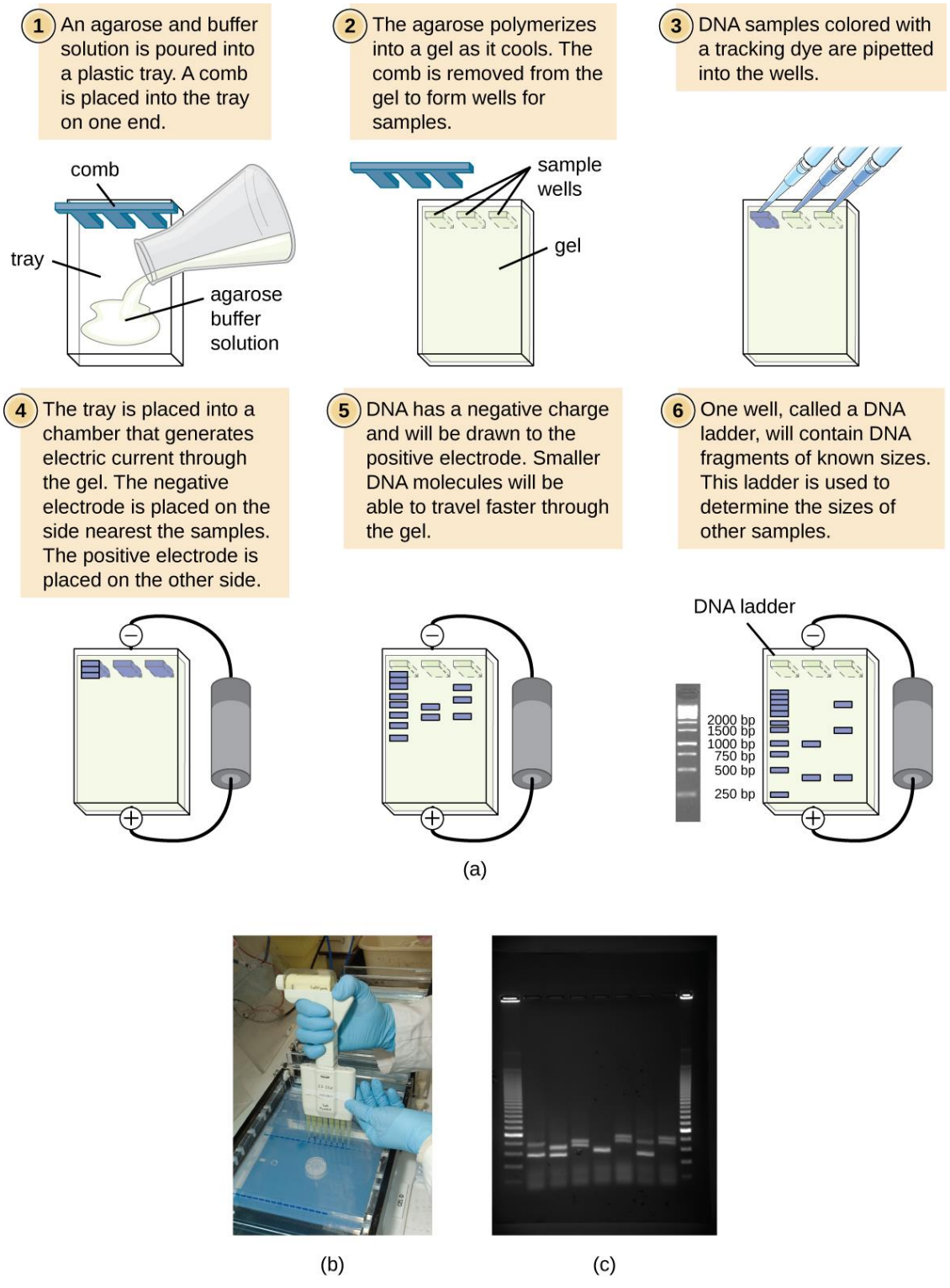


Figure 83- (a) The steps involved in preparing and running agarose gel electrophoresis. (b) DNA samples mixed with loading dye are loaded into the gel. (c) The gel is run then exposed to UV light and visualized with a gel documentation system (Parker et al. OpenStax CC BY 4.0 2016).

Gel Electrophoresis Protocol

Equipment:

- Microwavable glass flask or beaker
- Electrophoresis tank
- Power supply
- Stir plate
- Gel casting tray
- Fixed-wall gel caster
- Well combs
- Gel box
- Gel documentation system (e.g. Gel Doc XR Bio-Rad Laboratories)
- UV light source
- Microwave

Figure 84- A gel documentation system required to visualize DNA fragments (Bio-Rad 2017).

Consumables and Reagents

- 10% bleach solution or commercial DNA-removing decontaminant (e.g. DNAerase)
- 70% ethanol
- 1.5 mL sterile microcentrifuge tubes
- Agarose powder (molecular biology grade; e.g. Fisher BioReagents BP160100)
- 0.5X Tris Borate EDTA Buffer (TBE; Thermo Scientific™ #B52) OR 1X Tris Acetate EDTA Buffer (TAE; Thermo Scientific™ #B49)
    - OR if making up buffer solutions from scratch:

        - EDTA- 0.5 M, pH 8.0
        - NaOH (sodium hydroxide)
        - Tris base (Sigma-Aldrich #10708976001)
        - Boric acid (for TBE; Sigma-Aldrich #B0394) or acetic acid (for TAE; Sigma-Aldrich #A6283)
    - NOTE- TBE buffer is normally used for extended/long runs or repeated runs in the same buffer. TAE has better conductivity than TBE and DNA fragments will migrate faster through the gel.
- DNA intercalating dye (Ethidium Bromide- powdered or aqueous form; e.g. Sigma-Aldrich #E7637)
- 6X DNA Loading Dye (containing bromophenol blue, e.g. Thermo Scientific™ #R0611)
- 1kb DNA ladder (Promega #G7541)

Preparing Reagent Stock Solutions (WHO & CDC 2011)

<u>EDTA; 0.5 M, pH 8.0 (100 ml):</u>

1. Dissolve 18.6 g EDTA in 100 mL distilled H_2O.
2. Adjust pH to 8.0 with 10 M sodium hydroxide (NaOH) (~5 mL).
3. Store at room temperature.

<u>TAE (Tris/acetate/EDTA) electrophoresis buffer, 50X stock solution*</u> (if making up solution from scratch)

1. To 750 mL of distilled H_2O add:
2. 242 g Tris base
3. 57.1 mL of acetic acid

4. 100 mL of 0.5 M EDTA pH 8.0
5. Add distilled H_2O to 1000 mL and mix well on a stir plate.
6. Store at room temperature.

*TAE stock solution should be diluted to 1X in H_2O before use.

TBE (Tris/borate/EDTA) electrophoresis buffer, 10X stock solution* (if making up solution from scratch)

1. To 900 mL of distilled H_2O add:
    a. 108 g Tris base (890 mM)
    b. 55 g boric acid (890 mM)
    c. 40 mL 0.5 M EDTA, pH 8.0 (20 mM)
2. Add distilled H_2O to 1000 mL and mix well on a stir plate.
3. Store at room temperature.

*TBE stock solution should be diluted to 0.5X in H_2O before use.

Ethidium bromide (EtBr), 10mg/mL (if using powdered form)

1. Dissolve 0.2g EtBr in 20 mL distilled H_2O
2. Mix well and store in the dark in 1 mL aliquots at 4°C.

Gel Electrophoresis Protocol

Making a Standard 1% Agarose Gel (Addgene 2018; Kasibhatla et al. 2006; Lee et al. 2012; Sambrook & Russell 2001)

1. Weigh out 1 gram of agarose powder.

2. Mix agarose powder with 100 mL x1 TAE or 0.5X TBE in a microwavable flask. Be sure to use the same buffer as the one in the gel box. Different buffers should not be mixed.
3. Microwave until the agarose is completely dissolved (approximately 1-3 minutes). Take caution not to over boil the solution.

NOTE: **The flask can get very hot and the solution has a tendency to boil over. Stop and gently mix the flask periodically while heating to prevent this from occurring.**

4. Let the agarose solution cool to approximately 50-60 °C (approx. 3-5 minutes).
5. Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (2-3 µL per 100 mL gel). EtBr binds to the DNA and allows for visualization of the DNA using UV light.

NOTE: **EtBr has potential carcinogenic or teratogenic effects. Gloves, lab coat, and eye protection should be worn at all times. Nitrile gloves are more efficient than latex gloves at protecting against EtBr. Make sure to let the agarose cool to between 50°C and 60°C before adding the dye in order to minimize inhalational exposure (the compounds can produce a gas if the gel is too hot). Stock solutions of 10 mg/mL EtBr are commercially available. This should be kept in the dark at room temperature and can be re-used up to 10 times within 1 month. This should be discarded using institutional and local guidelines for hazardous waste. Do not pour the solution directly down the drain. EtBr can also be treated using de-staining bags (e.g. Amresco E732-25) or charcoal filtration. The treated solutions can then be discarded down the drain. Do not place gels in the normal waste disposal. Gels containing EtBr should be placed in sturdy plastic bags or containers to prevent leakage and disposed of as hazardous chemical waste (CDC 2017).**

6. Secure the gel tray in the fixed-wall gel caster with the well comb in place. Pour the agarose into the casting tray. Be sure to pour the solution slowly to prevent any bubbles from forming. If necessary, these can be pushed to the sides of the gel or popped using a pipette tip.

7. Let the gel sit at room temperature for 30-45 mins until it has completely solidified. Likewise, the gel can also be placed at 4 °C for 10-15 minutes.

Loading DNA Samples and Running the Gel

1. Once solid, place the agarose gel into the gel box and carefully remove the comb.
2. Fill gel box with 1xTAE or 0.5XTBE so the gel is completely covered.
3. Load 5 µL of the DNA ladder into the first lane of the gel.
4. Dispense 1-2 volumes (5-10 µL) of 6X DNA loading dye per 5µl sample onto a square of wax paper (i.e. Parafilm). This will produce a dot/bead.
5. Add each of the DNA PCR products to be tested to a single drop/bead of loading dye.
6. Mix the DNA samples and loading dye by pipetting up and down, then carefully load 5 µL of the mixed sample into a well of the gel. Repeat this for all DNA PCR products. **Note-** When loading the sample into the well, place the pipette into the buffer just above the well. Carefully push the sample out to fill the well. Be sure all of the sample is unloaded, before raising the pipette out of the buffer.

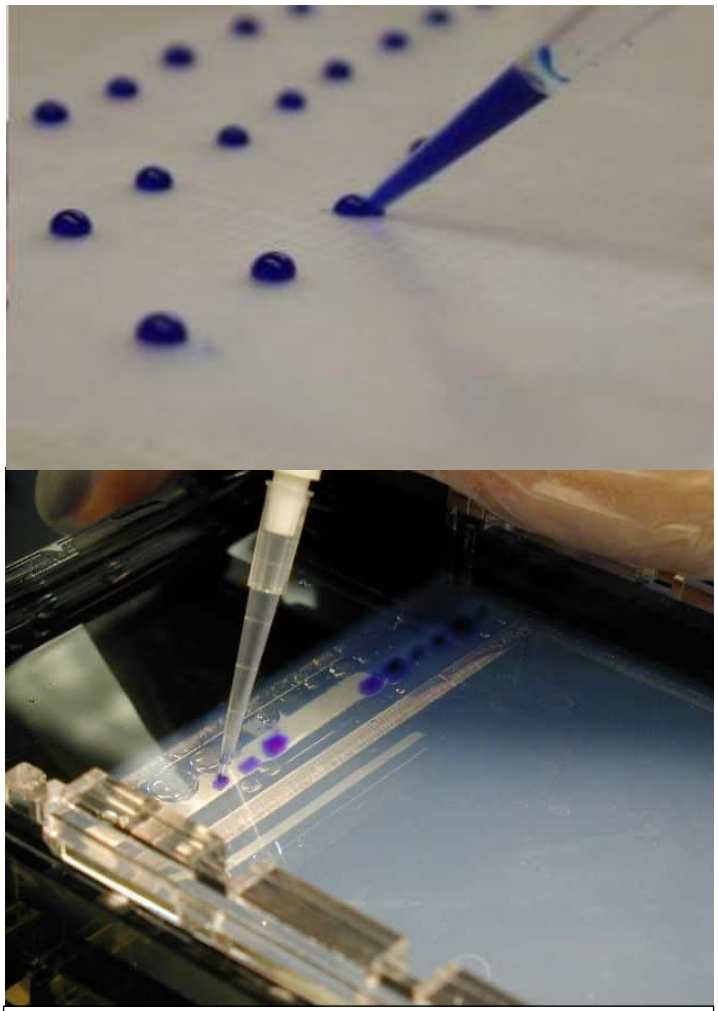


Figure 85- Loading dye (purple) is added to the DNA samples before being carefully loaded into the wells of the gel (Anderson 2019).

7. Connect the terminals from the power source to the appropriate terminals of the Gel Tank. The DNA is negatively charged and will run towards the positive (red) electrode.
8. Turn on the power source and adjust to an appropriate voltage (this varies based on many factors, but 80-120 V is recommended). Run the gel until the dye line is approximately 75-80% of the way down the gel. The time depends on the product size. Larger bands will need to run longer while smaller bands will need to run for less time. A typical run time is about 40 minutes to 1.5 hours, depending on the gel concentration, voltage and product size. Track the samples using the loading buffer to ensure the samples do not run off the gel.
9. Turn off the power, disconnect the electrodes and remove the gel from the gel box. Be sure to thoroughly rinse and clean the gel box with distilled water and allow to dry.

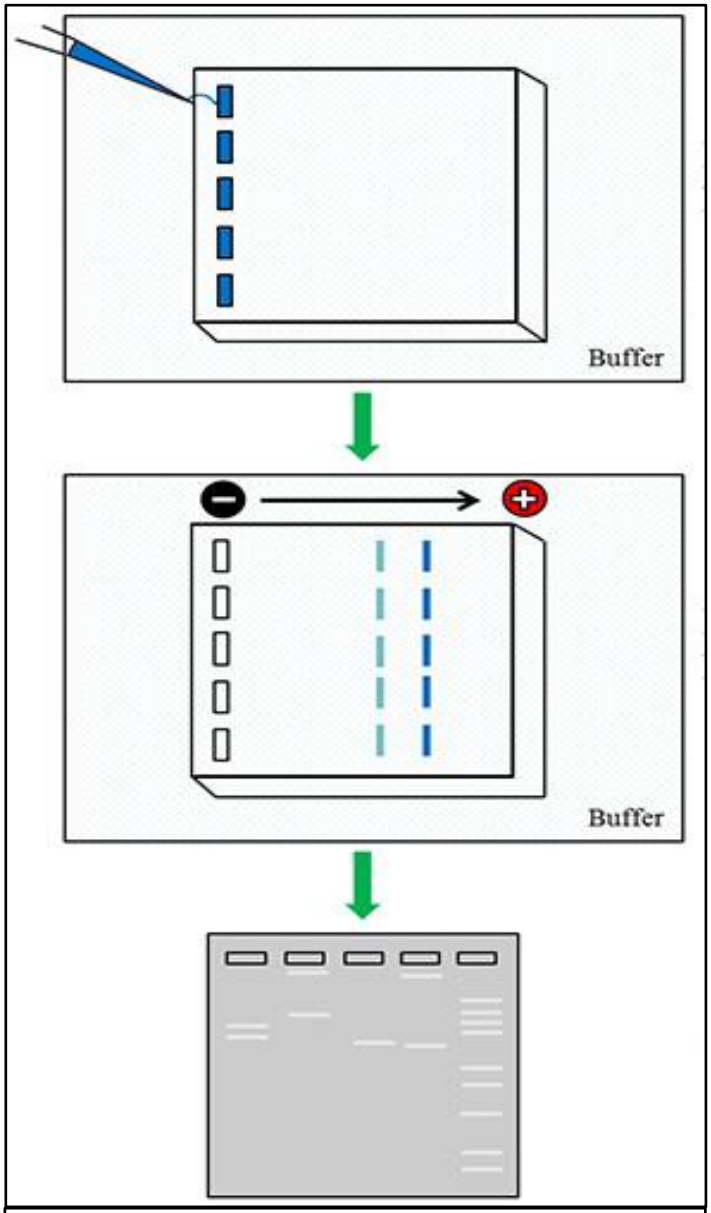


Figure 86- Agarose gel electrophoresis. DNA samples are loaded into the gel with a pipette. An electrical field is applied to separate samples. The stained gel is then visualized using a UV light source (Sambrook et al. 1989).

Analysis of PCR Products on an Agarose Gel

1. Visualize or photograph DNA fragments using any gel documentation imaging system with UV light (e.g. Gel Doc XR Bio-Rad Laboratories).
2. The negative control should not be amplified. The negative and positive control should have a banding pattern as expected.

Note- When using UV light be sure to wear safety goggles or a face shield, as well as gloves and a lab coat.

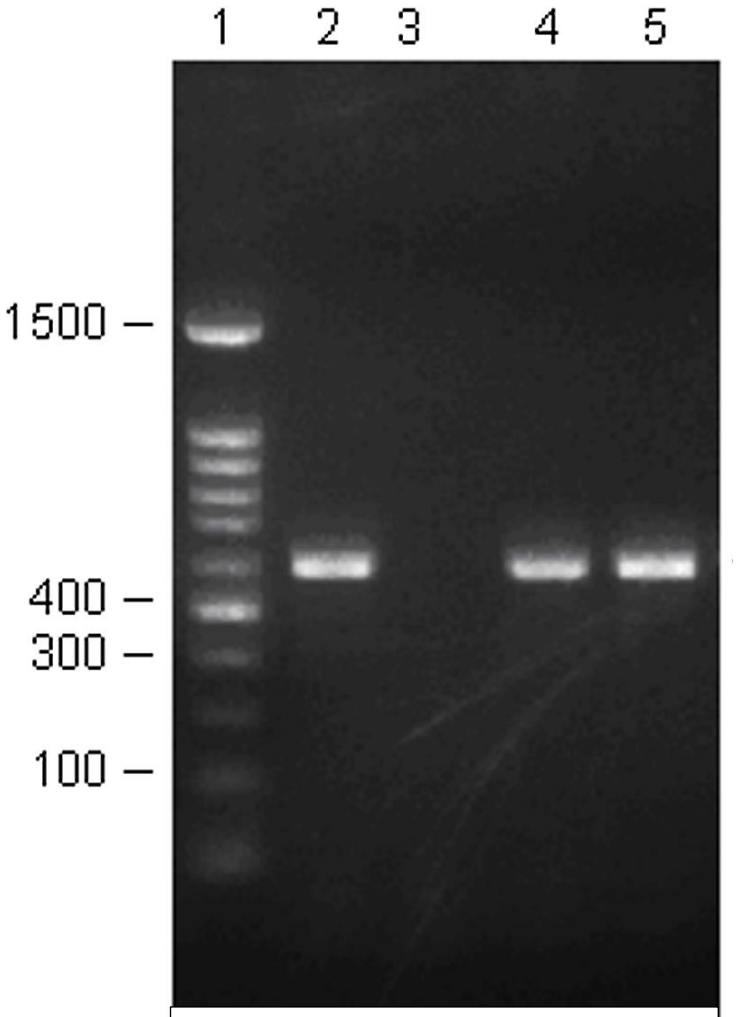


Figure 87- Gel electrophoresis PCR analysis of *B. pseudomallei* 550bp 16S gene. Lane 1- DNA ladder, 2- Positive control, 3- Negative control, 4-5- Sample DNA (Jilani et al. 2016).

Troubleshooting and Modifications to Conventional PCR and Gel Electrophoresis

There are several methods that can help to increase the resolution of DNA bands, such as running the gel at a lower voltage for longer or loading less DNA into the wells. If bands are running too close together, the agarose percentage of the gel can also be adjusted so they have better separation. A higher percentage agarose gel can be used to distinguish smaller bands while a lower percentage gel can be used to help separate larger bands. Using too much running

buffer may also distort bands, while using too little will cause the gel to partially dry out. An uneven gel can also cause issues with the electrical current and rapid migration of DNA at edges, which can lead to bands having a smiling or sad (frowning) shape (Blair 1991; Voytas 2000). See examples of problems associated with gel electrophoresis in Figure 88 below.

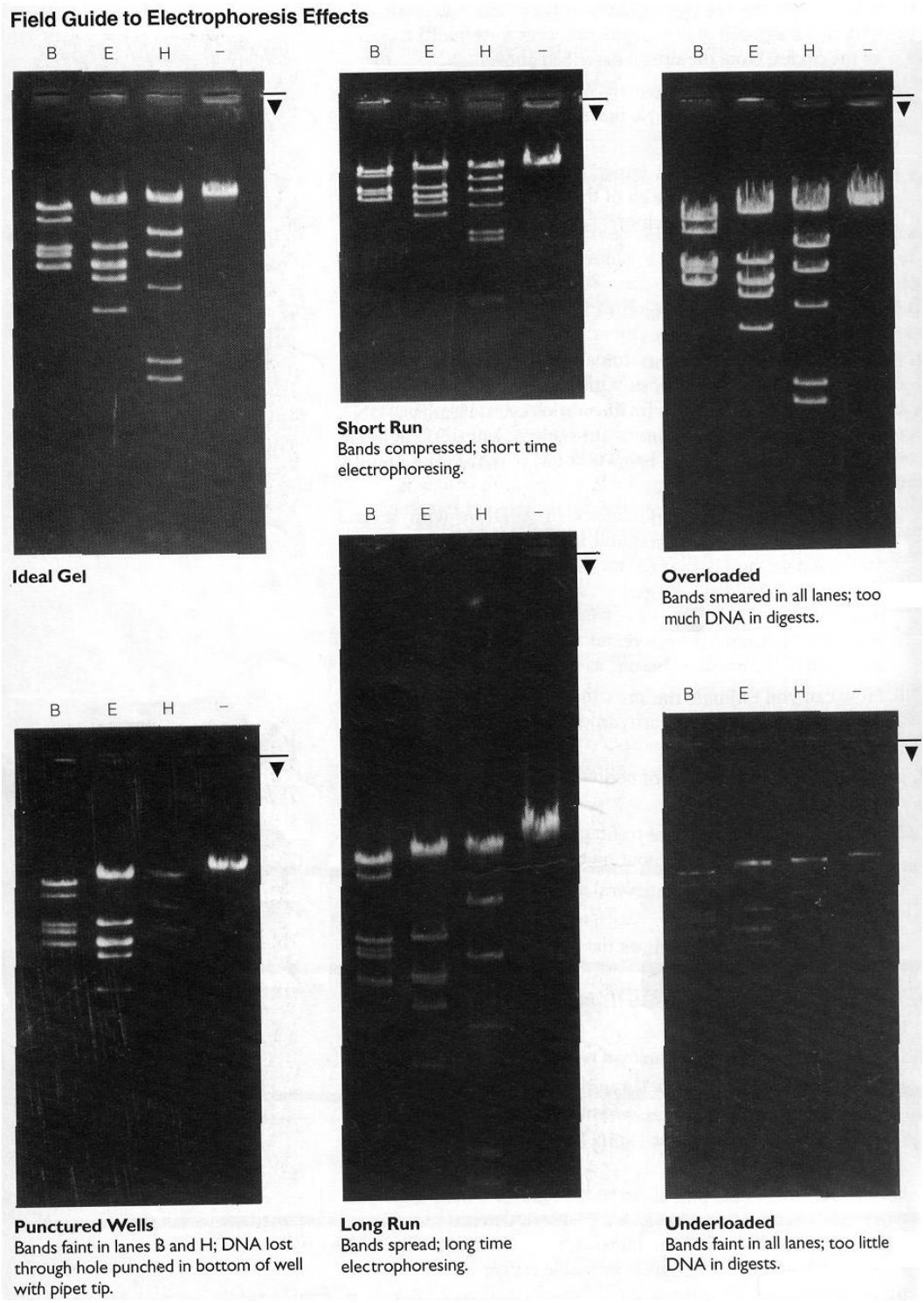


Figure 88- Several unexpected band effects may be seen when visualizing the finished gel product. These issues are often remedied by rerunning the gel again at a different voltage or using more or less of the DNA product (University of San Diego 2018). See additional conventional PCR and electrophoresis troubleshooting methods in Table 18 below.

Table 18- Possible causes and recommendations for common problems associated with conventional PCR and gel electrophoresis (Bio-Rad 2021).

Observation	Possible Cause	Recommendation
No bands or faint bands	Extension time is too short	Double check the cycle profile- generally use 1min/kb.
	Annealing temperature is too high	Double check the primers for proper T_m (melting temperature). Optimize temperature as needed.
	Template is degraded	Degraded template will usually have a smear in the lane. Re-extract DNA and repeat.
	PCR contains inhibitors	Dilute existing template DNA and repeat.
	Component was left out of master mix	Ensure that all reagents were added and repeat. If this still fails, throw out reagents and make fresh stock solutions.
Nonspecific bands	Too many cycles	Use fewer cycles if the template concentration is high.
	Thermocycler ramping speed is too low	The ramp speed should always be set to the maximum.
	Too much primer	Double check dilutions or use a lower concentration.
	Contamination of the master mix	Make new master mix and repeat.
	Contaminants of the PCR water	If bands appear in negative control, toss out water and repeat with fresh stocks.
	Too much template	Dilute existing template and repeat.

Smeared bands	Template is degraded	Re-extract DNA and repeat.
	Contamination	Use fresh reagents and repeat.

Chapter Summary

Conventional PCR is a sensitive and specific method used for *B. pseudomallei* detection. With conventional PCR, PCR products must be further examined after the reaction has run to ensure the correct product has been amplified after completing the reaction cycle. This is usually done by analyzing the size of the DNA product by means of gel electrophoresis. The preceding chapter describes a method for conventional TTS1-PCR detection of *B. pseudomallei* and agarose gel electrophoresis for DNA product confirmation. *B. pseudomallei* strain typing and bacterial species identification using conventional PCR techniques are described in Chapter 16.

Chapter 15: Real-Time Polymerase Chain Reaction (PCR) Detection of *B. pseudomallei*

Chapter Overview and Introduction to Real-Time PCR

Real-time PCR (quantitative PCR, qPCR) is a popular method used for the detection of microbial pathogens. Fluorescence is measured after each cycle, allowing the user to monitor the progress of the PCR as it occurs (i.e., in real time). The increase in fluorescence is proportional to the amount of amplified product and is quantitatively measured throughout the PCR run. Specialized thermal cyclers that are able to detect fluorescence are used to detect a signal while amplification occurs in "real-time" (Bustin 2000; Kubista et al. 2006).

The main advantages of real-time PCR are that it provides a fast and high-throughput (large-scale) means of detection through simultaneous amplification and visualization of newly formed DNA products, eliminating the need for a post-processing gel electrophoresis step. Real-time PCR also helps to prevent contamination of samples downstream, since no further manipulation is required to visualize the products after amplification. Determination of the bacterial load by real-time PCR can also be used to indicate a patient's response to antimicrobial treatment in real-time (Kralik & Ricchi 2017; Kubista et al. 2006).

Since most clinical and environmental samples contain low numbers of bacteria, a pre-enrichment step using an enrichment culture media (such as Ashdown media in the case of *B. pseudomallei*) is recommended prior to DNA extraction to promote the growth of the bacteria for downstream real-time PCR detection. This also helps to eliminate possible PCR inhibitors or competing microbiota that may be present in the sample (see Chapter 10 and Chapter 13 for further detail) (Kralik & Ricchi 2017).

Types of Real-Time PCR Assays

There are two primary methods used for the visualization of amplified DNA during real-time PCR: 1) Non-specific fluorescent DNA dyes such as SYBR® Green I, which use DNA-targeted intercalating fluorescent dyes that show enhanced fluorescence upon binding and 2) Fluorescently-labelled sequence-specific DNA probes, which use a sequence-specific fluorescent probe designed to bind to the target DNA between the two PCR primers (Figure 89) (Holland et al. 1991; Kubista et al. 2006; ThermoFisher 2014).

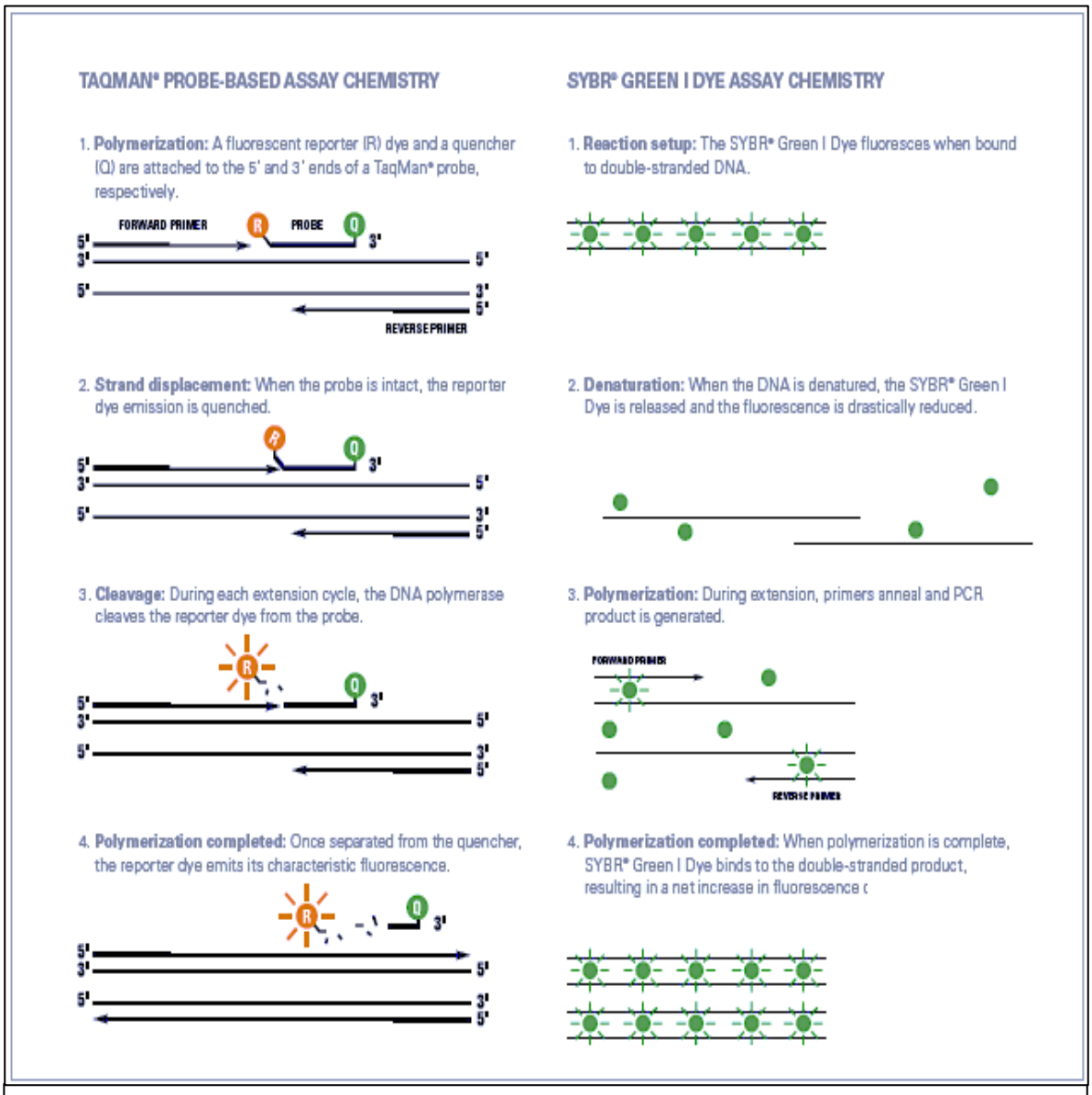


Figure 89- Comparison of TaqMan® and SYBR® Green real-time PCR processes (ThermoFisher 2014).

1. SYBR® Green I PCR: SYBR® Green I is a fluorescent DNA binding dye that binds to any double-stranded DNA. Once incorporated into double stranded DNA, the dye emits a stronger signal then when in solution alone (Figure 89). As the amount of the template DNA increases with each amplification cycle, the number of bound fluorophores and intensity of the fluorescent signal increases. A SYBR® Green real-time PCR protocol follows the conventional PCR amplification profile. This type of real-time PCR lacks in specificity as the dye will incorporate into any double stranded DNA, whether it is target or non-target product.

2. Fluorescently Labelled Sequence-Specific Probes: Fluorescently labeled probes, such as TaqMan® or fluorescence resonance energy transfer (FRET) probes, provide a highly sensitive and specific method of detection. A probe is a short, gene-specific sequence that is designed to bind the target DNA between the two PCR primers. With TaqMan® assays, a reporter dye is attached to the 5' end of the probe. On the 3' end is a quencher dye, which stops fluorescence of the reporter dye when the probe is intact. During PCR, the primers and probe anneal to the target DNA. DNA polymerase extends the primer upstream of the probe and eventually the 5' exonuclease activity will cleave it (break it apart). Once the probe is cleaved, this causes the reporter dye to fluoresce. This signals that amplification has occurred and allows for accumulated PCR product to be visible over time. (Figure 89).

   PCR with fluorescence resonance energy transfer (FRET) probes utilize two labelled probes that bind to the PCR product (Figure 90). When the two probes bind to the DNA, the fluorophores come into close proximity, enabling energy to transfer from a donor fluorophore to an acceptor fluorophore. This results in a fluorescent signal that is proportional to the amount of target sequence (QIAGEN 2010).

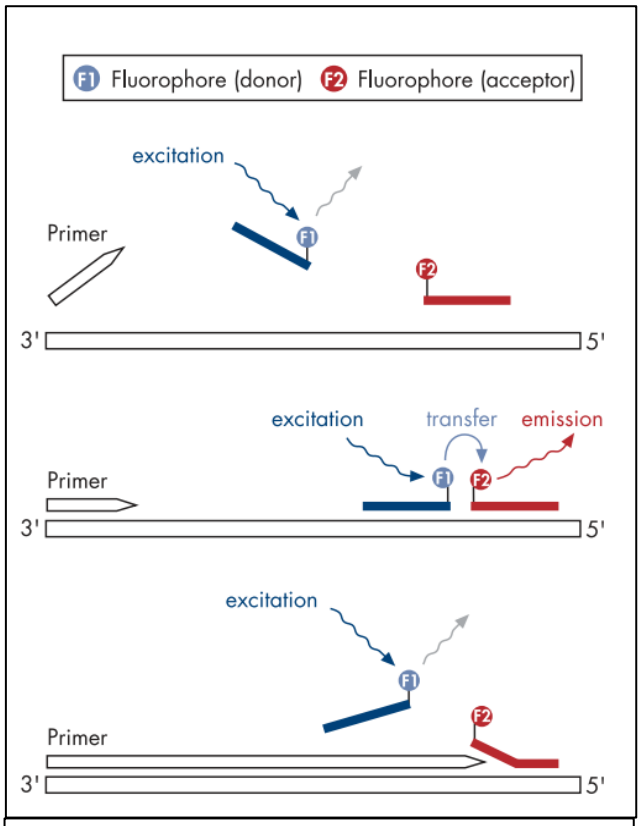


Figure 90- FRET probe method: No fluorescence is detected when not bound to the target sequence. During the PCR annealing step, both probes hybridize to the target sequence, bringing the donor and acceptor fluorophores into close proximity. This

Real-Time PCR Data Analysis

At the start of the qPCR reaction, fluorescence remains at a low level and is not detectable. Eventually, enough amplified product will accumulate and will give off a detectable signal. The cycle number at which this occurs is called the cycle threshold, or C_t, or the number of cycles required for the fluorescent signal to cross the threshold level (ThermoFisher 2014). The C_t value is proportional to the quantity of template present at the start of amplification and is inversely proportional to the amount of target DNA. This means that the lower the C_t value, the greater the amount of target DNA present (Figure 91). If a large amount of template is present at the start of the reaction, fewer amplification cycles are required to produce a fluorescent signal above the threshold and the reaction will have a low, or early C_t value. Likewise, if less template is present at the beginning of the reaction, more cycles are necessary for the fluorescence signal to rise above background and the

reaction will have a higher/later C_t value. Generally, C_t values of ≤ 29 indicate an abundant amount of the DNA target. Values between 30-37 indicate a moderate amount of target, and 38-40 indicate minimal amounts of target (ThermoFisher 2014).

The change in fluorescence over the course of the reaction is measured by a thermocycler machine that combines thermal cycling with fluorescent dye visualization. By plotting fluorescence against the cycle number, the thermocycler produces an amplification plot that displays the amount of amplified product over the duration of the entire PCR run. The amplification plot portrays the exponential phase and a plateau phase of the reaction. During the exponential phase, the amount of PCR product increases with every cycle. However, PCR reagents are consumed as the reaction progresses and will enter the plateau phase (Figure 91).

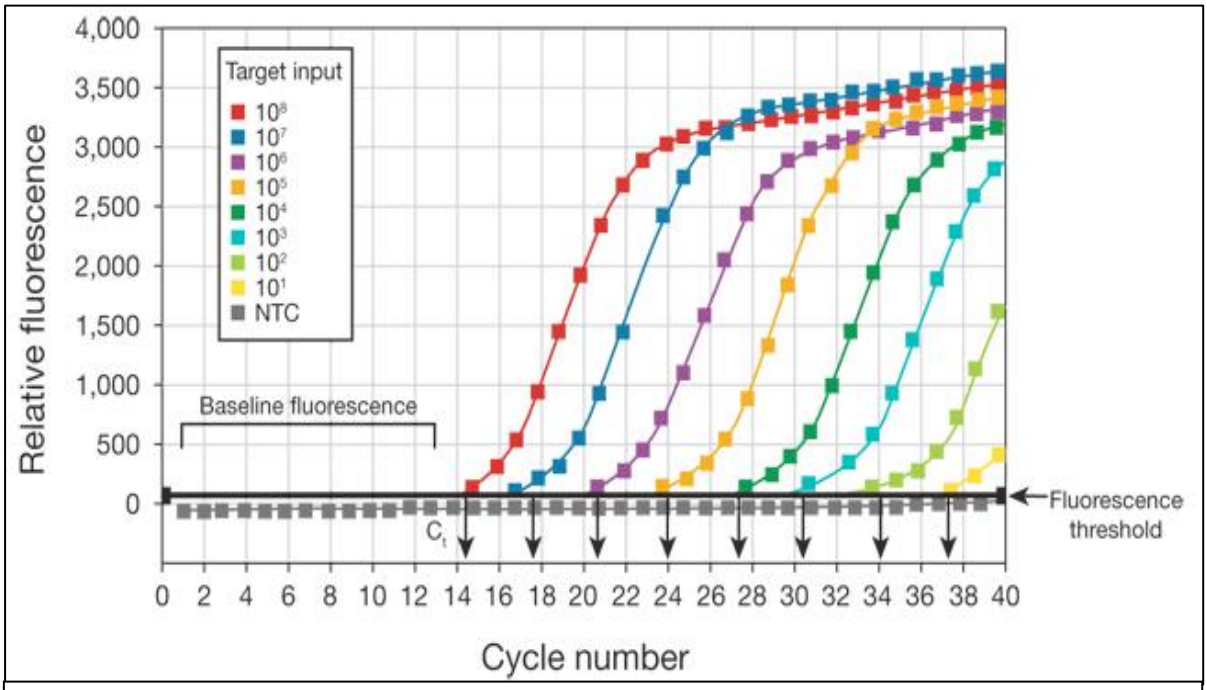


Figure 91- Amplification plot showing relative fluorescence vs cycle number. In this plot, the number of PCR cycles is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the reaction tube, is shown on the y-axis (ThermoFisher 2014).

There are two approaches that can be used for data analysis of real-time PCR: absolute and relative. Absolute quantification is used when the quantity of nucleic acid, called the "copy number", per amount of sample is required (ThermoFisher 2014). To perform this type quantification, a template of known concentration is serially diluted and amplified alongside the unknown samples. The data is then graphed to generate a standard curve (Figure 92). The standard curve is plotted as target concentration vs resulting C_t value. The sample C_t values can then be used to determine the DNA product copy number (i.e. amount of DNA product).

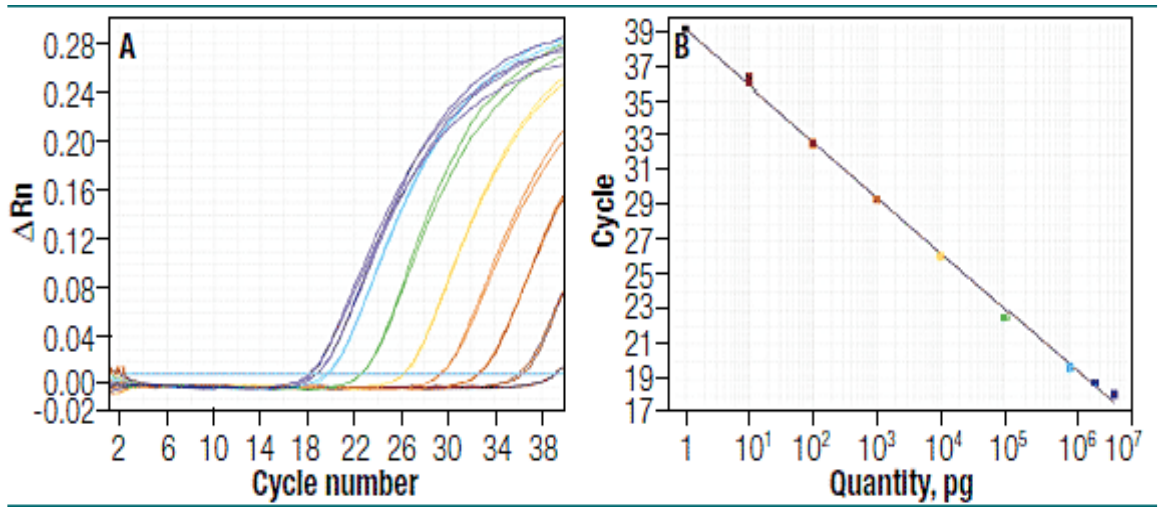


Figure 92- Absolute quantification analysis: Amplification plot showing a dilution series (A) used to generate a standard curve based on C_t values (B) (ThermoFisher 2014).

Relative quantification is used when the relative expression level of a gene of interest is wanted. With relative quantification, changes in gene expression are measured against another reference sample (such as an untreated control sample). In this type of analysis, a standard curve is not generated as the change can be calculated from the C_t values of the unknown and reference samples. Instead, the result output is a ratio: the relative amount (difference) of a target nucleic acid versus equivalent amount of test and control sample. An example of when relative quantification would be used is when measuring gene expression levels in response to a drug. Here, the level of

gene expression in a particular gene of interest in a treated sample is compared to the level of gene expression in an untreated sample (Figure 93) (ThermoFisher 2020a).

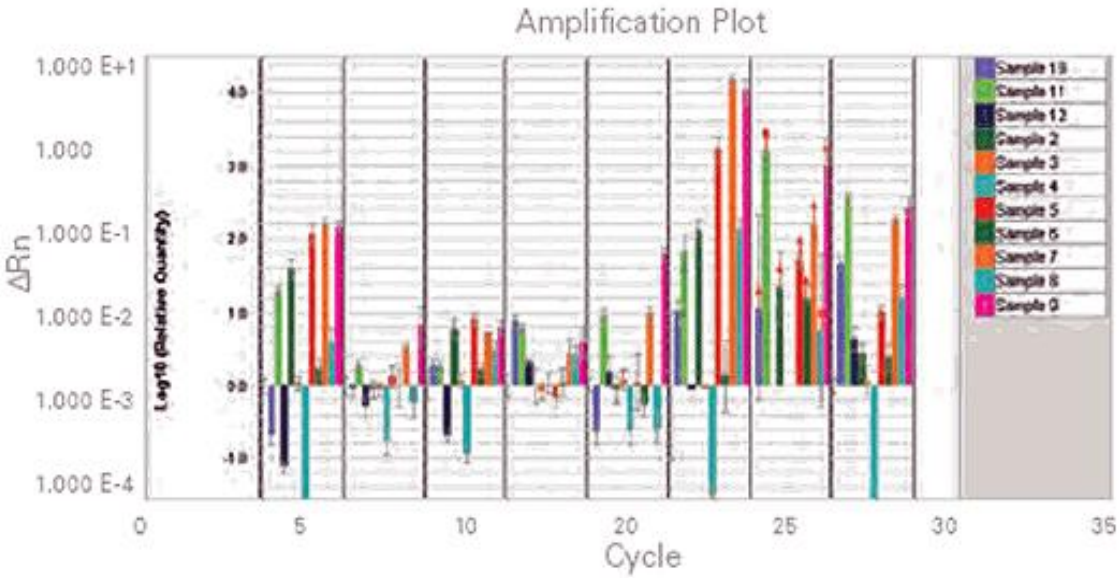


Figure 93- Relative quantification analysis where changes in gene expression level in a given sample are measured against a reference sample (ThermoFisher 2020a).

For further information about real-time PCR set-up and data analysis:

1. ThermoFisher Real-Time PCR Handbook available at: https://www.thermofisher.com/au/en/home/global/forms/life-science/digital-experience.html
2. QIAGEN: Critical Factors for Successful Real-Time PCR available at: https://www.qiagen.com/au/resources/resourcedetail?id=f7efb4f4-fbcf-4b25-9315-c4702414e8d6&lang=en.

NOTE- As a result of the very high specificity and sensitivity of real-time PCR method, protocols described here must be followed as written. Reagents and procedures should not be altered without prior testing and appropriate assay validation. There are also high initial setup and

training costs associated with the procedure that labs should account for beforehand. If a lab is going to conduct real-time PCR testing, they must be able to follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed below.

Specifics of the *Burkholderia pseudomallei* Real-Time PCR

A number of PCR assays have been developed and evaluated for the detection of *B. pseudomallei,* including several conventional PCR methods (Gal et al. 2005; Lew & Desmarchelier 1994; Winstanley & Hart 2000). These are discussed in further detail in Chapter 14. The most widely used real-time PCR assay utilizes primers and probes that are specific to a 115-base-pair region within *orf2* (open reading frame 2) of a *B. pseudomallei* type III secretion system (TTS) gene cluster, called TTS1. This has been demonstrated to be a highly sensitive and specific gene target that is able to distinguish *B. pseudomallei* from other closely related *Burkholderia spp.* in both clinical and environmental samples (Kaestli et al. 2007; Novak et al. 2006).

Primer and Probe Sequences-

- BPTTS4176F Forward: 5'- CGTCTCTATACTGTCGAGCAATCG -3'
- BPTTS4290R Reverse: 5'- CGTGCACACCGGTCAGTATC -3'
- BPTTS4208P Probe: FAM-CCGGAATCTGGATCACCACCACTTTCC-BHQ

Quality Controls (CLIA 2010; U.S. EPA 2004)

One of the most important aspects of any laboratory method is the use of the quality controls to ensure that the procedure has worked correctly and the results are reliable. Laboratories should test positive and negative controls on a regular basis to check that the PCR is working as expected. Routine PCR controls should include:

4. Negative control- PCR negative controls are used to show that no contamination has been introduced into the master mix or during PCR analysis. No amplification of nucleic acids should be detected in these wells. Two types of PCR negative controls that should be used include:
    a. Non-template control- PCR grade water should be used as a non-template control (NTC). NTCs should be included in each PCR run. It is also advisable to include one NTC for work done in both the clean PCR room/area and dirty PCR room/area.
    b. DNA extraction blank- This is used to examine if there is any contamination during sample processing and PCR analysis. Extraction is performed using sterile water and this is processed alongside the test samples. The same extraction and PCR procedures should be used as the test samples.
5. Positive control- PCR positive controls are used to show that PCR reagents have been prepared appropriately. These should be run with each PCR batch. Positive controls are typically performed through adding an external control to the master mix. This can be:
    a. Purified DNA from a known isolate of *B. pseudomallei* containing the sequence of interest. Care must be taken to ensure no cross-contamination occurs between the positive controls and the samples or negative controls. *B. pseudomallei* control DNA should be diluted in sterile PCR-grade water to minimize the risk of cross-contamination.
    b. A specific template containing the gene sequence to be amplified, including primer binding sites.
    c. A related sequence that has been shown to amplify with an efficiency that is comparable to the target sequence.

A protocol for pLepBaBp+, a plasmid which contains DNA sequences that act as a positive control for PCR detection of several bacterial species, including *Bacillus anthracis*, pathogenic *Leptospira spp.* and *Burkholderia pseudomallei*, is described below. Specifics on pLepBaBp+ plasmid propagation can also be found at the end of the chapter.

6. Internal positive control (IPC)- The presence of PCR inhibitors or errors during sample extraction are common causes of false negative PCR results. These can be easily examined using an Internal Positive Control (IPC), which is used to confirm DNA amplification, detect false negative results and if there are any inhibitory substances in a sample. IPC should be added to each reaction mix and should always show a positive result. This is particularly important if testing DNA from human clinical specimens. If the IPC is negative, it indicates the specimen may contain PCR inhibitors and will need to be diluted until the inhibitor is no longer affecting the C_t values. It may also indicate a problem with specimen collection or the DNA extraction. PCR assays often utilize a DNA dilution factor of 1 in 10 (1:10) or 1 in 100 (1:100), however this may vary depending on the level of inhibition.
    a. A protocol using the TaqMan® Exogenous Internal Positive Control (IPC) is described in the real-time PCR protocol below. If an exogenous IPC is not available, RNase P can be used as an internal control for human clinical specimens. For further information about RNase P and use of the RNase P internal control with human clinical specimens see: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F4316848_TaqMan_RNaseP_Cntrl_PI.pdf&title=UHJvZHVjdCBJbmZvIFNoZWV0OiBUYXFNYW4gUk5hc2UgUCBDb250cm9sIFJlYWdlbnRzIEtpdA==.

General Considerations and Safety Precautions

The high sensitivity of real-time PCR significantly increases the risk for cross-contamination. False positive results may occur if the specimen or reagents become contaminated. Good laboratory practice should be used during preparation of samples to minimize the risk of cross-contamination. The following precautions are recommended:

1. Maintain separate areas, equipment and supplies for pre- and post- PCR processes. Workflow in the laboratory should occur in a unidirectional manner. Never bring amplified PCR products into the master mix set up area.
2. Change gloves regularly, especially if contamination is suspected to have occurred.
3. Open and close sample tubes and plates carefully. Tubes should be centrifuged before opening. Keep tubes closed when possible.
4. Use positive-displacement pipettes or aerosol-barrier filter pipette tips.
5. Clean lab benches and equipment with 10% bleach solution or commercial DNA decontaminant, followed by 70% ethanol after every set up. UV light may also be used with proper precautions.
6. All patient specimens and positive controls should be considered potentially infectious and handled with appropriate safety precautions. Perform all manipulations of samples within a biological safety cabinet (BSC) where available.
7. Use appropriate PPE including gloves, eye protection, and lab coats while handling samples, reagents, pipettes, and other equipment and reagents.
8. PCR reagents should be kept on a cold block or on ice during use to ensure stability.

Reagent Storage and Handling

- Store all dried primers and probes and the positive control at 2-8°C until re-hydrated for use.
- Once the primers and probes have been diluted to the correct working concentration for PCR, they should be stored at -20°C.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes and master mix enzyme must be thawed before use and kept on a cold block or ice.

Consumables and Reagents

- 10% bleach (10:1 water: concentrated bleach- prepare fresh weekly) or commercial DNA-removing surface decontaminant liquid (such as DNase or RNase AWAY™)
- 70% ethanol
- 1.5 mL microcentrifuge tubes (PCR grade)
- 96 well polypropylene plates, tube strips or individual PCR tubes
- Pre-sterilized aerosol-barrier filter tips (2-20 µL, 10 µL, 200 µL, and 1000 µL)
- PCR cap strips or adhesive plate seals (e.g. MicroAmp® Optical Adhesive Film)
- Adhesive plate seals applicator (e.g. MicroAmp® Adhesive Film Applicator)
- Commercial real-time PCR master mix (e.g. LightCycler® FastStart DNA Master HybProbe Kit)
- Probe (see sequence listed above)
- Forward and Reverse Primers (see sequences listed above)
- DNA extracted from samples to be tested
- TaqMan® Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems ™)
- Positive control pLepBaBp+ plasmid DNA at a concentration of 10-12g/µL or other known positive control
- NTC and negative DNA extraction “blank” control
- PCR grade water
- Laboratory pens/markers
- Kimwipes or other laboratory tissue
- Personal Protective Equipment (lab coat, gloves, eye protection, shoe covers, hair net)

Equipment

- Vortex
- Racks/holders for 1.5mL microcentrifuge tubes, tube strips or PCR plates

- Microcentrifuge
- Real-time PCR thermocycler machine (e.g. Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument or Applied Biosystems 7500 Fast Real-Time PCR System)
- Pipettes (1-10 µL, 2-20 µL, 20-200 µL, and 100-1000 µL)

NOTE- The primary type of equipment is the real-time PCR thermocycler machine. When selecting an appropriate machine, be sure to consider the fluorescent filters used. These filters should be able to detect the wavelengths of light that are emitted by the fluorophores coupled to the probes. A desktop or laptop computer and the relevant real-time PCR thermocycler software are also necessary to view and analyze results. It is recommended that real-time PCR machines and computer used for data analysis are plugged into a backup battery with surge protection. This will protect data and keep PCR reactions running in the event power is lost (Csako 2006).

Preparation of DNA Template for Real-Time PCR

Commercial kits such as the QIAamp DNA Mini Kit (QIAGEN), Chelex©, or crude boil-prep extraction methods can be used for the extraction of genomic DNA for real-time PCR detection (See Chapter 13 for further detail and protocols). However, it is important that DNA extraction from clinical specimens is performed in a separate room or area than the PCR reaction assembly area. If separate rooms are not possible, separate laboratory benches should be used, along with separate pipettes, laboratory coats, and gloves. Decontamination of surfaces and equipment with 10% bleach followed by 70% ethanol should be done after handling any DNA template.

Extracted DNA is typically very concentrated, which may lead to PCR inhibition and non-specific background signal. DNA quality control should be performed after extraction to check for purity and concentration as described in Chapter 13. An Internal Positive Control (IPC) can be added to each reaction mix to indicate possible assay inhibition. The IPC should always have a positive PCR result. If

the IPC is negative, it typically indicates that the DNA may contain PCR inhibitors and will need to be diluted. PCR assays often utilize a DNA dilution factor of 1 in 10 (1:10) or 1 in 100 (1:100), however this may vary depending on the level of inhibition.

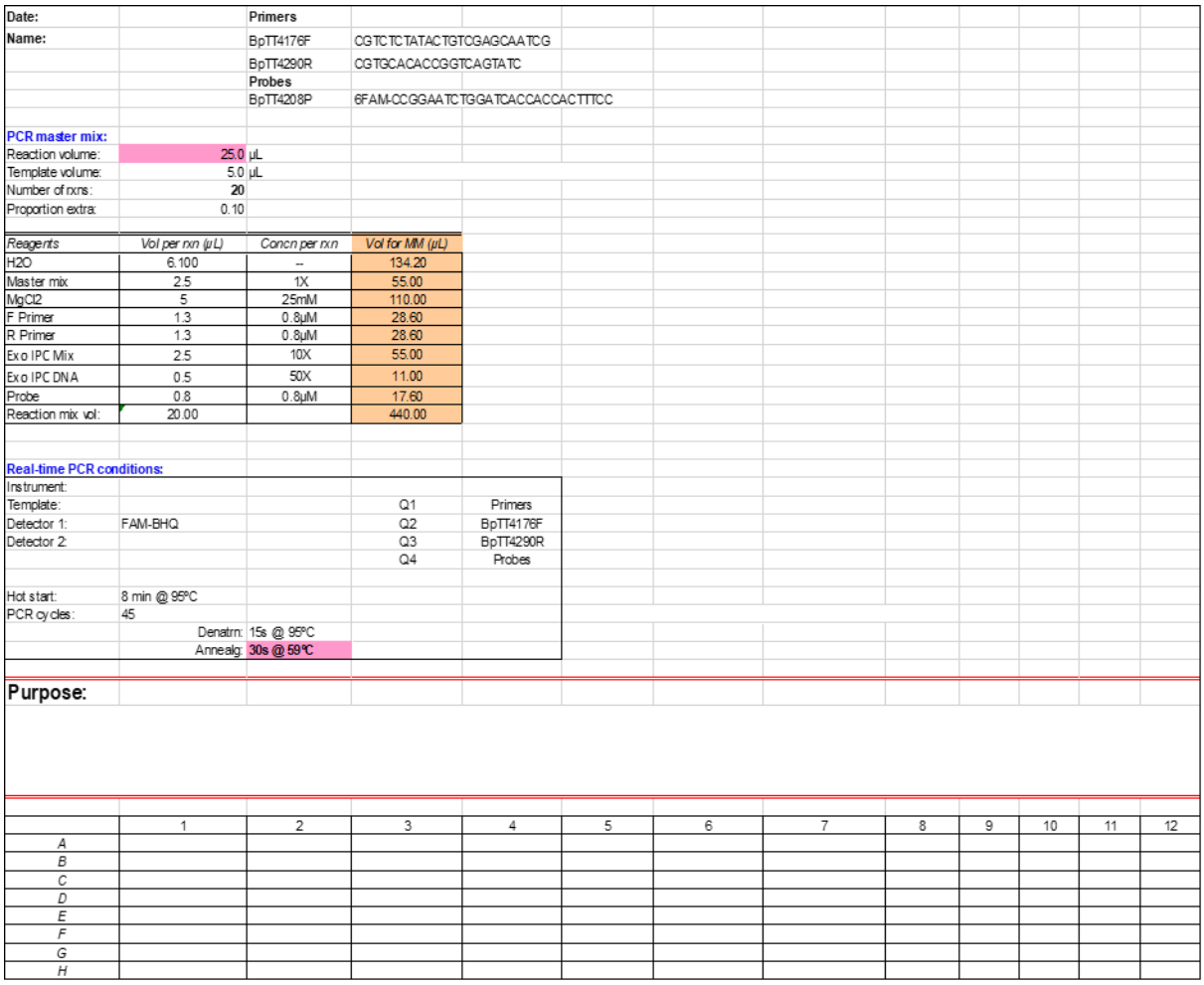
Technical Resources

- LightCycler® FastStart DNA Master HybProbe Kit Manual https://lifescience.roche.com/en_us/products/lightcycler-faststart-dna-master-hybprobe.html
- TaqMan® Exogenous Internal Positive Control (IPC) Manual http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041040.pdf

***B. pseudomallei* TTS1 Real-Time PCR Protocol** (adapted from Novak et al. 2006)

Before Starting the Procedure

A. Prior to starting, plan the experiment by filling out and printing a PCR template worksheet (see Figure 94 below for an example template).
B. Be sure sufficient quantities of working stocks of primers and probes to be used are available and ensure primers and probes have been diluted to the appropriate working concentration.



Date:		**Primers**		
Name:		BpTT4176F	CGTCTCTATACTGTCGAGCAATCG	
		BpTT4290R	CGTGCACACCGGTCAGTATC	
		Probes		
		BpTT4208P	6FAM-CCGGAATCTGGATCACCACCACTTTCC	
PCR master mix:				
Reaction volume:	25.0	µL		
Template volume:	5.0	µL		
Number of rxns:	20			
Proportion extra:	0.10			

Reagents	*Vol per rxn (µL)*	*Concn per rxn*	*Vol for MM (µL)*
H2O	6.100	--	134.20
Master mix	2.5	1X	55.00
MgCl2	5	25mM	110.00
F Primer	1.3	0.8µM	28.60
R Primer	1.3	0.8µM	28.60
Exo IPC Mix	2.5	10X	55.00
Exo IPC DNA	0.5	50X	11.00
Probe	0.8	0.8µM	17.60
Reaction mix vol:	20.00		440.00

Real-time PCR conditions:				
Instrument:				
Template:			Q1	Primers
Detector 1:	FAM-BHQ		Q2	BpTT4176F
Detector 2:			Q3	BpTT4290R
			Q4	Probes
Hot start:	8 min @ 95ºC			
PCR cycles:	45			
	Denatrn:	15s @ 95ºC		
	Annealg:	30s @ 59ºC		

Purpose:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 94- Example of a PCR template worksheet. This should be filled out prior to starting the protocol.

Primer and Probe Dilutions

Primers and probes must be diluted from the concentrated stocks into working stocks. When diluting primers and probes, use sterile filter tips and be sure to work in a sterile area that is free from possible extraneous template DNA in order to avoid cross-contamination. It is best to work in a clean PCR cabinet or hood if this is available.

Concentrated stocks of primers and probes should be stored at -20°C. Working stocks can be stored at 4°C if used regularly. Ensure probes are stored in the dark as they are light-sensitive. Primer and

probe stocks should be aliquoted when they first arrive in the laboratory before freezing for longer-term storage. The length of time that primers and probes should be stored will vary. Using positive controls with each reaction will help to determine when primers and probes should be replaced (QIAGEN 2010).

For the real-time *B. pseudomallei* TTS1 PCR assay, primer and probes should be diluted to 8 µM before being used in the reaction. To do this:

From Concentrated Liquid Stock:

This is done is two steps:

1. Find the volume of primer stock needed:

   (Concentration of Stock)*(Volume of Stock) = (Concentration Wanted)*(Volume Wanted)

2. Find the volume of nuclease-free water to add:

   (Volume Wanted) – (Volume of Stock)

Example: Want a primer concentration of 8 µM in 200 µL. The given primer concentration is 400 µM.

1. Find the volume of primer stock needed:
   A. Concentration Wanted: 8 µM
   B. Volume Wanted: 200 µL
   C. Concentration of Stock: 400 µM
   D. Volume of Stock (µL) =

      (400 µM)*(x µL) = (8 µM)*(200 µL)

      (400 µM)*(x µL) = 1600 µM*µL

      x µL = (1600/400) µL

      x = 4 µL of stock primer

2. Find the volume of nuclease free water to add:

200 µL - 4 µL = 196 µL of nuclease-free water

Answer: Adding 4 µL of stock primer to 196 µL of nuclease-free water will yield a solution of 200 µL at 8 µM concentration.

From Lyophilized Stock (Freeze-Dried Precipitate Powder)**:**

Lyophilized primers and probes must be resuspended in solution before they can be used. The lyophilized primers will come with a technical data sheet that will list the quantity of primer in nanomoles (nmol). This quantity should be used to determine the concentration of the primer stock wanted. This concentration can be used to create a 100µM suspension.

*This calculation works for a 100µM final concentration. *

Example: The primer quantity given is 40 nmol.

1. Multiply the nmol concentration of the lyophilized primer or probe by 10 to get the resuspension volume in microliters:

   40 x 10 =400 µL

   Answer: Adding 400 µL of either nuclease-free water or TE buffer (see note below) to the lyophilized primers will give a concentration of 100µM.

2. Add the water or buffer directly to the tube of lyophilized primers. Ensure that all the material is rehydrated by pipetting up and down or vortexing. Look for any faint pellets or smears still at the bottom of the tube. Once resuspended, allow the primers to sit for a few minutes at room temperature, then centrifuge to the bottom and dilute to 8µM (the working stock required for the TTS1 PCR- See Dilutions from Concentrated Liquid Stock above).

NOTE: Weak buffers such as TE (10mM Tris pH 7.5-8.0, 1mM EDTA) or Tris (10mM Tris-HCl pH 8.0) are usually preferred with TE as the best choice. These buffers give a more stable environment for the primers/probes. If these options are not available, sterile, nuclease-free water can also be used.

Real-Time TTS1 PCR Protocol

Set-Up Procedure:

1. Map out where the samples will be placed in the 96-well plate using a template worksheet. Label the plate accordingly. There should be one reaction well per sample, plus the negative DNA extraction "blank" control, two no template controls (NTC) (one for both the clean and dirty PCR set-up rooms/areas) and the PCR positive control.
2. Enter the DNA clean room or designated area. Gather PCR reagents including the PCR master mix, primers, probes, and PCR grade water. Allow working stocks of primers and probes to thaw completely before use If they are stored at -20°C. Briefly vortex each tube before use to bring down any droplets.
3. Prepare master mix according to the table below. Combine Vials 1a and 1b to make the final Vial 1 mix. Make enough mix for all samples, plus two extra samples (for example, if you have 12 samples to test including all controls, make enough master mix for 14 samples).

Master Mix Reagent	µL Per Reaction
Water	6.1
Vial 1 Mix (Vials 1a and 1b combined from Lightcycler® FastStart DNA Master Hybprobe kit (Roche))	2.5
$MgCl_2$ 25 mM (Vial 2 from Lightcycler FastStart DNA Master Hybprobe kit)	5.0
Forward Primer [8 µM]	1.3
Reverse Primer [8 µM]	1.3
Probe [8 µM]	0.8
10X Exo IPC Mix (from TaqMan® Exogenous IPC Kit)	2.5
50x Exo IPC DNA (from TaqMan® Exogenous IPC kit)	0.5
Total	**20**

4. Gently mix the master mix and briefly spin in a microcentrifuge.
5. Load 20 µL of master mix into the appropriate well, according to the worksheet created in Step 1. Do this for each reaction.
6. Add 5 µL sterile PCR-grade water to the “clean room” NTC well. Use the adhesive plate film or caps to cover the wells.
7. Return all reagents to the appropriate storage area and wipe working surfaces with 10% bleach or DNA decontaminant, followed by 70% ethanol.
8. Take the plate to the dirty room/area. Put on a new laboratory coat and keep the same pair of gloves on. Carefully remove the adhesive cover or caps from plate to prevent splashing or spraying of samples.
9. Add 5 µL to the appropriate well of the following in this order:
    - Extracted sample DNA
    - Extracted negative “blank” control
    - Dirty room no template control (NTC)
    - pLepBaBp+ plasmid DNA or other known positive control template
10. Cap the columns of wells as you go, covering the wells to protect them from the positive control. Use the roller tool to secure caps or plate seal applicator to secure plate seal film. Ensure caps or film are tightly fitted to avoid evaporation from wells during PCR.
    - NOTE- Incorrectly capped or uncapped wells may cause a reaction to fail and contaminate the real-time PCR machine. If this happens, wipe down the interior of the machine with 70% ethanol.
11. Wipe down the workspace with 10% bleach or DNA decontaminant, followed by 70% ethanol and turn on the UV light for 1 hour, if it is available. Remove laboratory coat and gloves and discard the gloves.

12. If possible, spin the plate at 500xg for a few seconds to bring down any droplets and collect reagents to the bottom of the wells.
13. Transport the plate directly to the real-time PCR machine and place in the machine.

Software Procedure:

The following instructions are detailed for the Applied Biosystems (ABI) 7500 instrument. If another instrument is being used, follow the manufacturer's instructions for setting up the software and for data analysis. The thermal cycler conditions will stay the same regardless of the machine being used.

1. Turn on ABI 7500 Real Time PCR System instrument.
2. Open the plate tray and place test plate into instrument. Close the tray.
3. Launch the Applied Biosystems 7500 System Software.
4. Click "Create New Document".
5. Change "Run Mode" to "Standard 7500".
6. Next to "Plate Name", enter the experiment number. Click Next.
7. Select appropriate detectors for FAM-BHQ (for TTS1) and VIC (for the ABI IPC). Add them to the Detection in the Document window. NOTE: Refer to the 7500-programming document to create the FAM-BHQ detector.
8. Next to "Passive Reference", select "None". Click Next.
9. Using the mouse, click and drag to select boxes corresponding to the wells containing samples.
10. Next to the detector box, check the "Use" box for both FAM-BHQ and VIC ABI IPC.
11. Change the task menu to mark "U" for unknown, "S" for positive "standard" controls or "NTC" for no template controls. Enter sample names into the appropriate wells. Click Finish.
12. Select the Instrument tab. Modify the thermal cycler conditions according to the adjacent table.
13. Other settings:

	Cycles	Temp	Time
Initial denaturation	1	95°C	8 min
Amplification	45	95°C 59°C	15 sec 30 sec

a. Verify that the number of cycles is 45.

b. Change the sample volume from 20 µL to 25 µL.

c. Verify that "Run Mode" is "Standard 7500"

14. Click File→Save As, and name and save the file in an appropriate project folder.
15. Enter information about the run in the "Reason for Change Entry" screen and click OK.
16. Click Start.

Data Analysis

1. When the run is complete, click on the Results tab.
2. Click on the Amplification Plot tab to view the raw data.
3. Highlight all the samples so that all the curves appear on the graph.
4. Click the radio button next to "Manual C_t" and enter a number corresponding to the scale on the left (19200, for example).
5. Using the mouse, click and drag the red threshold line until it lies above any background noise and within the exponential phase of the fluorescence curves.
6. Click "Analyze". The red threshold line will turn green, indicating the data has been analyzed.
7. Click File → Save to save the file again. Enter information about the run in the "Reason for Change Entry" screen.
8. Click the Report tab to display the C_t values. Record or print results according to your institution's reporting process.

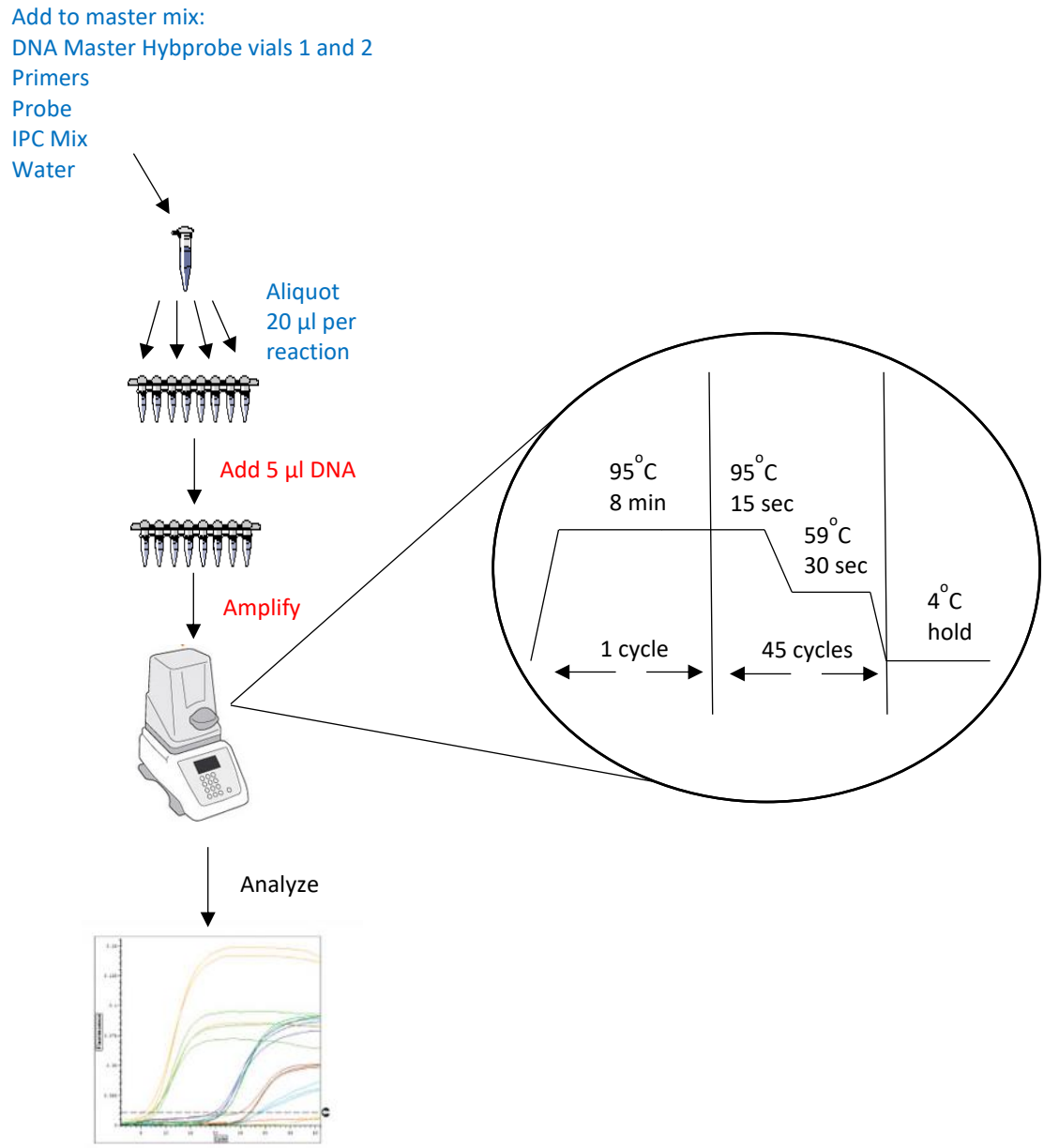


Figure 95- Work flow chart for Real-Time PCR Procedure: All steps in blue are completed in a "clean room" while steps in red are completed in a "dirty room"

Interpretation of Results and DNA Sample Storage

The readout of the data generated by real-time PCR machines typically generates an amplification plot as well as PCR C_t sample values (Figure 96). The curves that are generated in the amplification plot should be sigmoidal (characteristic "S"-shaped), ideally leveling out and reaching a plateau as the last cycle is approached. This indicates that all the reagents have been used. If a plot is lacking

these characteristics, it should be considered negative or retested. Each curve should be carefully examined, and all C_t values should be documented. The electronic data files created for each run should be saved for future reference (Nolan et al. 2013).

The cycle number at which the fluorescence curve for each sample crosses the fluorescence threshold (this can be manually set or generated automatically by the data analysis software) is called the cycle threshold value (C_t value). The plate readout shows the C_t value generated by each reaction. NTCs and negative controls should produce straight lines close to zero and give a "No C_t" result. Positive controls should have C_t values that are less than 35 and the curves generated should all be sigmoidal (WHO & CDC 2011).

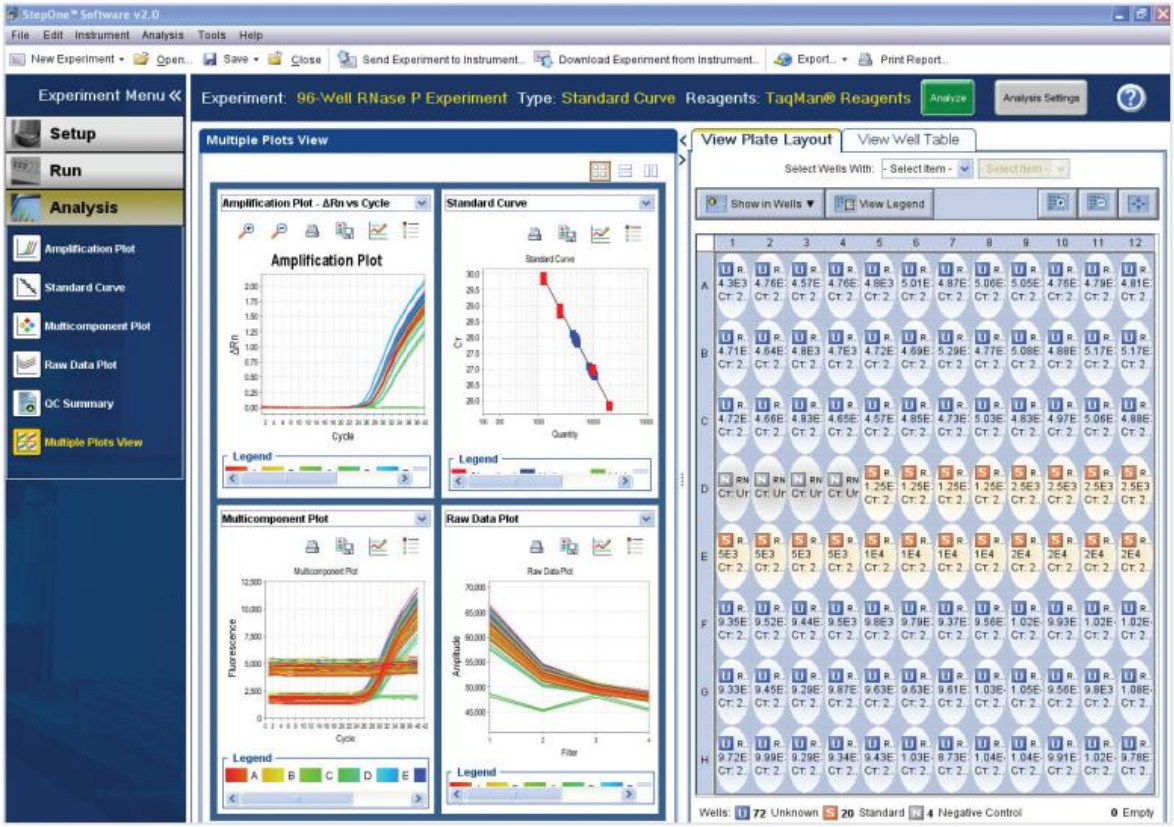


Figure 96- Typical real-time PCR readout depicting an amplification plot and plate layout of tested samples.

For the *B. pseudomallei* TTS1 assay:

1. Positive: C_t values ≤ 35 for TTS1 and IPC and appropriate results for positive and negative controls in the run.
2. Negative: C_t value negative or > 40 for TTS1 and IPC, and appropriate results for positive and negative controls in the run.
3. C_t values between 36 and 40 are considered equivocal. These samples should be diluted 1:10 using PCR-grade water to reduce the presence of inhibitors and retested. Other troubleshooting suggestions are described below.

Extracted DNA should be stored at ≤ -20°C for short-term storage and at ≤ -70°C for long-term storage.

NOTE- Labs that are setting up real-time PCR detection assays for the first time should consider pairing with a reference laboratory to help with results interpretation and ensure appropriate Quality Control.

Real-Time PCR Troubleshooting

If any NTC wells generates a C_t result or a curve that crosses the threshold, the water from that room should be thrown out. If the extracted water negative control generates an amplification curve that crosses the threshold, contamination has likely occurred during DNA extraction. Reagents used for DNA extraction should be replaced and the extraction space should be thoroughly cleaned (ThermoFisher 2014).

Additional factors to consider when troubleshooting real-time PCR reactions:

1. Ensure separate rooms, laboratory coats, gloves, pipettes and sterile barrier filter tips are being used for master mix preparation and template addition.

2. Ensure all work areas are appropriately cleaned and decontaminated.
3. Reaction plates should be covered to protect during transport from the clean room to the dirty room.
4. Consumable reagents (e.g. pipette tips, plates, caps, adhesive films, etc.) should not be reused.
5. Ensure the DNA extraction process was performed correctly.
6. If unsure of a result, the specimen or extraction can be transferred to a reference laboratory for confirmation.

Table 19- Real-time PCR troubleshooting (ThermoFisher 2014).

Observation	Possible Cause	Recommendation
Amplification curve shows abnormal values (the increment change of fluorescent signal at each time point, "ΔR_n", is abnormal)	The baseline was set improperly	Refer to the manual baseline set previously and ensure that all sample C_t values are higher than the baseline
	An amplification signal is detected too early	Dilute the DNA sample and re-run
Amplification curve shows no amplification across all samples	Degraded reagents and/or probe	Dispose of all reagents and start again with fresh stocks. Make sure to store and handle reagents properly to avoid early expiration.
	Degraded or contaminated template	Re-extract DNA and repeat PCR.
	Inhibitors present in reaction	If the TaqMan® IPC control is negative, repeat using diluted DNA to ensure no inhibitors are present.
	One or more of the reaction components were not added	Verify that all components were added.
Duplicate samples show different amplification	Inaccurate pipetting	Follow proper pipetting practices and repeat.
Negative controls show amplification*	Contaminated reagents	Repeat PCR with new reagents. Use proper handling to ensure no further contamination. Never use DNA in a clean room.
	Contamination through pipetting	Re-run and be careful to not contaminate negative wells with any DNA
Positive controls show no amplification*	Degraded or contaminated template	Re-extract control DNA and repeat PCR.
	One or more of the reaction components were not added	Verify that all components were added.
Noisy signal above the threshold	Evaporation	Check the seal of the optical adhesive cover for leaks.
	Inaccurate pipetting	Check calibration of pipettes. Follow proper pipetting techniques.

*If any of the control samples result in curves that are not expected (e.g. positive control shows no amplification or negative control shows amplification) the sample results cannot be confirmed and the entire PCR should be repeated before results can be reported.

Propagation of Plasmid pLepBaBp+

Background

Plasmid pLepBaBp+ contains short target sequences that act as positive controls for certain real-time PCR assays. This plasmid was developed using a pUC plasmid transformed into an *Escherichia coli* strain named TOP10 (Invitrogen – catalogue number C4040) (Figure 97). pUC is a series of plasmid cloning vectors that is a circular double stranded DNA with 2686 base pairs. It is a widely used vector since the recombinant plasmid-containing cells (cells with DNA that have been modified to include genes from multiple sources) can be distinguished from the non-recombinant cells based on the color differences of bacterial colonies on selective culture agar (see below for detail) (Yanisch-Perron et al. 1985).

The plasmid contains short target sequences for PCR based detection of a few bacterial species: *B. anthracis*, pathogenic *Leptospira spp.* and *B. pseudomallei*. The plasmid also contains a sequence for RNase P which serves as an internal positive control for human DNA. To produce more plasmid, grow the *E. coli* in selective media (Luria Burtani) containing ampicillin. DNA can then be extracted from a colony using any plasmid DNA extraction method (CDC 2020).

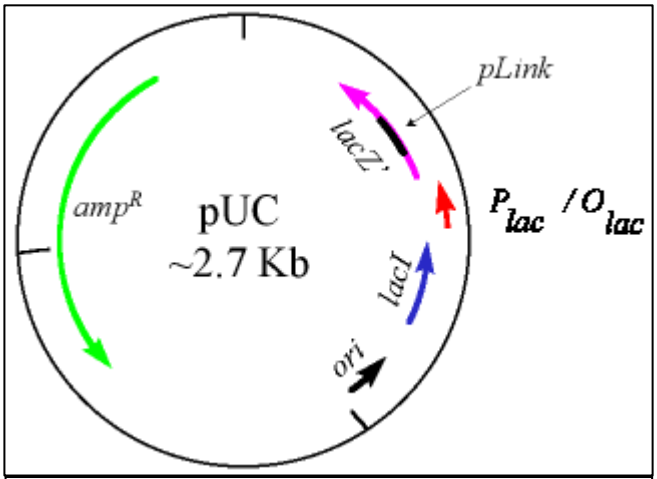


Figure 97- Map of the pUC plasmid used to create the pLepBaBp+ control in *E. coli*. This plasmid contains an ampicillin resistance cassette allowing it to be grown in selective media (Blaber 1998).

pLepBaBp+ Plasmid Propagation Protocol

For the real-time PCR procedure above, the plasmid DNA should be used at a concentration of 10^{-12} g/µL (i.e. 1 picogram/µL). Interference of signal can occur if used at too high of a concentration.

Equipment and Reagents

- Sterile inoculating loops or sterile toothpicks
- Erlenmeyer Flask (50 mL)
- Luria Burtani (LB) AMP100 Broth (i.e. with 100µg/mL ampicillin)
- LB Agar AMP100 plates (i.e. with 100µg/mL ampicillin)
- *Escherichia coli* strain TOP10 (Invitrogen – catalog number C4040)
- Incubator set to 37°C
- Shaking Incubator
- Spectrophotometer
- Plasmid Extraction Kit (various brands available)
- Parafilm
- Personal Protective Equipment (lab coat, gloves, eye protection, shoe covers)

Protocol

The plasmid is in an *E. coli* TOP10 15% glycerol stock that is best stored at -80°C. For maximum preservation, do not thaw out the tube at any time.

1. Warm LB agar AMP100 plate to 37°C prior to streaking.
2. Open the tube of *E. coli* TOP10 and scrape the surface of the frozen broth with either a microbiological loop or toothpick.
3. Streak LB agar AMP100 plate with the bacteria.
4. Incubate LB agar AMP100 plate overnight (12 to 18 hours) at 37°C.
5. Examine plate to verify colonies present.

6. Select a single colony using a microbiological loop or toothpick.
7. Inoculate Erlenmeyer Flask containing at least 5 mL of LB AMP100 broth.
8. Incubate at 37°C in shaking incubator at 200 rpm for 12 to 18 hours or mix thoroughly and place in a standing incubator if not available.
9. Prepare plasmid using any preferred miniprep DNA extraction kit.

NOTE- Plate may be preserved up to one week. Use parafilm to seal plate and leave at 4°C. The *E. coli* with plasmid stock tube should be stored in 15% glycerol at -80°C. For maximum preservation, do not fully thaw out the tube at any time.

Chapter Summary

Real-time PCR is a highly a sensitive and specific method used for the detection, quantification and typing of multiple pathogens, including *B. pseudomallei*. The main advantages of real-time PCR are that it provides a fast and high-throughput method of detection through simultaneous amplification and visualization of newly formed DNA products. The preceding chapter describes a method for real-time PCR detection using the *B. pseudomallei* type III secretion system (TTS1) gene cluster. As a result of the very high specificity and sensitivity of the detection assay, real-time PCR protocols must be followed exactly as described. If a lab is going to conduct real-time PCR testing, they should be able to follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed above. Labs that are setting up real-time PCR detection assays for the first time should consider pairing with a reference laboratory to help with results interpretation and ensure appropriate Quality Control.