Chapter 12: Polymerase Chain Reaction (PCR) Overview

Chapter Overview

Polymerase chain reaction (PCR) is a laboratory technique used to amplify strands of DNA or RNA (ThermoFisher 2012). It is relatively simple to use and generates results rapidly. The technique is also highly sensitive and specific with results in just a few hours. PCR is increasingly used to detect bacterial DNA in clinical and environmental samples. In some melioidosis-endemic regions, PCR is routinely used to confirm or identify suspected *B. pseudomallei* colonies grown on selective agar that are also oxidase-positive, colistin resistant and co-amoxiclav susceptible. It is also commonly used to screen environmental samples and can help to determine which samples to attempt more extensive bacterial culturing.

The following chapter presents an overview of the PCR process, its essential components and briefly describes the main types of PCR used to detect *B. pseudomallei* in clinical and environmental specimens. Good laboratory practice guidelines and PCR laboratory setup are also explained. Additional details about DNA extraction and specific PCR protocols can be found in Chapters 13-15.

Introduction to PCR

Every human, animal, plant, parasite, bacterium and virus contains unique DNA or RNA sequences. Using PCR, these unique sequences can be copied or “amplified”. PCR amplification is achieved by mimicking the replication process that occurs naturally in cells, where DNA is duplicated using the enzyme DNA polymerase and free nucleotides (Figure xxx). Samples are exposed to cycles of heating and cooling (termed “thermal cycling”). During these cycles, DNA primers bind to the target DNA sequence, allowing the DNA polymerase to generate copies of the target sequence in large quantities (ThermoFisher 2012).

PCR is used to generate enough target DNA so that it can be further analyzed, including sequencing, visualization by gel electrophoresis, or cloning into a plasmid for further experiments. PCR is used in many areas of molecular biology, research, forensic science, medical diagnostics and evolutionary ecology. In clinical diagnostic labs, PCR enables rapid and highly specific diagnosis of infectious pathogens (Cai et al. 2014). PCR also allows for the identification of non-culturable or slower-growing microorganisms such as mycobacteria or anaerobic bacteria, discriminates between non-pathogenic and pathogenic strains through the identification of specific genes, and can confirm the purity of an isolated sample (Salis 2009).

Despite this, there are several limitations of PCR for clinical diagnostics that should be noted, including supply and equipment costs and initial staff training expenses. Additionally, previous information about the target sequence is necessary to create the appropriate primers. Here, the precise upstream sequence of the target region on each of the two single-stranded template strands must be known to ensure that the DNA polymerase can bind and add the correct complementary bases to DNA strands during DNA synthesis (Garibyan & Avashia 2013). Another limitation of PCR is that even a small DNA contaminant can be amplified, resulting in confusing or false-positive results. False-negatives due to too low concentration of target DNA can also occur (Podnecky et al. 2013). It is important to recognize that unlike bacterial culture detection, PCR can only detect the presence of DNA, not active growth of an organism.

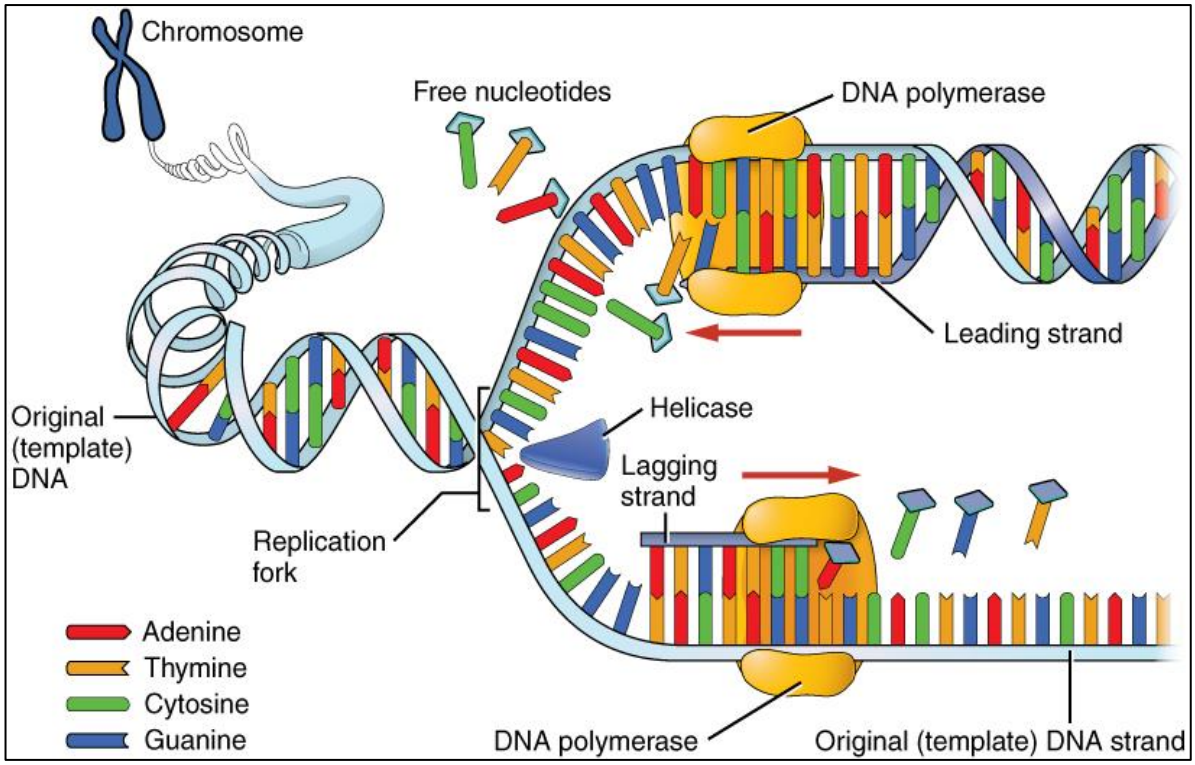


Figure 52- Typical DNA replication process. The enzyme DNA polymerase moves along the DNA template strand and adds free nucleotides to create a duplicate copy. This phenomenon is the basis for PCR, where specific DNA sequences are amplified (Samiksha 2016).

PCR Components

PCR mimics the DNA replication process that occurs naturally in cells. It uses a small quantity of DNA that serves as the initial template, a pair of primers that binds to each end of the target sequence, DNA polymerase, four deoxyribonucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), and a few essential ions (e.g. magnesium) and salts (ThermoFisher 2012). The DNA is then exposed to cycles of heating and cooling. Figures 53-54 give an overview of how these components work together to replicate the template DNA.

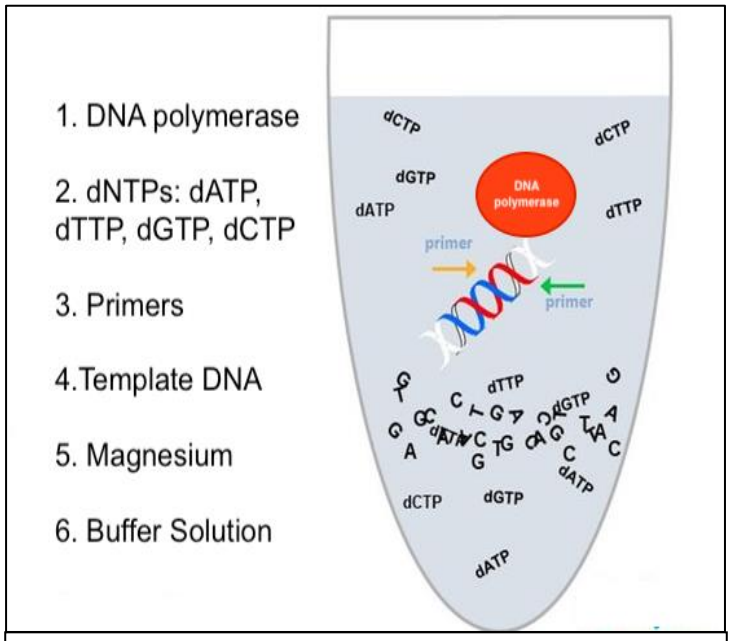


Figure 53- Solution of components making up a PCR reaction inside a test tube (Adapted from Christensen 2018).

1. DNA polymerase: An enzyme that synthesizes DNA molecules from dNTPs. DNA polymerase cannot initiate synthesis of a new strand of DNA, it can only add to an existing DNA strand. During replication, the polymerase "reads" the template DNA and creates a new strand to match it by adding new nucleotides, one by one.
2. Deoxyribonucleotide triphosphates (dNTPs): dNTP is a solution of nucleotides. These are used as the building blocks of DNA. The mixture contains equal amounts of the bases adenine (dATP), thymine (dTTP), guanine (dGTP) and cytosine (dCTP). These nucleotides are added by the DNA polymerase and bind to their corresponding nucleotides as in normal replication: adenine pairs with thymine (AT bond) and guanine pairs with cytosine (GC bond).
3. Primers: Primers are short sequences of single-stranded DNA that are complementary to the template DNA. Since DNA polymerase can only add to existing strands of DNA, primers must first bind to the template DNA first and initiate replication. There is a forward primer that complements the upstream end of the template DNA and a reverse primer that is the reverse complement of the downstream end. Primers should be 18-24 nucleotides in length and specific to the target sequence.
4. Template DNA: DNA that contains the region to be copied. Ideally, there should be 10-1,000 copies of the template DNA for each PCR reaction. Excess template can reduce the efficiency of the assay.

5. Magnesium: Magnesium is an essential cofactor for DNA polymerase. It is used to stabilize double stranded DNA and limit DNA denaturation during PCR. $MgCl_2$ typically comes in the prepared buffer solution with any PCR kit.
6. Buffer Solution: This is a solution that provides a stable chemical environment for the optimal activity of the DNA polymerase. It normally comes with the PCR kit being used (Bustin 2004; Hollis 2013).

PCR Process

PCR amplification relies on the concept of thermal cycling, where samples are exposed to cycles of heating and cooling to induce DNA replication (ThermoFisher 2012). To automate this process, a thermocycler machine is used to initiate each stage of the reaction by raising and lowering the temperature at specific times for a specified number of cycles. There are three major steps that make up each PCR cycle. Most protocols incorporate a total of 35-40 cycles, resulting in exponential replication of the target DNA (Figure 55) (Garibyan & Avashia 2013; Lorenz 2012).

1. Denaturation: During denaturation, the solution is first heated. This temperature is normally between 94° and 98°C. As the heat increases, it breaks apart the two double strands of DNA and separates into two single strands. The amount of time needed depends on the guanine and cytosine (GC) bond content of the DNA. GC bonds are much stronger than adenine and thymine (AT) bonds and therefore take more energy to break. The higher the GC content of the template DNA, the longer the denaturation step should be.
2. Annealing: During annealing the reaction is held for several minutes and is then cooled to between 30° and 65°C. This temperature is based on the melting temperatures of the primers being used. This mixture is held at this temperature for around one minute, which allows primers to bind, or anneal, to the complementary sequence.

3. Elongation (also called Extension): During the extension stage, the sample is heated again. This is typically between 60°and 75°C and the reaction is held at this temperature for less than one minute. The time of extension is dependent on the length of the target, with longer templates needing a longer elongation time. The DNA polymerase will then generate a new DNA strand by binding to the primers and adding dNTPs to the template strand, producing a complimentary strand of DNA. This step allows for the DNA polymerase to extend the primers that have hybridized by adding dNTPs (Lorenz 2012).

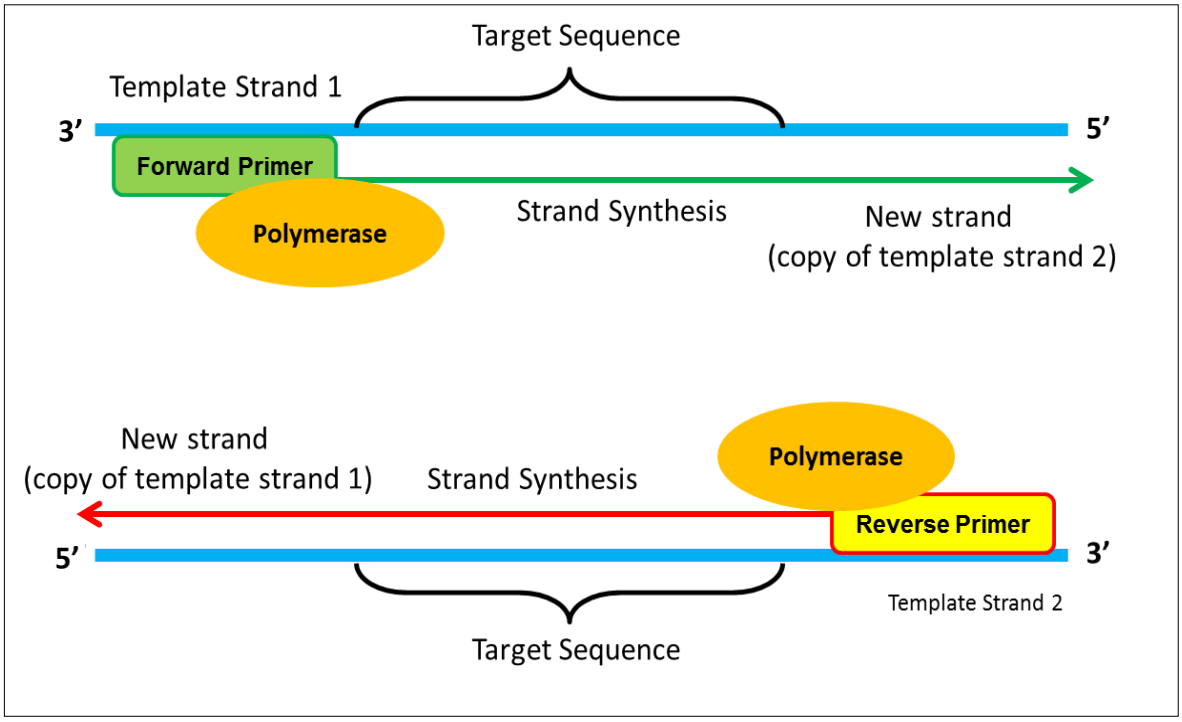


Figure 54- Overview of the components of PCR. First, primer pairs must bind to the template DNA strand. Then DNA polymerase can begin to replicate the DNA sequence by adding dNTPs to the primer. This results in a replicate copy of the template DNA sequence (Hollis 2013).

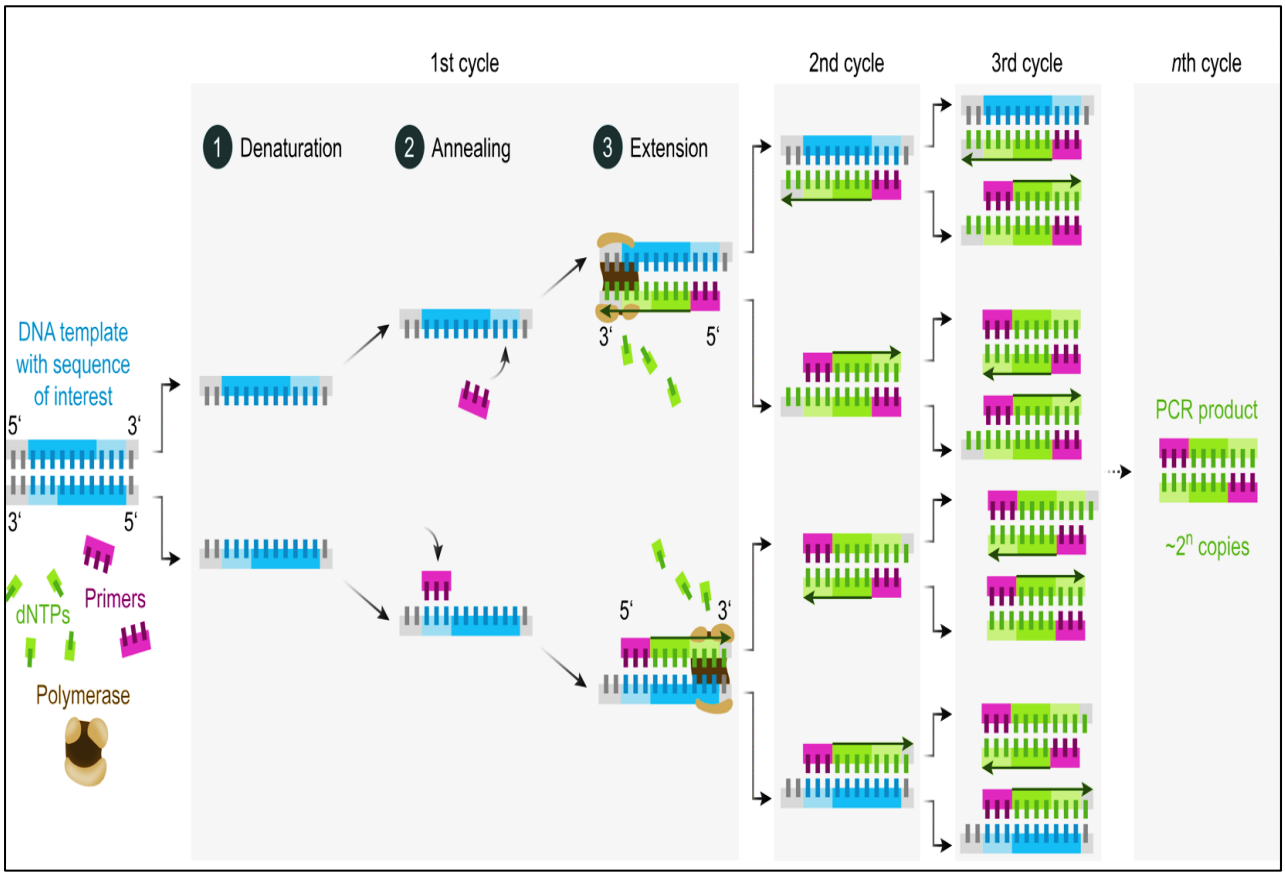


Figure 55- Exponential replication of target DNA. After several cycles of denaturation, annealing and elongation, the DNA template is amplified exponentially (Enzoklop, CC BY-SA 4.0 Wikimedia Commons).

PCR Types: Conventional vs Real-Time PCR

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). A modification of conventional PCR detection, called quantitative PCR (qPCR) or real-time PCR enables researchers to amplify a particular DNA sequence and to quantify it simultaneously (Garibyan & Avashia 2013).

Real-time PCR enables the amount of a given sequence present in a sample to be analyzed by measuring the increase of DNA product after each round of PCR amplification. There are two main methods of DNA quantitation used during real-time PCR. These are shown in Figure 56 below. One uses DNA-targeted intercalating fluorescent dyes that show enhanced fluorescence upon binding. As the quantity of the template DNA increases with each amplification cycle, the number of bound fluorophores and intensity of the fluorescent signal increases. The second method uses fluorophore-labeled sequence-specific DNA probes. The most widely utilized type is the TaqMan probe, which uses the 5'-3' exonuclease activity of Taq polymerase to break apart the fluorophore from the quencher, producing fluorescence (Figure 56) (Bonetta 2005; Smith & Osborn 2009).

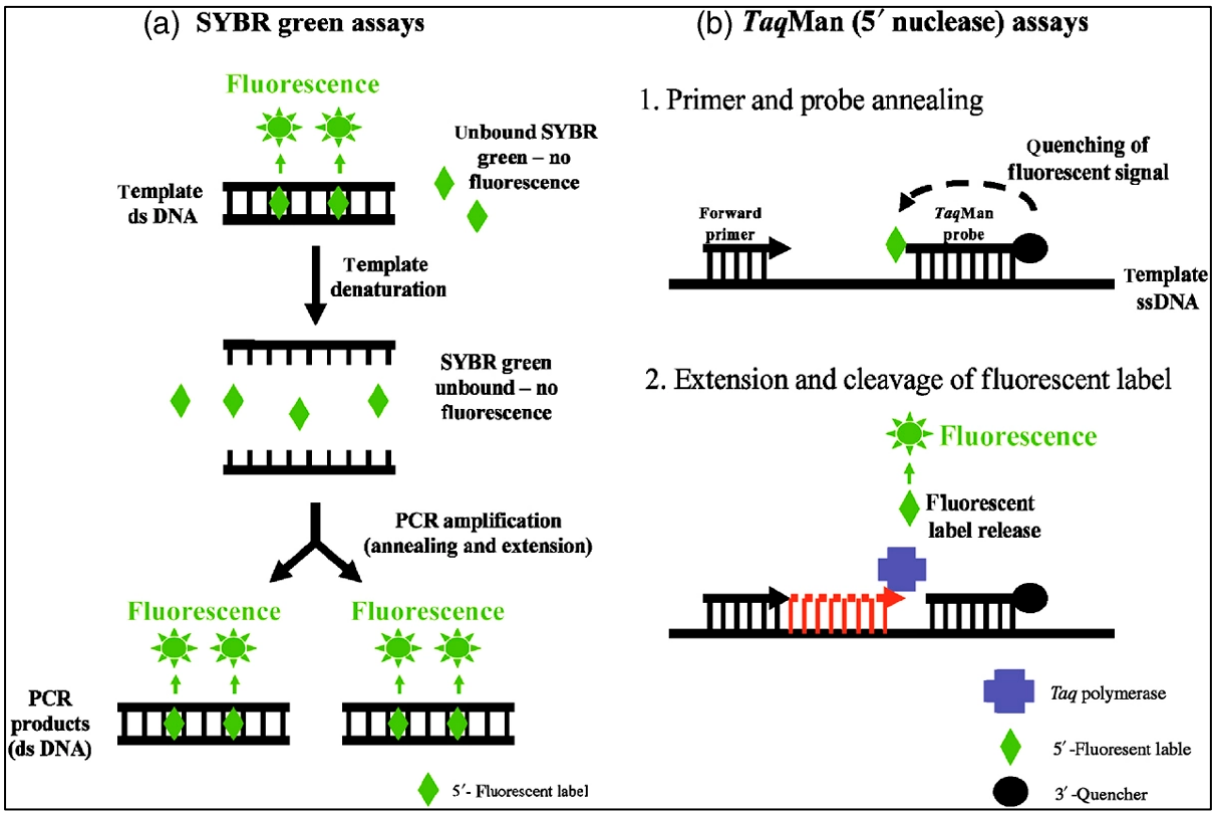


Figure 56- Detection processes for real-time PCR: (a) DNA-targeted intercalating fluorescent dyes, such as SYBR Green and (b) fluorophore-labeled sequence-specific DNA probes, such as TaqMan assay (Smith & Osborn 2009).

As the number of DNA copies increases, the fluorescent signal increases. By plotting fluorescence against cycle number and comparing the results against a standard curve, the user is able to determine the quantity of DNA at each step of the PCR reaction (Smith & Osborn 2009). Additional details and *B. pseudomallei*-specific protocols for conventional and real-time PCR assays can be found in Chapters 14 and 15.

Pros and Cons of Real-Time PCR (qPCR) Over Conventional PCR Assays

Advantages of real-time PCR

1. **Speed:** DNA is detected while the PCR reaction is occurring. Amplified products do not need to be visualized with a secondary procedure after the PCR has finished (e.g. on an agarose gel).
2. **Quantification:** Allows for quantification (measurement) at the exponential phase that depends on initial sample quantity.
3. **Sensitivity:** real-time PCR can distinguish as few as two-fold increases in the quantity of target DNA generated and can detect as little as a few molecules of staring DNA.
4. **Throughput:** real-time PCR can process a large number of samples quickly, particularly when using automated extraction systems.
5. **Replicability**: results are normally very reproducible (Čepin 2017; Smith & Osborn 2009).

Disadvantages of qPCR

1. **Cost:** the equipment required for fluorescence detection means that real-time PCR thermocyclers are five to ten times more expensive than conventional PCR thermocyclers. Highly specific reagents and consumables are also required because of the sensitive fluorescent detection method. qPCR kits often contain expensive master mix solutions (a premixed concentrated solution that includes all components for a real-time PCR reaction that are not sample-specific).

2. **Prone to contamination:** real-time PCR is an extremely sensitive method and very prone to error and contamination. Several controls, such as a no template control (a sample containing no DNA) and buffer control need to be included when performing the assay to assure quality control checks in every run.
3. **Complexity:** interpretation of results is more complex and requires further data analysis than with conventional PCR assays (Čepin 2017; Smith & Osborn 2009).

Table 16- Comparison of real-time versus conventional PCR (ThermoFisher 2012).

	Real-time PCR	Conventional PCR
Process	Measures PCR amplification as it occurs. Data is collected during the exponential growth phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid.	Measures the amount of accumulated PCR product at the end of the PCR cycles.
Applications	• Quantitation of gene expression • Quality control and assay validation • Pathogen detection • Copy number variation	Amplification of DNA for: • Sequencing • Genotyping • Cloning • Pathogen detection
Advantages	• Increased dynamic range of detection • No post-PCR processing • Higher sensitivity and specificity • Closed system reduces the risk of contamination • An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated • Shorter turnaround time • No post-PCR processing	• Less expensive reagents and equipment • Less complex results interpretation • Widely used molecular diagnostic format
Disadvantages	• Cost of equipment and consumables • Data analysis and interpretation of results are more complicated • More sensitive to errors and PCR inhibition	• Lower sensitivity and specificity • Lower resolution • Non-automated • Results are not expressed quantitatively • Post-PCR processing takes additional time

PCR Laboratory Setup and Good Laboratory Practice

The capacity for PCR to generate many copies of a target sequence from a small quantity of DNA has made PCR a useful and widely implemented diagnostic tool. This also means that certain precautionary measures must be used to prevent false positive results and contamination of the assay or laboratory space. False positive or misleading results can occur through cross-contamination of samples and different reactions prepared at the same time, or through contamination of stock solutions. Other microorganisms present within the laboratory environment are also a significant source of contamination. When conducting PCR experiments, measures can be undertaken to avoid the contamination of reagents, laboratory equipment and bench space. Good laboratory practices should be used to help reduce the likelihood of contamination (Mifflin 2007; Public Health England 2018; U.S. EPA 2004). The following general precautions will help to reduce the chance of contamination.

1. Organization of PCR Workspace and Equipment

The prevention of PCR contamination starts with the distribution of PCR laboratory workspaces. Workspaces should be organized to ensure that the flow of work occurs in one direction, from clean areas (pre-PCR) to dirty areas (post-PCR). Ideally, the air in clean areas should be positively pressurized (air forced out of the room) to ensure that airborne microorganisms or particles do not contaminate the samples or reagents in that room. Dirty areas should be negatively pressurized (air forced in) to help prevent the escape of amplified products (Figure 57) (Mifflin 2007; WHO 2018).

Where possible have separate designated rooms, or at minimum, physically separate areas for:

a. Sample/specimen preparation and nucleic acid extraction
b. Pre-PCR: Reagent/master mix preparation
c. Pre-PCR: Nucleic acid template addition
d. Post-PCR: PCR amplification, handling of the amplified product and product analysis e.g. gel electrophoresis.

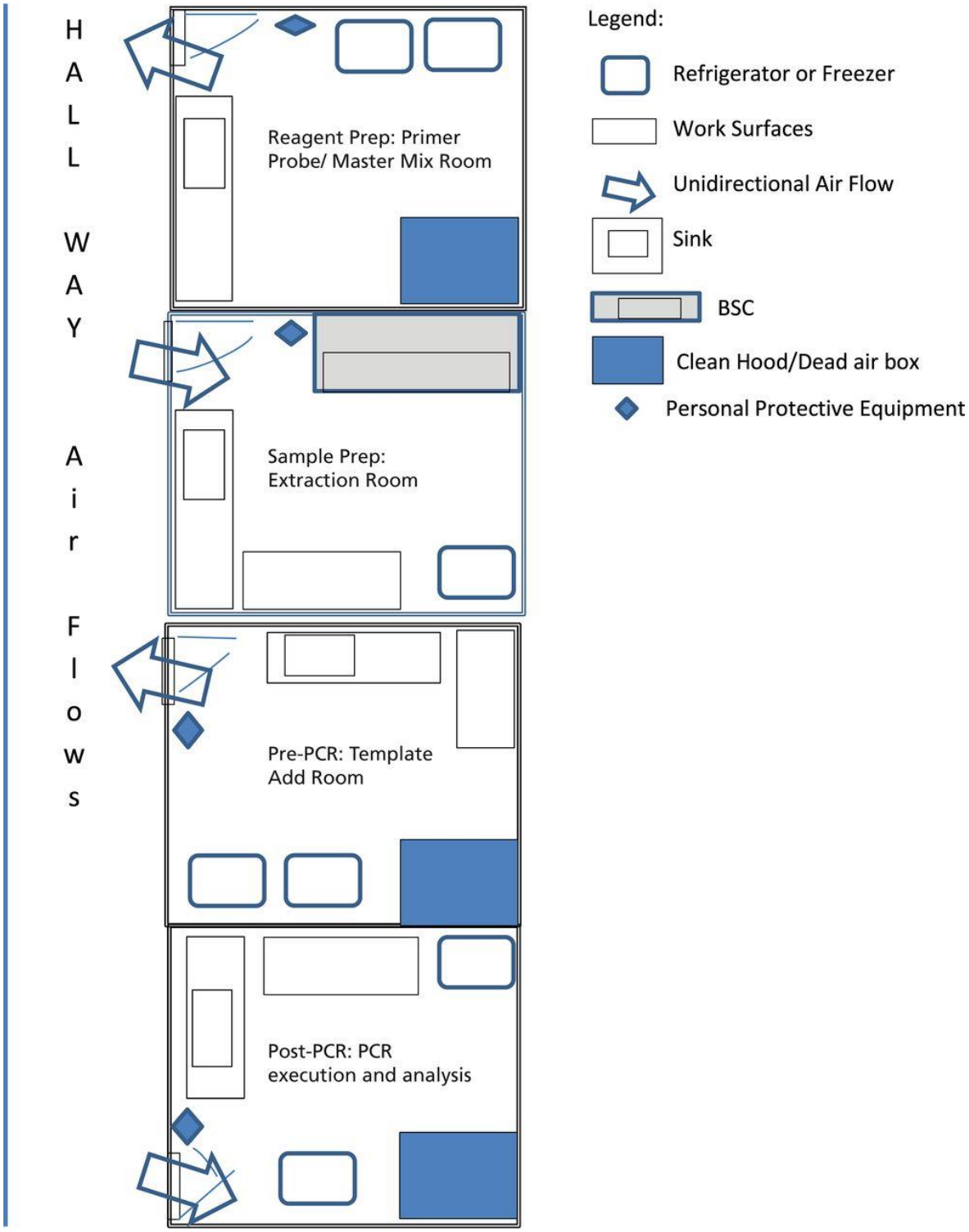


Figure 57- Sample floor plan for a molecular laboratory to ensure the segregation of specimens and nucleic acid template from clean reagents for PCR (Carey et al. 2018).

Sample/specimen preparation and nucleic acid extraction area

A sample preparation area should be used for sample processing and for preparing PCR controls. Laboratories can also include a designated space in this area for sample/specimen reception and storage. Clean gloves, laboratory coats and closed footwear should be worn at all times and separate pipettes and laboratory coats also should be designated for all work done here to control contamination from this room to any other location. This room should also be kept under negative pressure, which will help to prevent contamination outside of this space. Where possible, equipment from this room should not be removed and used in the reagent/master mix preparation area (U.S. EPA 2004).

Pre-PCR: Reagent/master mix preparation area

This area should be positively pressurized and should be the cleanest of all PCR preparation areas. Specimens, extracted DNA and amplified PCR products should not be handled in this area. Where possible, a laminar flow cabinet equipped with a UV light should be used in this area. A laminar flow cabinet is a hooded and enclosed bench that is used to prevent contamination of biological samples where air is drawn through a HEPA filter and blown towards the user. Due to the direction of air flow, the sample/reagents are protected from the user while the user is not protected from the sample/reagents (Figure 58) (U.S. EPA 2004).

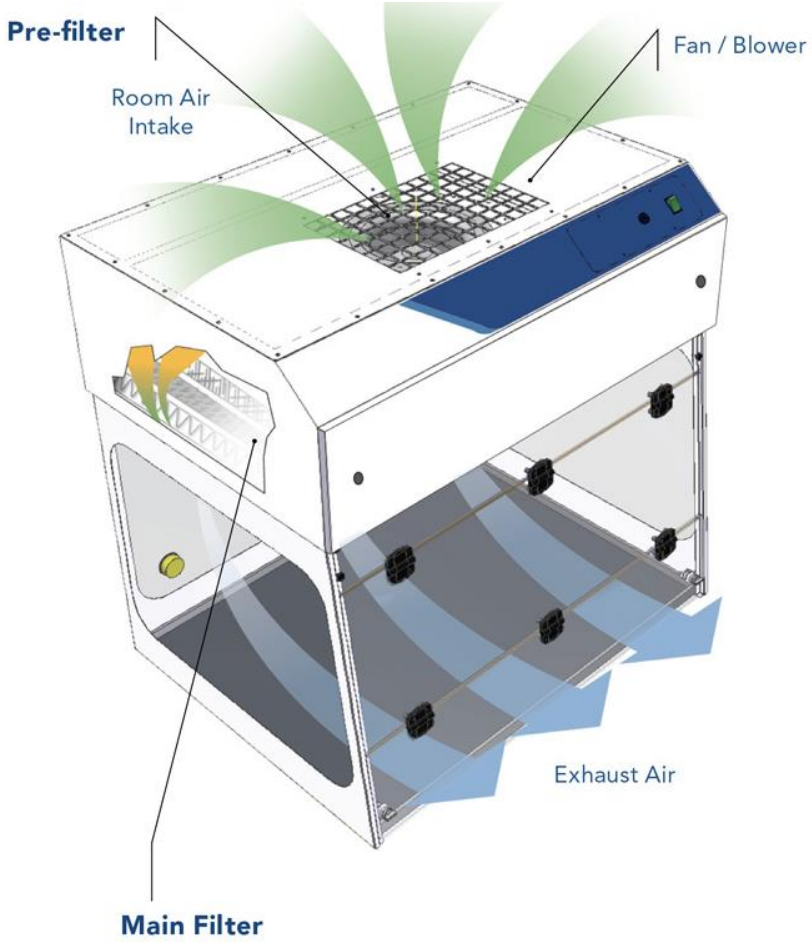


Figure 58- A laminar flow cabinet is a workspace enclosed on three sides that prevents cross contamination between samples and offers UV lights for sterilization (Air Science 2021).

PCR reagents should be kept in a freezer or refrigerator according to the manufacturer specifications. Fridges and freezers should not contain any template DNA or specimens, as this can lead to contamination of the reagents. Gloves should be changed regularly but especially when entering the pre-PCR area and laminar flow cabinet. The pre-PCR area and laminar flow cabinet should be cleaned before and after use as below:

1. Wipe down all items in the cabinet, e.g. pipettes, tip boxes, vortex, centrifuge, tube racks, pens, etc. with 10% bleach solution or a commercial DNA-destroying decontaminant (such as DNA-Erase), followed by 70% ethanol, and allow to dry.

2. If working in a laminar flow cabinet turn on the UV light for 30 minutes.

Pre-PCR: Nucleic acid (DNA/RNA) template addition area

This area should be used for the addition of extracted DNA/RNA and controls to master mix tubes or plates. Nucleic acid must be handled using a separate set of pipettes, filter tips, tube racks, fresh gloves, lab coats and other equipment. To prevent contamination of extracted DNA, laboratory staff should change gloves regularly and before handling controls. Ensure equipment has been wiped clean with 10% bleach or commercial DNA-destroying decontaminant before adding controls. Sample template nucleic acid and controls should be stored in separate designated fridges or freezers in this area. The sample workspace should be cleaned in the same way as the reagent/master mix preparation area (U.S. EPA 2004; Viana & Wallis 2011).

Post-PCR: PCR amplification, handling of the amplified product and product analysis

This space is used for post-amplification practices and typically contains thermocyclers and real-time PCR machines. The air should be negatively pressurized to help prevent the escape of amplified products and be separate from the pre-PCR areas. The risk of contamination is high in this area and PCR reagents and the extracted nucleic acid should not be handled here where possible. This area should also have a separate set of gloves, lab coats, PCR plate and tube racks, pipettes, filter tips, and other equipment to minimize contamination (WHO 2018).

Post-PCR product analysis can also be performed here, although where resources and space permit, this should preferably be done in a separate contained post-PCR area. Product detection equipment, e.g. gel electrophoresis tanks, power packs, gel documentation system and reagents (e.g., loading dyes, molecular marker, agarose gel, and buffer components) should be kept here. The area should also contain a separate set of gloves, lab coats, plate and tube racks, pipettes,

filter tips, bins and other equipment. Workspaces and equipment should be cleaned as described above (WHO 2018).

In resource or space-limited settings, having four separate rooms or spaces may not be possible. In this instance, PCR setup can include two work spaces: a pre-PCR clean room/area that contains no DNA (only master mix) and a post-PCR dirty room/area where extracted DNA is added to the master mix, such as in Figure 59.

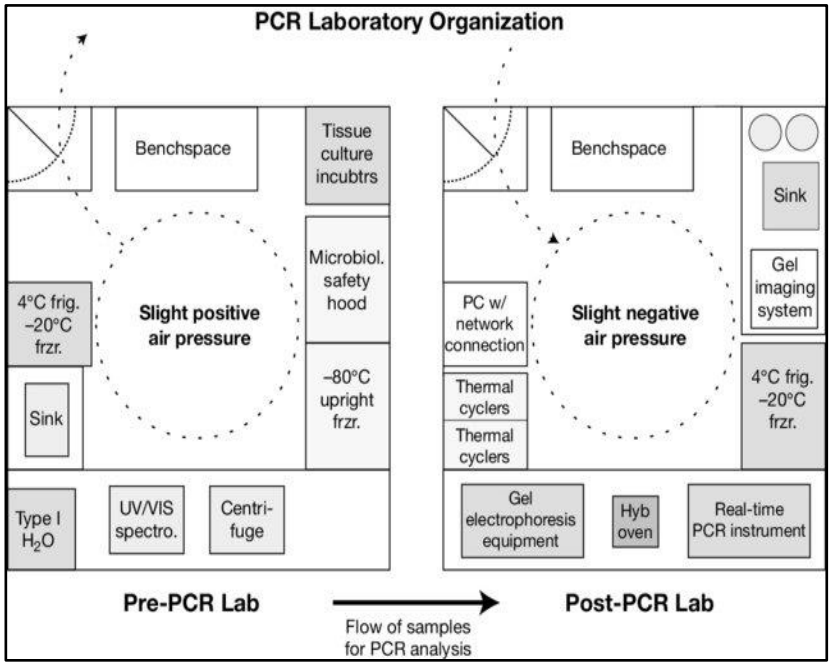


Figure 59- Organization of a PCR laboratory with two separate pre-and post-PCR rooms (Mifflin 2007).

If separation of workspaces cannot be achieved, extra care must be taken to avoid DNA contamination of primer and probe reagents. Here, a possible (though less desirable) option is to do the master mix and reagent preparation in a containment cabinet, such as laminar flow cabinet (Mifflin 2007; Viana & Wallis 2011).

2. Unidirectional Workflow

Ideally, laboratory staff should abide by a unidirectional workflow. Pre-PCR rooms, particularly those used to prepare/aliquot PCR stock reagents, should not be entered on the same day after working in rooms where products and clinical specimens are handled. If one must go against the unidirectional workflow, care should be taken as described below:

a. Hands should be washed and gloves and lab coats changed when moving between areas.

b. Reagents and equipment should not be moved from a dirty area to a clean area. If this is unavoidable, they should be cleaned with 10% bleach or a commercial DNA decontaminant, followed by sterile water or 70% ethanol (only on non-plastic surfaces). Ultra-violet (UV) light should be where if available.

c. Lab books and paperwork must not be taken into the pre-PCR rooms if they have been used in the post-PCR rooms. If necessary, make duplicate print-outs of protocols/sample IDs, etc. (Mifflin 2007; Viana & Wallis 2011; WHO 2018).

3. Good Laboratory Practices and Handling of Reagents

a. PCR reagents should be aliquoted as they are received to avoid unnecessary freeze-thawing and to prevent stock reagents from contamination as soon.

b. If contamination is detected, all work should be suspended until the source of contamination has been determined and eliminated. A full investigation should be carried out to determine why the contamination occurred. Occasionally, discarding all suspected reagents is necessary.

c. All reagents, stocks, tubes etc. should be clearly labelled. Do not keep reagents past the expiration date.

d. Briefly spin/centrifuge reagent and sample tubes before opening to avoid the generation of aerosols. Open and close all tubes and reaction plates carefully so samples do not splash. Close tubes immediately after use to avoid the introduction of contaminants.

e. Bench work areas in PCR laboratories should be cleaned with disinfectant solution before and after use.

f. Use powder-free gloves to avoid assay inhibition and change frequently.

g. Protect reagents containing fluorescent probes from light to avoid degradation (U.S EPA 2004; Viana & Wallis 2011; WHO 2018).

4. Pipetting Technique

Appropriate pipetting technique is important to ensure both the accuracy and quality of PCR results. Correct pipetting technique can also minimize contamination and help to prevent false positive or ambiguous results (see Figure 60 below). Pipette reagents and samples using filter tips, which will help to prevent cross-contamination of samples. Confirm with the manufacturer that the filter tips fit the brand of pipette to be used prior to purchase (U.S EPA 2004; WHO 2018). All equipment should also be calibrated regularly according to the manufacturer guidelines. If manufacturer instructions permit, pipettes should be sterilized by autoclave on a regular basis. If pipettes cannot be autoclaved or no autoclave is available, clean them with 10% bleach (followed by a thorough wipe down with sterile water) or with a commercial DNA-destroying decontaminant followed by UV exposure after use (Viana & Wallis 2011; WHO 2018). Good practice for accurate pipetting can be found at:

- Gilson guide to pipetting: https://www.gilson.com/pub/static/frontend/Gilson/customtheme/en_US/images/docs/2019_Guide_to_Pipetting_LT800550-F_compressed.pdf.

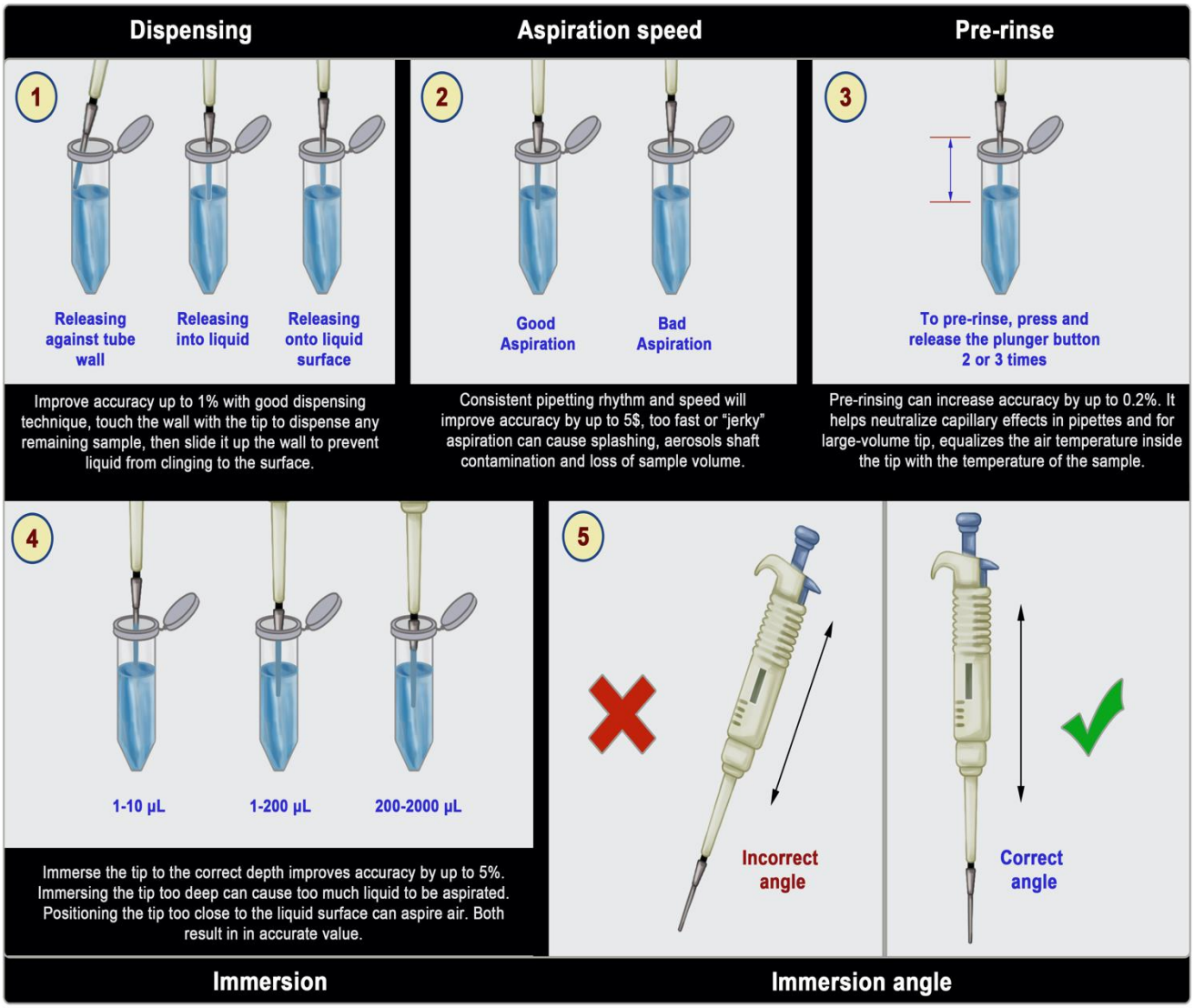


Figure 60- Good laboratory practice for accurate pipetting during PCR molecular reactions.

5. Quality Controls

Quality controls are important for ensuring that contamination of reagents or workspace has not occurred and that the assay is working as expected. Non-template controls (NTCs) contain all reagents except for template nucleic acid. Rather, PCR-grade water is normally added to these PCR wells. It is recommended that water used both in the clean and dirty area is added to at least two

NTC reaction wells. If any of these NTC well generates a positive PCR result, the water should be replaced and reactions should be run again using new NTCs.

PCR reaction positive controls should also be run using DNA from a known positive *B. pseudomallei* isolate or using non-*B. pseudomallei* template DNA (plasmid DNA) that contains the PCR target gene of interest, which will help to minimize cross-contamination between reaction wells. If using DNA from a known positive *B. pseudomallei* isolate, this should be diluted in PCR-grade water to decrease the likelihood of contamination of the PCR workspace or reaction wells, helping to avoid false positives (Viana & Wallis 2011; WHO 2018).

When extracting DNA from human clinical specimens RNAse P can be used as an internal positive extraction control to monitor sample quality and the presence of PCR reaction inhibitors (Esakova & Krasilnikov 2010). An additional control for contamination at the DNA extraction step is the extraction of water (see Chapter 13 for further detail on DNA extraction methods and controls). If the extracted water negative control generates an amplification curve that crosses the threshold, then contamination has occurred during the DNA extraction process. Reagents should be replaced and the extraction workspaces and equipment should by cleaned thoroughly.

Chapter Summary

Polymerase chain reaction (PCR) is a laboratory technique used to amplify strands of DNA or RNA (ThermoFisher 2012). It is a highly specific method of *B. pseudomallei* detection that can generate results rapidly. In some melioidosis-endemic regions, PCR is used to confirm or identify oxidase-positive colonies with a morphology typical of *B. pseudomallei* on selective agar that are also resistant to colistin and susceptible to co-amoxiclav. PCR is also frequently used for direct detection from environmental specimens. The preceding chapter presents a general overview of PCR, PCR laboratory setup and briefly describes the main types of assays used to detect *B. pseudomallei* in

clinical and environmental specimens. Additional details about DNA extraction and specific PCR protocols can be found in Chapters 13-15.

Chapter 13: *B. pseudomallei* DNA extraction from Clinical and Environmental Specimens

Chapter Overview

Extraction of nucleic acid (DNA or RNA) serves as a starting point for multiple downstream applications, including clinical diagnosis and experimental research. The following chapter presents an overview of the typical DNA extraction process, DNA extraction quality control, and protocols for DNA extraction from *B. pseudomallei* clinical and environmental specimens. DNA isolated from these protocols can be used for subsequent molecular PCR detection and diagnosis of *B. pseudomallei* infection. Conventional and real-time PCR procedures are discussed in Chapters 14 and 15.

DNA Extraction Introduction

DNA extraction is a technique used to isolate DNA from the inside of a cell. Once extracted, DNA can be used for downstream molecular analyses, including PCR and genetic sequencing. There are three primary steps involved: lysis, separation and purification (Elkins 2013).

1. Lysis- During lysis, the cell membrane walls and cell nucleus are broken open, releasing the DNA. This can be accomplished several different ways. It can be done enzymatically using lysozyme, which digests peptidoglycans in the cell wall, or using detergents like sodium dodecylsulfate (SDS) to dissolve the cellular proteins. It can also be achieved mechanically utilizing glass beads, which are vortexed with the sample. Mechanical lysis is generally faster than enzymatic methods.
2. Separation- During separation, DNA is separated from any cellular debris. This often involves the use of sodium (Na+) ions to neutralize the negative charge present in DNA molecules. This makes the DNA less water soluble and more stable.

3. Purification- After separation of DNA from the aqueous solution, alcohol (e.g. isopropanol or ethanol) is added. This leads to precipitation of DNA out of the solution as DNA does not dissolve in alcohol. It is then rinsed to remove cellular debris.

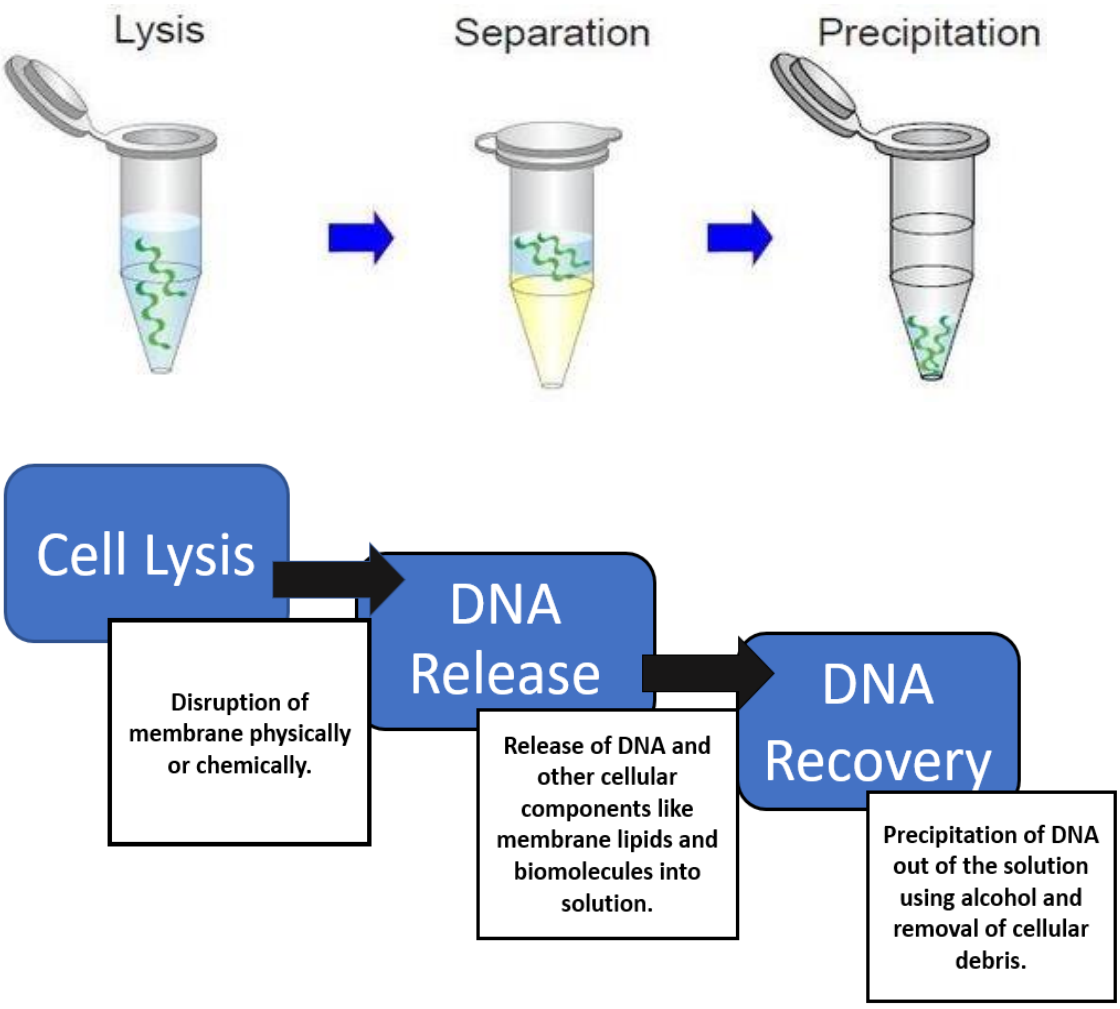


Figure 61- Steps involved in basic DNA extraction. Three major processes involved are cell lysis, separation of DNA from other cellular components and debris, followed by precipitation and purification of the DNA from the aqueous solution (Bio-Helix 2016).

Methods of DNA Extraction

Common DNA extraction methods include organic chemical extraction, Chelex® extraction and solid-phase extraction (Elkins 2013).

1. Organic chemical extraction- Organic chemical extraction involves the addition of several chemical solutions and nomally incorporates a lysis step, a phenol chloroform extraction, an ethanol precipitation, and washing steps (Elkins 2013). Organic extraction is inexpensive, simple and yields large quantities of pure DNA. However, there are still many steps involved and the procedure may still take longer than other extraction methods. It also uses toxic chemicals such as phenol and chloroform and there is an increased risk of contamination because multiple tubes are used to transfer the DNA (Tan & Yiap 2009).
2. Chelex® extraction- Here, Chelex® resin beads are added to the sample and this solution is then heated, vortexed and centrifuged. The cellular materials bind to the Chelex® beads, while the DNA is available in the supernatant (Singh et al. 2018). The Chelex® method is faster, simpler and relatively inexpensive compared to other extraction techniques, and it only requires one tube, which decreases the risk of DNA contamination. However, the DNA is lower quality and degrades quickly after being extracted. It is typically only used for more rapid PCR-based analyses (Butler 2005).
3. Solid-phase extraction- Solid-phase extraction methods work through the binding of nucleic acids to solid support membranes, such as magnetic beads coated with silica or other materials. These are washed with alcohol to remove contaminants and then with a liquid that makes the nucleic acids soluble again, freeing the DNA from the beads/support (termed “elution”). This produces high-quality, double-stranded DNA that can be used for PCR and additional downstream analyses such as genetic sequencing. Many solid-phase extraction commercial kits are available through different commercial companies. However, solid-phase extraction kits tend to be more expensive than organic chemical extraction or Chelex® extraction (Elkins 2013; Singh et al. 2018).

   Solid-phase extraction methods can also be fully automated using advanced laboratory instruments such as the QIAcube or EZ1 (QIAGEN) or MagNA Pure LC Instrument (Roche

Diagnostics), which typically come preinstalled with multiple protocols for purification of plasmid DNA, genomic DNA, RNA, viral proteins, as well as DNA and RNA clean-up (Butler 2005). Automated instruments often use the same kits as manual solid-phase nucleic acid extraction but can dramatically decrease the time and effort required to extract the DNA. Automated kits also require less time for set-up, increasing throughput. However, testing of only one or a few samples using automated systems can be costly. The initial cost of setting up automated systems is also more than with manual extraction kits since a robot system must be purchases to run the kits (Meumann et al. 2006; Podnecky et al. 2013). See more information about automated kits and the QIAcube or EZ1 at www.qiagen.com, or MagNA Pure LC (Roche Diagnostics) available at https://www.lifescience.roche.com/en_au/products/magna-pure-.

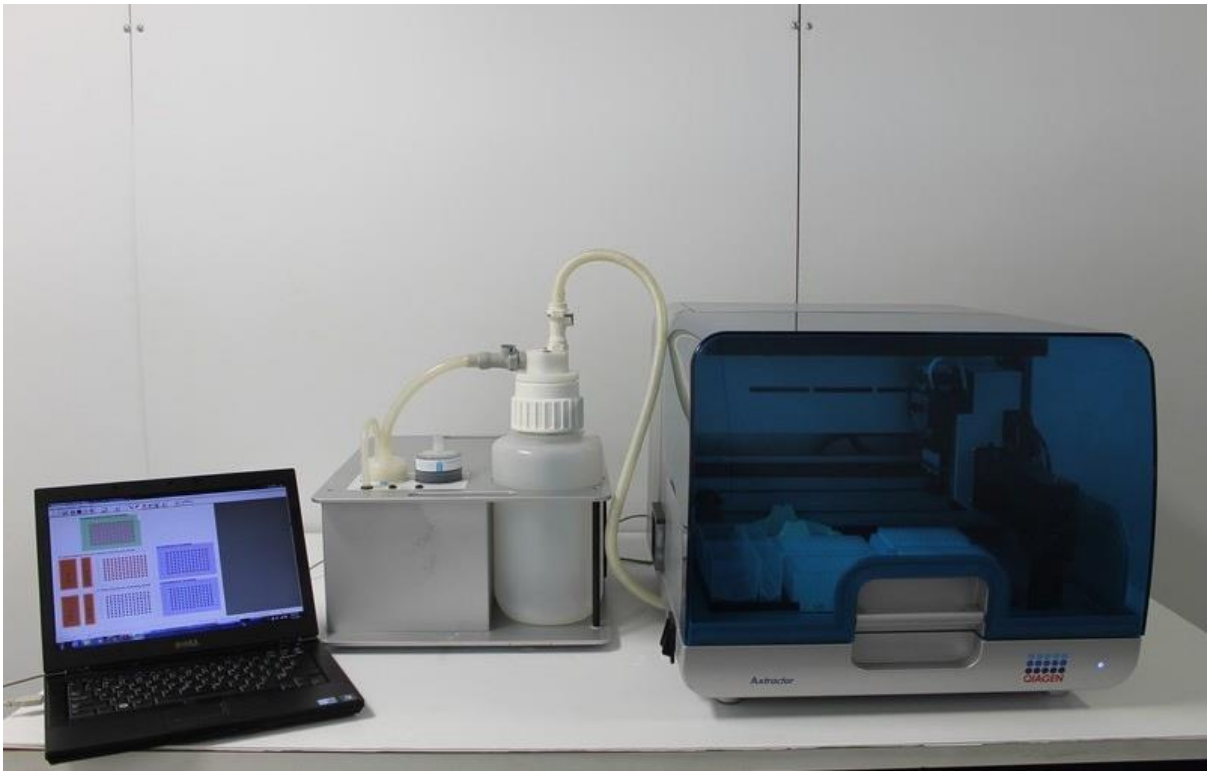


Figure 62- Automated QIAcube extraction robot and typical laboratory setup.

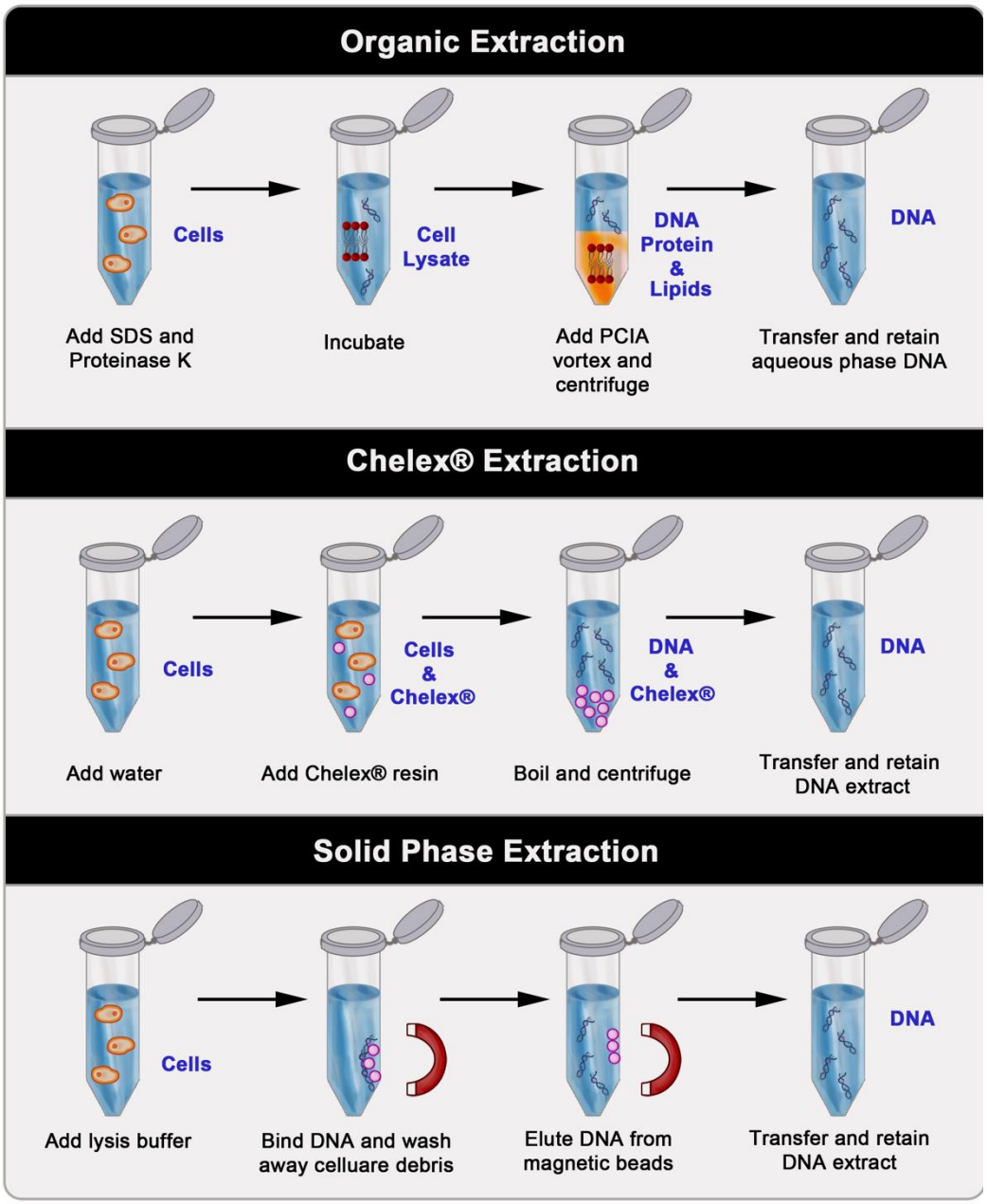


Figure 63- The three major methods of DNA extraction. Top: Organic extraction using sodium dodecylsulfate (SDS), Proteinase K and phenol:chloroform:isoamyl alcohol (PCIA) separates DNA from lipids and proteins. Middle: Extraction using Chelex® resin. This releases DNA by boiling and vortexing and inactivates degradative enzymes such as nucleases. Bottom: Solid phase extraction selectively binds DNA to a solid support such as silica in the presence of a high salt solution.

There are multiple factors to consider when selecting the most appropriate DNA extraction method, including (Dhaliwal 2013):

1. Sample type and storage method- DNA can be extracted from many sources, including human tissues, blood, bacteria, yeast, fungi, stool, body fluids, spores, soil, and other clinical samples (Thatcher 2015). Sample preparations may be fresh or previously frozen, paraffin-embedded or formalin-fixed tissue, ethanol-fixed, etc. (Mullegama et al. 2019).
2. Intended use- The quality and quantity of the extracted DNA should be adequate for the intended downstream purposes, e.g., sequencing, molecular fingerprinting, or PCR detection.
3. Humic content- Humic substances are naturally occurring organic compounds that arise from the decomposition of plant, animal, and microbial material. They are a natural component of soils. If the sample has humic content a kit/method that removes these substances should be used since they can inhibit downstream applications like PCR (Maccarthy 2001).
4. DNA yield- The amount of DNA extracted from a sample will vary depending upon the sample type, inhibitors, and the method of extraction.
5. Simplicity- The best extraction method may depend on the experience of the user and the amount of control the user desires during extraction.
6. Cost- Manual and automated commercial kits are often more expensive than organic or Chelex® extraction methods and may not be practical in poorly-resourced laboratories for routine specimen diagnostics. These systems also require access to technical help and should take maintenance and repair costs into consideration.

B. pseudomallei DNA Extraction

Solid-Phase Extraction: Commercial Kits for *B. pseudomallei* DNA Extraction

The use of solid-phase extraction commercial kits for the isolation of *B. pseudomallei* DNA from clinical and environmental samples has many advantages, including the wide availability of kits, ease

of use, comparatively low cost as well as the standardization of reagents and protocols (Podnecky et al. 2013). Commercial kits vary in a several ways, including components that make up the lysis buffer, the mechanisms used to separate DNA, the type of membrane used, the wash buffer used and the way the DNA is recovered. Different extraction methods can generate different yields and purity. While no DNA extraction method has been shown to be optimal for all specimen types (Rantakokko-Jalava 2002) many have been evaluated and validated for *B. pseudomallei*-specific applications such as soil and water DNA extraction (Draper et al. 2010; Kaestli et al. 2007; Knappik et al. 2015), whole blood (Podnecky et al. 2013) and extraction from other melioidosis clinical specimens (Meumann et al. 2006; Richardson et al. 2012). The ability to detect *B. pseudomallei* with PCR has been shown to vary for different DNA kits and is dependent on the bacterial load, the quantity and quality of the bacterial DNA extracted and the elimination of PCR inhibitors (Meumann et al. 2006; Podnecky et al. 2013). Therefore, selecting the best kit and protocols are crucial.

Common commercial kits used for the extraction of *B. pseudomallei* DNA from clinical and environmental specimens include:

For clinical specimen extraction-

1. QIAamp DNA Mini Kit (QIAGEN)- The QIAamp DNA Mini Kit is a widely used and well-validated kit used to extract *B. pseudomallei* DNA from clinical specimens (Richardson et al. 2012). It offers a fast and easy method for purification of total DNA for reliable PCR testing. Genomic DNA can be extracted from many sample types including whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells, and tissue. The QIAamp procedure can be used on fresh or frozen whole blood or blood that has been treated with citrate, heparin, or EDTA. It also requires no phenol/chloroform extraction or alcohol precipitation and very little handling. The QIAamp procedures can also be automated on the QIAcube (Dhaliwal 2013; QIAGEN 2016).

2. High Pure PCR Template Preparation Kit (High Pure, Roche Diagnostics)- Here, cells are lysed by a short incubation with a lysis buffer and Proteinase K in the presence of a chaotropic salt (i.e. a molecule that can disrupt the hydrogen bonds between water molecules). DNA binds to specific glass fibers present in the filter tubes and a low salt elution releases the DNA from the glass fibers (Merk et al. 2006). This kit can be used on a wide variety of specimens, however *B. pseudomallei* DNA yields from whole blood have been low in previous studies, likely due to reduced concentrations of circulating bacteria in blood (Podnecky et al. 2013).
3. DNeasy UltraClean Microbial DNA (QIAGEN)- This kit allows for the extraction of high-quality genomic DNA from many different microorganisms, such as yeast, fungi, Gram-negative and Gram-positive bacteria and spores. Microorganisms are lysed through a combination of heat, detergents, and mechanical force. The freed DNA then binds to a silica filter, and the DNA is washed and eluted in a DNA-free Tris buffer (Dhaliwal 2013).
4. DNeasy Blood and Tissue (QIAGEN)- This kit can be used to extract genomic DNA from multiple sample types including fresh or frozen, formalin-fixed or paraffin-embedded tissues and cells, as well as blood, and bacteria cultures. A silica-based method is used to extract the DNA and can be completed in around 20 minutes following cell lysis (Boyle & Lew 1995; Merk et al. 2006).

For environmental sample extraction:

1. DNeasy PowerSoil (QIAGEN): This can isolate *B. pseudomallei* DNA from soil and other environmental samples. It uses a humic content removal step that eliminates PCR inhibitors and generates high-quality DNA that can be used for many downstream applications (Dhaliwal 2013; Kaestli et al. 2007).
2. QIAamp Fast Stool Kit (QIAGEN): This kit allows for rapid purification of high-quality bacterial genomic DNA from samples containing high levels of inhibitors, such as soil. InhibitEX Buffer is used to efficiently remove PCR inhibitors commonly present in environmental samples (Hall et al. 2019).

Specimen Types

Previous studies have demonstrated that certain specimens with higher bacterial loads (e.g. sputum, pus and urine) are particularly useful clinical specimens for diagnostic PCR testing in patients with melioidosis and that standard DNA extraction methods are robust for these samples (Meumann et al. 2006; Richardson et al. 2012). DNA extraction of *B. pseudomallei* from direct whole blood remains challenging due to the low bacterial concentration and increased presence of inhibitors (Lau et al. 2010; Podnecky et al. 2013), however DNA extracted from plasma, buffy coat blood fractions and from cultured blood specimens have shown enhanced proportional isolation of *B. pseudomallei* DNA (Richardson et al. 2012). Additional steps that degrade inhibitory human DNA and facilitate bacterial cell lysis may improve the quality and quantity of DNA extracted from whole blood and other clinical specimens.

B. pseudomallei Clinical Specimen Extraction Protocols Using Solid-Phase Commercial DNA Extraction Kits

Extraction Quality Controls

Quality extraction controls are important in ensuring that procedures are valid and the results are reliable. To control for false positive and false negative results both a negative and positive extraction control should be utilized (Hornung et al. 2019):

1. Negative Control (extraction blank/contamination control)- This sample contains no sample DNA, only water. This control will ensure that no contamination occurred during the extraction and that any DNA used in the PCR is from the specimen samples only.
2. Internal Positive Control (amplification control)- The presence of PCR inhibitors or errors during sample extraction or thermocycler malfunctions are the most common causes of false negative PCR results and can be controlled by including an Internal Positive Control (IPC). This is used to confirm that DNA amplification has occurred, detects false negatives, and

qualitatively detects the presence of amplification inhibitory substances in a sample. There are two main types of IPCs:

A. Endogenous IPCs- These occur naturally in the specimen, such as a sequence of the from normal microflora genomes (e.g., 16s) or RNAse P in the human genome.

B. Exogenous IPCs- These are external templates that are spiked into samples either during DNA extraction or before PCR amplification. They are simultaneously extracted and then amplified with the target nucleic acid and serve as sensitive indicator of loss or degradation of the specimen DNA either during sample processing or due to inhibition. There are many exogenous IPCs available commercially (e.g., TaqMan® Exogenous Internal Positive Control (IPC)) Reagents- See specific real-time PCR protocol in Chapter 15). 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. If IPC is not available, RNase P (RP) can be used as an internal control for human clinical specimens. For further information about 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 Safety and Handling Measures (BMBL 6th ed. 2020)

A. Patient specimens should be handled at the BSL-2 safety level.

B. Do not pipette by mouth.

C. Wear disposable powder-free gloves, lab coat and goggles while handling reagents or specimens. Wash hands thoroughly after performing the test.

D. All materials should be disposed so that infectious agents are inactivated:

   i. Solid wastes should be autoclaved or sterilized using a pressure cooker if an autoclave is not available.

   ii. To liquid wastes: Add 10% bleach solution. Allow 20-30 minutes for decontamination before disposal.

   iii. All wipes/cleaning material should be treated as hazardous waste.

E. Spills should be wiped thoroughly with a 10% bleach solution. The wiped area should be covered with absorbent material, saturated with the 10% bleach solution and allowed to stand for 20-30 minutes.

F. Do not use the reagents beyond the expiration date.

G. The DNA extraction procedure and Quality Control protocols must be followed closely to obtain reliable test results.

H. It is important to pipette the exact reagent volume and to mix thoroughly after the reagents have been added. Failure to do so could result in inaccurate results.

I. Do not interchange equipment or reagents from different kits.

Equipment and Reagents

- Commercial DNA extraction kit (e.g. QIAamp DNA Mini Kit) containing Buffers and Protease or Proteinase K
- Centrifuge filter columns (such as Microcon YM-100 Columns or Millipore Ultrafree-MC Centrifugal Filter Devices, 0.1 µm)
- 2.0 mL microcentrifuge tubes with O-rings
- Capped 1.5 mL microcentrifuge tubes (e.g., Cat. No. T6050G, Marsh Biomedical Products)
- Tube racks for microcentrifuge tubes

- Biosafety cabinet (BSC)
- Pipettes and barrier/filter pipette tips for volumes 50–1000 µL
- Vortex mixer
- Microcentrifuge with aerosol-tight, autoclavable motor (or similar centrifuge capable of 20,000 ×g/14,000 rpm)
- Personal Protective Equipment (lab coat, eye protection and gloves)
- Water bath or heat block set to 56°C
- Waterproof markers
- KayDry or Kimwipe tissues or similar lab towels
- Biohazard waste disposal (bags and pans)
- Forceps/tweezers
- Distilled water
- 10% bleach solution, made fresh daily (be sure to check the concentration of hypochlorite to determine the correct dilution to use)
- Ethanol, 100%
- Ethanol, 70%
- Phosphate Buffered Saline (PBS)
- Specimens

1. Before Starting the Extraction

Before beginning with the DNA extraction be sure to do the following:

1. Bring specimens to room temperature.
2. Heat a water bath or heat block to 56°C.
3. Ensure that all reagents provided in the extraction kit (e.g. buffer AW1, buffer AW2, Proteinase K) have been prepared according to the manufacturer instructions and are within the expiration date.

4. Prior to beginning the extraction protocol, turn on BSC. Disinfect before use by spraying with a 10% bleach solution and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.
5. Label all tubes with the appropriate unique specimen/sample identifier.
6. Disinfect all equipment before use by spraying with a 10% bleach solution and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.
7. Place the following items in the cabinet: a plastic bag-lined discard waste pan or bucket, pipettes and pipette tips, PBS, Buffers AL and Proteinase K or Protease from the extraction kit, vortex mixer, appropriate number of 2 mL O-ring tubes and labeled 1.5 mL centrifuge tubes (including positive and negative extraction controls) placed in microcentrifuge rack.
8. Prepare enzymatic lysis buffer detergent with lysozyme as described below (if using).
9. Prepare specimens as described below before beginning the extraction. Specimen preparation will vary depending on the source material.

<u>Enzymatic lysis buffer detergent</u>

While not required, using an enzymatic lysis buffer detergent with lysozyme pre-treatment is recommended when performing DNA extractions from *B. pseudomallei* specimens. It may be particularly useful for specimens with lower *B. pseudomallei* bacterial loads, such as whole blood. Lysozyme is an enzyme used to break down the bacterial cell wall and increase protein or nucleic acid extraction concentation. Enzymatic lysis buffer (without lysozyme) can be made up in large batches and stored at 4°C. Lysozyme should be added immediately before use.

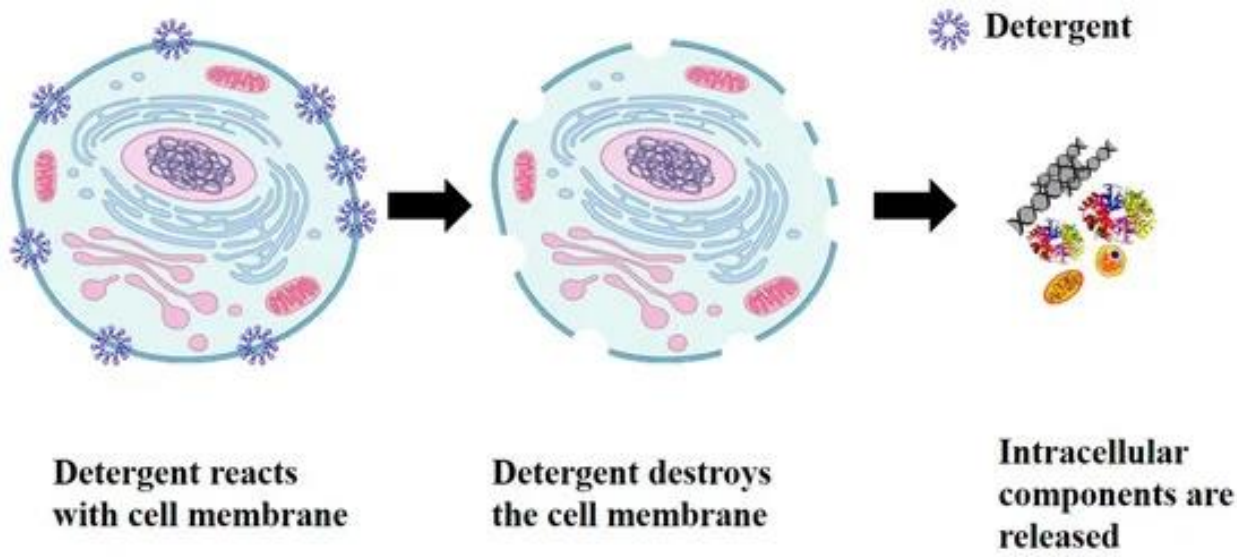


Figure 64- Cell lysis is an important step of the DNA extraction process that works to open the cell membrane and release the intracellular components. Enzymatic lysis buffer can help to increase the bacterial DNA concentration that is extracted (Shehadul Islam et al. 2017).

Lysis buffer detergent ingredients:

- 0.5M Tris–HCl, pH 8.0
- 0.5M EDTA
- 1.2% Triton
- Distilled water
- 20 mg/mL lysozyme powder

For 1 liter of lysis buffer:

1. Add 40 mL 0.5M Tris-HCL and 2 mL 0.5M EDTA and 946 mL of distilled water to a sealable glass bottle.
2. Mix well and autoclave or use a pressure cooker to sterilize.
3. Allow to cool to room temperature after autoclaving or pressure cooking. **Add triton only after completely cooled** (this is done to prevent a foam explosion from occurring!).
4. Store at 4°C until ready to use.
5. Aliquot/measure out enough enzymatic lysis buffer for the extraction batch prior to use (each sample requires 360 µL of lysis buffer) and add it to the correct measure of lysozyme (20 mg/mL powder). **This solution must be made fresh on the day.**
    a. Lysozyme (mg) = (number of samples to be extracted + 4) x (360/1000) x 20. NOTE- it is easier to add the liquid buffer to the small quantity of powder and mix than the other way around. Vortex to mix thoroughly.

2. Specimen Preparation

Preparation of Whole Blood and Plasma and Buffy Coat from Whole Blood

This method is based on whole blood collected in standard EDTA tubes (such as Vacutainer® tubes). Since the concentration of *B. pseudomallei* circulating in whole blood is relatively low, preparation of plasma and/or buffy coat from whole blood is recommended for DNA extraction from *B. pseudomallei*.

Whole blood

1. Mix the blood collection tube by inverting immediately after collection. Inadequate mixing of tubes with anticoagulants may allow micro-clots to form and interfere with extraction. Specimens intended for whole blood analyses should not be centrifuged.

Whole blood specimens stored in Vacutainer® EDTA tubes.

2. Transfer 200 µL of patient whole blood stored in EDTA tubes to a clean, labelled 1.5 mL microcentrifuge tube.

<u>For Plasma and Buffy Coat</u>

1. Centrifuge EDTA blood tubes containing whole blood for 15 minutes at 800xg (2200-2500 rpm). The centrifuged blood should appear similar to the Figure below (Figure 65):

   NOTE- Most blood samples require separation of plasma and buffy coat from cells within two hours of collection.

2. While blood is spinning, clean cabinet and set up workspace in cabinet as described above.

3. Carefully remove the collection tube from centrifuge.
4. Using a disposable transfer pipette, remove most of the plasma (top layer above the buffy coat and red blood cells), taking care not to disturb the buffy coat layer below. Leave approximately 1 cm of plasma above the buffy layer. If the buffy coat layer is disturbed, spin the cells again at 800xg for 2-3 minutes. Store plasma in labelled 2 mL microcentrifuge tubes.

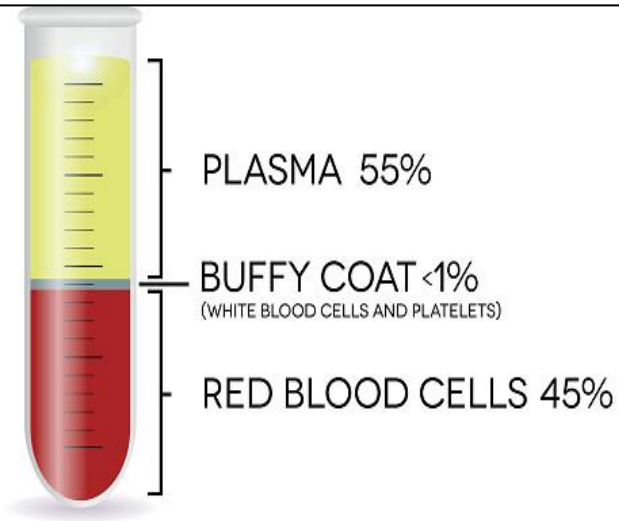


Figure 65- Schematic diagram of a fractioned whole blood sample. The buffy coat layer contains most of the white blood cells and platelets and is located between the plasma and red cells (SP&A Application Laboratory 2014).

5. Using a disposable transfer pipette, remove the buffy coat layer gently and place into a labelled 2 mL microcentrifuge tube. Try not to touch any of the red blood cells. If the buffy coat sample contains visible red cells, spin the 2 mL tubes in a centrifuge at 10,000xg for 30 seconds. Transfer the buffy coat layer into a clean 2 mL tube.
6. Transfer 200 µL of the buffy coat for each patient specimen being extracted into a 2 mL sterile screw cap O-ring microtube.

Body Fluids (e.g., wound abscess, pus)

1. Mix sample by inverting.
2. Transfer 200 µL of sample to sterile 2 mL microcentrifuge tube.

Urine

1. Transfer 200 µL of the sample to a clean 2 mL microcentrifuge tube.

2. Centrifuge the urine sample at 20,000xg/14,000 rpm for 10 minutes.
3. Discard the supernatant.
4. If the urine has already been pelleted than retain the whole pellet for DNA extraction.

Sputum

An initial step containing a sputolysin (such as Calbiochem CAS 578517) pre-treatment is recommended for DNA extractions from sputum specimens. This helps to breakdown the mucin matrix, releasing bacteria that may be trapped within the sputum (Nielsen et al. 2004).

1. Make up sputolysin solution as per manufacturer's instructions.
2. Add equal volume of sputolysin solution to the specimen.
3. Place in a shaking incubator at 37°C and 150 rpm for 20 minutes. If a shaking incubator is not available vortex the solution for 30 seconds to one minute until mixed thoroughly and place in a 37°C incubator for 20 minutes.
4. Centrifuge at 8,000xg for 20 minutes.
5. Discard the clear supernatant.
6. Calculate the volume of the resulting pellet and add 5x the volume of PBS.
7. Vortex to mix thoroughly.
8. Transfer 400 µL of the pellet/PBS solution to a sterile 2 mL microcentrifuge tube.

Rectal Swab

Stool samples and rectal swabs typically contain many compounds that can degrade DNA and inhibit downstream reactions. To ensure removal of these substances, reagents, such as InhibitEX Buffer (QIAGEN Cat No./ID: 19593) are recommended prior to DNA extraction. These work by adhering to inhibitory substances so that they can be removed early in the extraction process (QIAGEN 2010).

1. Add 1 mL InhibitEX Buffer to a 2 mL microcentrifuge tube with O-ring.
2. Using sterile forceps/tweezers and scissors, cut the shaft of the swab ~1.0 cm above the absorbent material. It is good practice to change gloves between samples after this step.
3. Place the cut swab in the 2 mL microcentrifuge tube containing the InhibitEX Buffer.
4. Vortex on high for 1 minute and leave for 10 minutes at room temperature, making sure the entire swab is within the InhibitEX Buffer. Centrifuge for 1 minute at 10,000xg.
5. Transfer 400 µL of the supernatant to clean 2 mL microcentrifuge tube.

All Other Swab Specimens (E.g., Nose, Throat, Wound, etc.)

1. Add 400 µL saline (0.9% NaCl) or PBS solution to a 2 mL microcentrifuge tube.
2. Using sterile forceps/tweezers and scissors, cut the shaft of the swab ~1.0 cm above the absorbent material. It is good practice to change gloves between samples after this step.
3. Place the cut swab in the 2 mL microcentrifuge tube containing the saline or PBS.
4. Vortex on high for 1 minute and leave for 10 minutes at room temperature, making sure the entire swab is within the saline or PBS. Centrifuge for 1 minute at 10,000xg.
5. Transfer 400 µL of the supernatant to clean 2 mL microcentrifuge tube.

Bacterial Cultures-

1. Culture the specimen as described previously in Chapter 10 on Ashdown agar or other *B. pseudomallei*-selective agar and incubate at 37^{o}C for 48 hours.
2. After 48 hours, transfer a small amount of the culture (approximately half (1/2) of a 10 µL disposable loop) to a 2 mL sterile screw-cap tube.
3. Add 360 µL enzymatic lysis buffer containing lysozyme (If using- see components above) to the 2 mL sterile screw-cap tube. Vortex for 30 sec.
4. Place into a 37^{o}C incubator; leave overnight, but not longer than 24 hours.

DNA extracted from bacterial cultures can also be used for downstream genetic typing or whole genome sequencing. However, it is important to ensure that the culture is pure and contains only a single *B. pseudomallei* isolate. To ensure it is a pure culture:

- From the initial culture plate, use a sterile loop to select a single colony suspected (or already confirmed) to be *B. pseudomallei*.
- Ensure that only the bacteria colony of interest is touched.
- Subculture the single colony to Chocolate or Ashdown agar and incubate at 37°C for 48 hours.
- After 48 hours, transfer a small amount of the culture (approximately half (1/2) of a 10 µL disposable loop) to a 2 mL sterile screw-cap (O-ring) tube.
- Add 360 µL enzymatic lysis buffer containing lysozyme to the 2 mL sterile screw-cap tube. Vortex for 30 sec.
- Place into the 37°C incubator; leave overnight, but not longer than 24 hours.

NOTE- DNA extracted from bacterial cultures can be from clinical or environmental specimens. **However, because of the increased volume/concentration of bacteria present when culturing specimens, DNA extraction from cultures that are already confirmed or suspected to be *B. pseudomallei* should be handled in a BSL-3 laboratory until the bacteria has been effectively lysed and is no longer viable/infectious (usually after incubation with Buffer AL).**

See more about appropriate biosafety techniques for handling *B. pseudomallei* culture specimens and additional information about BSL-3 laboratory specifications available from the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition available at: https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2020-P.pdf.

3. *B. pseudomallei* Clinical Specimen DNA Extraction- Manual Kit Procedure

NOTE- The following protocol is based on the QIAamp DNA Mini Kit. It is a summary of the procedures included in the kit manual provided by the manufacturer. Please refer to the manual for detailed product information and protocols: https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en

Similar well-validated kits that have been shown to yield a comparable concentration of high-quality genomic DNA (as described at the beginning of the chapter) from the same volume of specimen can be used if QIAamp is not available. Likewise, in resource-poor laboratories, DNA from clinical specimens can be extracted using Chelex®100 resin beads. Chelex® extraction can be done directly on bacterial cultures grown using selective enrichment media (Ashdown or TBSS-C50 as described in Chapter 10) or from bacterial growth on agar plates. See below for Chelex® 100 extraction details and protocols (de Lamballerie et al. 1992).

Manual Kit Extraction Protocol

1. Place 360 µL of enzymatic lysis buffer (if using; see above) into a 2 mL microcentrifuge tube.
2. Add specimen (amount depends on specimen type; see specifications above). **For culture specimens, the buffer should be added the day before (as above) and incubated overnight.**
3. Vortex samples for 30 seconds.
4. Incubate tubes in a heat block at 37°C for 30-60 mins (this does not need to be done for culture specimens as they will have been in the incubator overnight).
5. Briefly centrifuge tubes at 10,000xg for 30 seconds.
6. Add 20 µL Proteinase K and then 200 µL AL Buffer.

7. Vortex for 30 seconds and briefly centrifuge at 10,000xg for 30 seconds.
8. Incubate tubes using a preheated heat block or water bath at 56°C for 1-2 hours.
9. Briefly centrifuge tubes at 10,000xg for 30 seconds.
10. Add 200 µL of 100% ethanol.
11. Vortex for 30 seconds and briefly centrifuge at 6,000xg for 30 seconds.
12. Load 550 µL of the sample onto the spin column.
13. Centrifuge column at 20,000xg (~14,000rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
14. Place the spin column in a clean 2 mL collection tube.
15. Repeat steps 12-14 until the entire sample has been loaded into the column.
16. Add 500 µL of Buffer AW1 to the center of the column filter. Try not to wet the rim of the tube.
17. Centrifuge column at 20,000xg (~14,000 rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
18. Place the spin column in a clean 2 mL collection tube.
19. Add 500 µL of Buffer AW2 to the center of the column filter. Try not to wet the rim of the tube.
20. Centrifuge column at 20,000xg (~14,000 rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
21. Place the spin column in a clean 2 mL collection tube.
22. Centrifuge column at max speed (20,000xg/~14,000 rpm) for 3 mins to dry the column.
23. Place the spin column in a clean 2 mL collection tube.

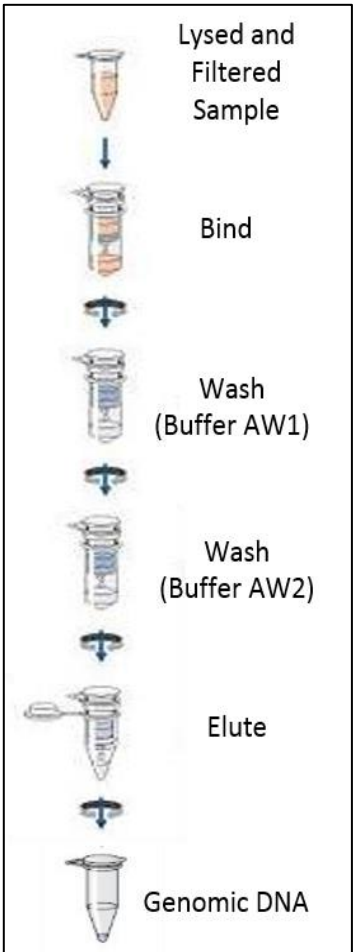


Figure 66- Flow chart of DNA extraction procedure (QIAGEN 2016).

24. Add 200 µL AE Buffer or distilled water to the spin column.
25. Leave at room temperature for 10 minutes.
26. Centrifuge at 6,000xg for 1 minute.
27. Re-load the 200 µL of eluate onto the same column to maximize the concentration of DNA recovered. Leave at room temperature for 10 minutes.
28. Centrifuge at 6,000xg for 1 minute. Retain collection tube and discard column.
29. Transfer DNA solution to a new labelled 1.5 mL screw cap tube and store at 4°C.
30. Disinfect all bench tops, centrifuge, vortex, pipettes and cabinet with a 10% bleach solution or DNAerase and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

B. pseudomallei Environmental Sample DNA Extraction Protocols Using Commercial DNA Extraction Kits

Direct DNA extraction from soil and sediment is especially challenging as particles can reduce the extraction efficiency of commercial kits (Kaestli et al. 2007; Young et al. 2014). The quality and quantity of DNA extracted is decreased by incomplete cell lysis in the soil mixture and also DNA adherence to soil particles (Krsek & Wellington 1999). Additionally, humic substances present in most organic material are strong inhibitors of PCR (Tsai & Olson 1992). A detection method that combines bacterial culture using selective enrichment broth, followed by DNA extraction and real-time PCR has been shown to be the most robust method of detection for *B. pseudomallei* in environmental specimens (Dance et al. 2018; Kaestli et al. 2007). Protocols combining bacterial culture and commercial kit-based DNA extraction methods for the detection of *B. pseudomallei* in soil, water and air specimens are described below. The protocols are based on the DNeasy PowerSoil kit (QIAGEN Cat# 12888-100). Please refer to the manual below for detailed product information and protocols:

https://www.qiagen.com/au/resources/resourcedetail?id=5a0517a7-711d-4085-8a28-2bb25fab828a&lang=en

Figure 67- QIAGEN DNeasy PowerSoil Kit with kit components and reagents.

Similar well-validated kits that have been shown to yield a comparable concentration of high-quality genomic DNA from the same volume of specimen can be used if PowerSoil kit is not available. Likewise, in poorly-resourced laboratories, DNA from environmental samples can be extracted using Chelex® 100 resin beads. This can be done directly from bacterial cultures using selective enrichment media (Ashdown or TBSS-C50 as described in Chapter 10) or from bacterial growth on agar plates. See below for details and protocols (de Lamballerie et al. 1992).

Solid-phase *B. pseudomallei* DNA Extraction from Environmental Samples

Equipment and Reagents-

- Vortex Adapter for 24 tubes (1.5-2 mL) (cat. no. 13000-V1-24)
- Vortex-Genie® 2 Vortex
- Centrifuge for 50 mL conical centrifuge tubes as well as 2mL tubes
- BSC
- Heat block or water bath set to 70°C
- Shaking incubator or standing/benchtop incubator set to 37°C
- DNeasy PowerSoil Kit (QIAGEN Cat# 12888-100)
- 50 mL conical centrifuge tube (e.g., Falcon tube)
- 2 mL Centrifuge filter columns (such as Microcon YM-100 Columns or Millipore Ultrafree-MC Centrifugal Filter Devices, 0.1 µm)
- 1.5 mL microcentrifuge tubes (e.g. Axygen™ MCT200LC or Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes)
- Pipette and pipette tips
- Disposable culture loops
- Ashdown or TBSS-C50 Broth (see Chapter 10 for components)
- Aurintricarboxylic acid (ATA- see below for components)
- Proteinase K (20 mg/mL- e.g., ThermoFisher Cat# AM2546)
- KayDry or Kimwipe tissues or similar lab towels
- Biohazard waste disposal (bags and pans)
- Tape or lab Parafilm®
- Waterproof labelling marker
- Distilled water
- 10% bleach, made fresh daily (this is a dilution of household bleach and water, be sure to check the concentration of hypochlorite to determine the correct dilution to use)

- Ethanol, 70%
- PPE (gloves, lab coat, goggles)

Aurintricarboxylic acid (ATA)

While not required, the addition of ATA to soil has been shown to significantly increase DNA yield. ATA is an inhibitor of nucleases and has been successfully used for *B. pseudomallei* DNA extraction from soil (Kaestli et al. 2007).

ATA components-

- ATA (Sigma-Aldrich A1895-5G)
- Distilled water

Protocol-

1. To 500 mL of distilled water: add 1.75 grams (3.5 mg/mL) ATA.
2. Adjust pH to 8 using sodium hydroxide (NaOH).
3. Autoclave or sterilize using a pressure cooker.
4. Aliquot/divide into 50 mL measurements.
5. Freeze at -20°C until ready to use. Ensure the solution is protected from light.

Extraction Controls

Quality extraction controls are important to ensure the procedure is valid and the results are reliable. DNA extraction protocols must include a negative control. Using a known *B. pseudomallei* positive control or an internal extraction positive controls is recommended:

1. Negative control (extraction blank)- This sample contains no sample DNA, only water. This control will ensure that no contamination occurred during the extraction and that any DNA used in the PCR is from the specimen samples only.

2. Positive control- A known *B. pseudomallei* positive control can be run concurrently with samples each time an extraction is performed. Care should be taken to ensure no cross-contamination occurs between the positive controls and the samples or negative controls. Likewise, a standardized internal extraction positive control may also be used (such as BIOLINE #BIO-35028 or Primerdesign™ Precision). When performing DNA extraction, it can be helpful to include an external source of DNA template that is added to the lysis buffer. This is purified alongside the sample DNA and can then be detected as a positive control for the extraction during the PCR run. Its successful amplification can be used to show that PCR inhibitors are not present or are not there in a high concentration.

General Safety and Handling Measures (BMBL 6th ed. 2020)

1. Samples should be handled at the BSL-2 safety level.
2. Do not pipette by mouth.
3. Wear disposable powder-free gloves, lab coat and goggles while handling reagents or specimens. Wash hands thoroughly after performing the test.
4. Before start of sample processing, prepare aliquots of all buffers and solutions to use per batch of samples.
5. All materials used should be disposed of so that infectious agents are appropriately inactivated:
    a. Solid wastes should be autoclaved or sterilized using a pressure cooker if an autoclave is not available.
    b. All wipes/cleaning material should be treated as hazardous waste.
    c. Spills should be wiped with a 10% bleach solution. The area should be covered with absorbent material, soaked with a 10% bleach solution and allowed to stand for at least 20-30 minutes.
6. Do not use the reagents beyond the expiration date.

7. Extraction procedure and Quality Control protocols should be followed closely to achieve reliable results.
8. It is important to pipette the exact reagent volume written in the protocol and to mix thoroughly after each reagent is added. Failure to do so could result in inaccurate results.
9. Do not interchange equipment or reagents from different kits.

B. pseudomallei **DNA Extraction Protocol from Soil Samples**

A. Preparation of Soil Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each soil sample:
    A. 3x 50mL labelled conical centrifuge tubes
    B. 1x power bead tube
    C. 7x 2mL collection tubes
    D. 1x spin filter
2. Weigh 20 grams of soil into a 50 mL labelled conical centrifuge tube (e.g., Falcon tube).
3. Add 20 mL of Ashdown or TBSS-C50 selective enrichment broth to each falcon tube.
4. Mix thoroughly using a vortex for 30 seconds.

5. Place 50 mL tubes in a shaking incubator and shake for a minimum of 24-48 hours (samples may be left in the incubator up to 7 days before being processed) at 37˚C at 220 rpm. If a shaking incubator is not available vortex tubes thoroughly and place in a 37°C standing or bench top incubator. Try to keep the time in the incubator consistent for all samples.

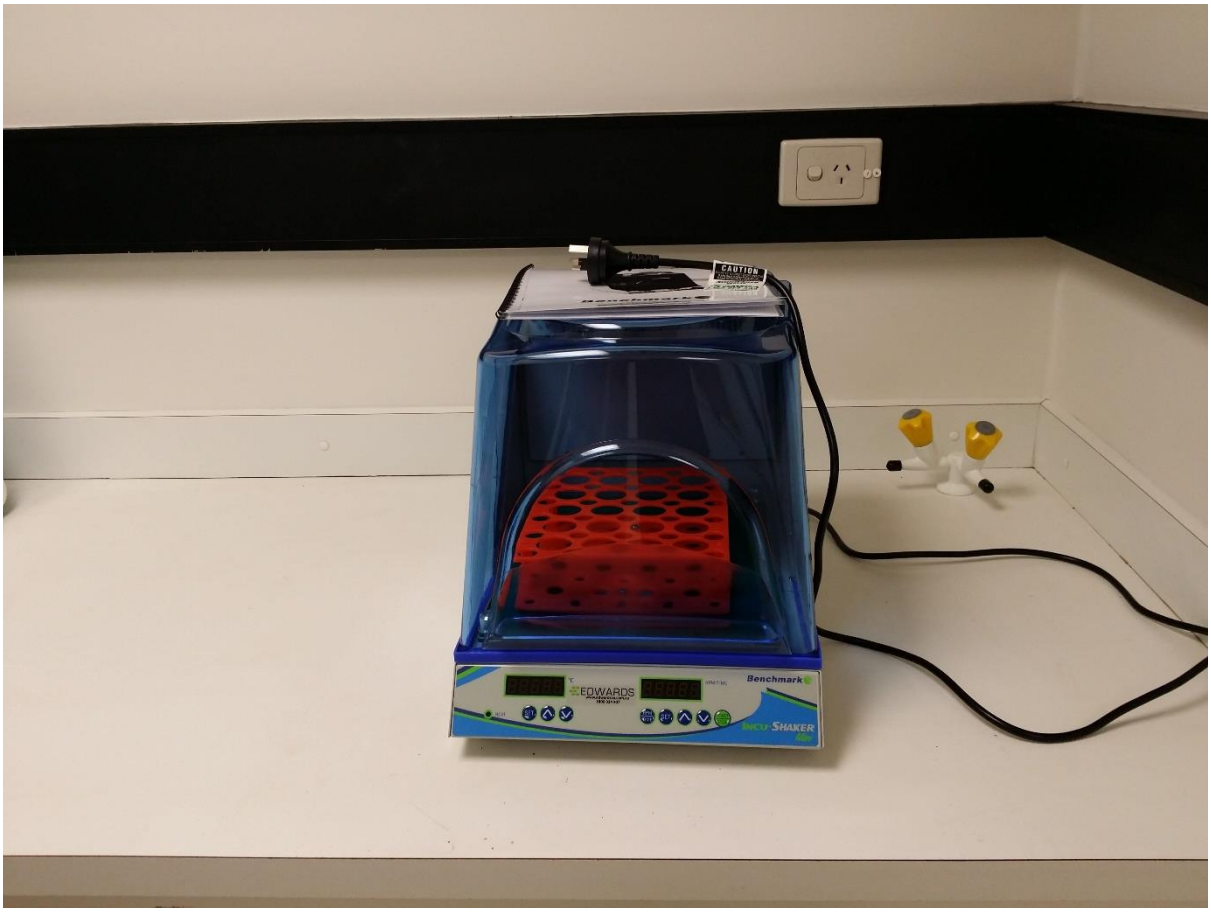


Figure 68- Example of a shaking incubator containing a fitted tube rack for 50 mL conical centrifuge tubes.

B. Soil Sample Processing

1. Remove soil samples from the incubator.
2. Pour the supernatant into a 50 mL labelled conical centrifuge tube.
3. Centrifuge the tube at 4,300xg for 30 seconds.
4. Transfer the supernatant into a clean 50mL tube containing 150 µL of ATA (if using; see above).

5. Seal the lid with tape or laboratory Parafilm®.
6. Centrifuge at 4,300xg for 45 minutes.
7. Discard the supernatant being careful not to disturb the pellet - if there is only a small soil pellet, leave a thin layer of supernatant on top of the pellet.
8. Pour contents of the PowerSoil PowerBead tube into the 50 mL conical centrifuge tube containing the soil pellet. Resuspend pellet with a clean loop and carefully transfer all back into the PowerBead tube. Discard the empty 50 mL tube.
9. Add 60 µL of Solution C1 to the sample and vortex briefly. If Solution C1 has precipitated, heat at 60°C until the precipitate dissolves.
10. Incubate the sample for 10 min at 70˚C in a heat block or water bath and transfer to ice for 2 minutes.
11. Add 20 µL of proteinase K (20 mg/mL) and incubate for 30 minutes in a heat block or water bath at 56˚ C.
12. Secure PowerBead Tubes horizontally using

Figure 69- Vortex-Genie 2 Vortex and the fitted vortex adaptor. The platform has been designed specifically for vigorous mixing and homogenization of multiple samples simultaneously.

a Vortex Adapter for 24 (1.5–2.0 mL) tubes (cat. no. 13000-V1-24). See Figure 69 above for an example the fitted vortex adapter and tube positioning.

13. Vortex at maximum speed for 20 min. Vortexing is critical for complete homogenization and cell lysis. Use of the Vortex Adapter will maximize sediment homogenization, which can lead to higher DNA yields. If this platform is not available, vortex EACH specimen vigorously for 1-2 minutes.
14. Centrifuge tubes at 10,000xg for 30 seconds.
15. Transfer the supernatant to a clean labelled 2 mL collection tube.
16. Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 2–8°C for 5 minutes.
17. Centrifuge the tubes at 10,000 x g for 1 minute.
18. Avoiding the pellet, transfer up to 600 µL of supernatant to a clean 2 mL collection tube.
    Note: The pellet contains non-DNA organic and inorganic material including humic acid, cell debris and proteins. To improve DNA quality, avoid transferring any of the pellet.
19. Add 200 µL of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
20. Centrifuge the tubes for 1 minute at 10,000 x g.
21. Transfer up to 750 µL of supernatant to a clean 2 mL collection tube, ensuring the pellet is not touched.
22. Mix Solution C4 by shaking thoroughly and add 1200 µL to the supernatant. Use a larger 50 mL tube to combine solution C4 with the supernatant from the previous step if necessary. Vortex for 5 seconds.
23. Load 675 µL onto the Spin Column and centrifuge at 10,000xg for 1 min. Discard the flow-through.
24. Repeat Step 23 until all of the sample has been processed.
25. Add 500 µL of Solution C5. Centrifuge for 30 seconds at 10,000xg.
26. Discard the-flow through. Centrifuge again for 1 min at 10,000xg.

NOTE- It is important that all traces of wash solution are removed as the ethanol in Solution C5 can inhibit downstream applications such as PCR and gel electrophoresis.

27. Carefully place the Spin Column into a clean 2 mL Collection Tube.
28. Add 100 µL of Solution C6 to the center of the white filter membrane to wet the membrane.
29. Centrifuge at room temperature for 30 seconds at 10,000xg. Discard the Spin Column ensuring the eluate (flow-through) is retained (this is the DNA).
30. The DNA is now ready for downstream applications or can be stored at 4˚C or frozen (-20°C to -80°C) for longer-term storage.

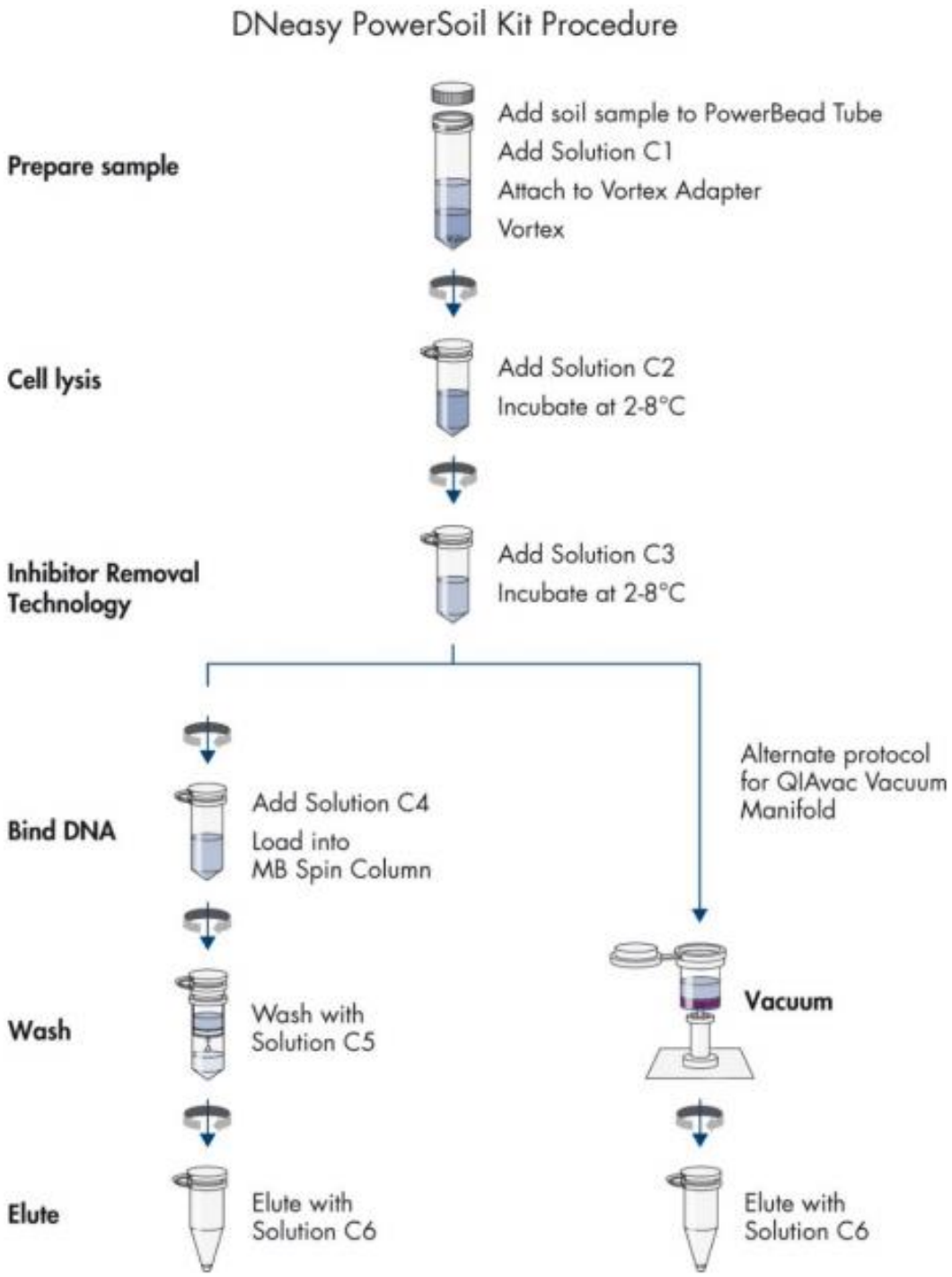


Figure 70- QIAGEN DNeasy PowerSoil protocol using spin columns (left; described above) and QIAvac Vacuum Manifold tubes (right; not described) (QIAGEN 2017).

***B. pseudomallei* DNA Extraction Protocol from Water Samples**

A. Preparation of Water Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each water sample:
    A. 3x 50mL labelled conical centrifuge tubes
    B. 1x power bead tube
    C. 7x 2mL collection tubes
    D. 1x spin filter
2. Filter 500 mL to 1L of water collected for each sample through 0.2-µm-pore-size, 47-mm-diameter cellulose acetate filters using an electrical, vacuum, or manual hand pump as described in Chapter 10.
3. Place the filter in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container.
6. Vortex for 15 seconds with the lid on and place the container in a shaking incubator for 24-48 hours (up to 7 days) at 37˚C at 220 rpm. If a shaking incubator is not available vortex and place containers containing filters in a 37°C standing or bench top incubator for 24-48 hours. Try to keep the time in the incubator consistent for all samples.

B. Water Sample Processing

1. After 48 hours transfer the samples to a BSC. Carefully pour the enrichment broth into to a sterile labelled 50 mL conical centrifuge tube and spin at 4,300×*g* for 30 seconds.
2. Transfer the supernatant to a clean labelled 50 mL conical centrifuge tube and spin at 4,300x*g* for 45 minutes.
3. Discard the supernatant.
4. Transfer the pellet to a clean labelled 2 mL screw-cap centrifuge tube.
5. Follow steps 8-30 of the DNA extraction protocol for soil samples as above.

***B. pseudomallei* DNA Extraction Protocol from Air Samples**

A. Preparation of Air Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each water sample:
    a. 3x 50mL labelled conical centrifuge tubes
    b. 1x power bead tube
    c. 7x 2mL collection tubes
    d. 1x spin filter
2. Place the air filter in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container as described in Chapter 10.
3. Vortex for 15 seconds with the lid on and place the container in a shaking incubator for 24-48 hours (up to 7 days) at 37˚C at 220 rpm. If a shaking incubator is not available vortex and place containers containing filters in a 37°C standing or bench top incubator for 24-48 hours. Try to keep the time in the incubator consistent for all samples.

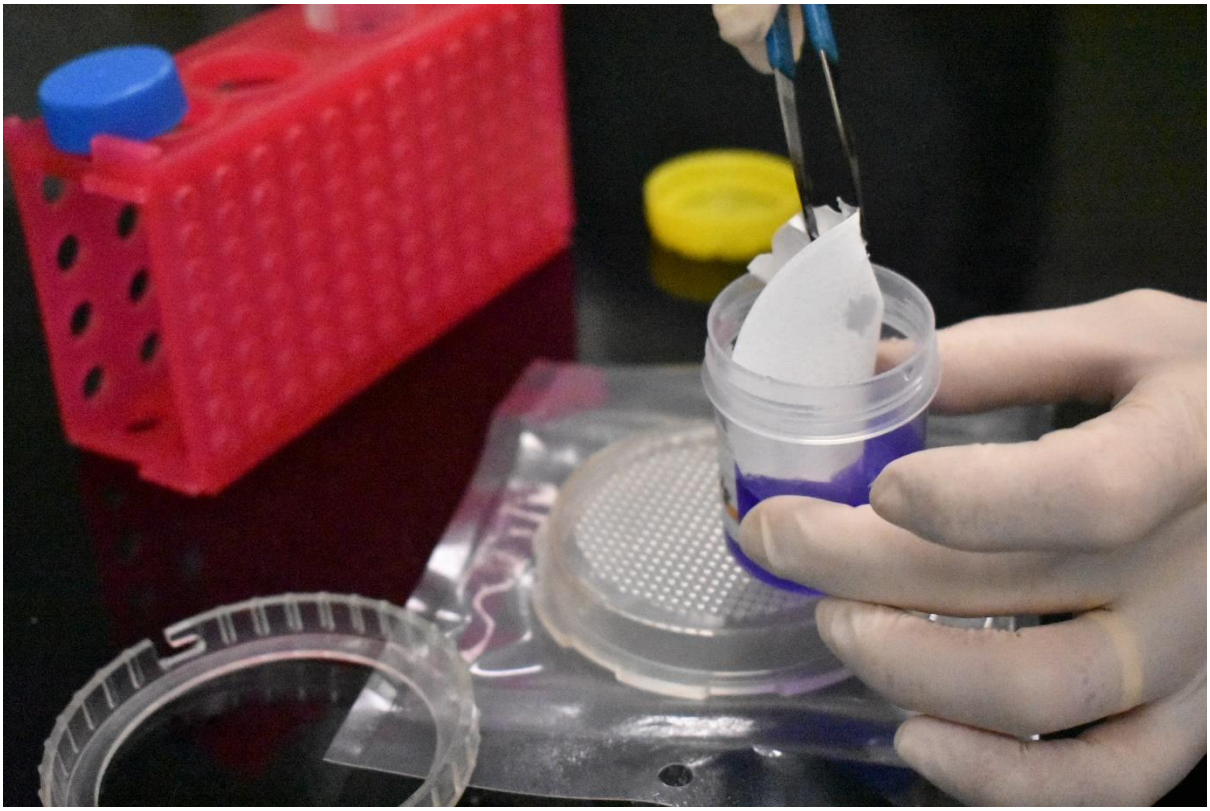


Figure 71- The air filter should be incubated in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container prior to beginning the DNA extraction protocol.

B. Air Sample Processing

4. After 48 hours transfer the samples to a biosafety cabinet. Carefully pour the enrichment broth into to a sterile labelled 50 mL conical centrifuge tube and spin at 4,300×*g* for 30 seconds.
5. Transfer the supernatant to a clean labelled 50 mL conical centrifuge tube and spin at 4,300x*g* for 45 minutes.
6. Discard the supernatant.
4. Transfer the pellet to a clean labelled 2 mL screw-cap centrifuge tube.
5. Follow steps 8-30 of the DNA extraction protocol for soil samples as above.

Chelex® 100 DNA Extraction from *B. pseudomallei* Bacterial Cultures

Chelex® 100 is a chelating material (a compound that binds tightly to metal ions) that can be used as a method of DNA extraction for downstream PCR analysis. Chelex® extraction works by adding Chelex® resin beads to a specimen, heating the solution, then vortexing and centrifuging the solution. The cellular materials bind to the Chelex® beads, while the DNA is available in the supernatant. DNA extraction procedures utilizing Chelex® 100 are simple, rapid, involve no toxic organic solvents, and do not require large amounts of reagents or expensive equipment. As a result, it is a more cost-effective approach to DNA extraction compared to commercial silica-membrane based kits (e.g. QIAamp DNA Mini Kit) (de Lamballerie et al. 1992; Walsh et al. 1991).

There are several drawbacks of Chelex® DNA extraction that should be noted. The high heat steps involved in Chelex® extraction denature the DNA double helix, resulting in single-stranded DNA that is less stable and degrades quickly. As a result, Chelex®-extracted DNA should only be used for PCR-based analyses and not for other applications such as genetic sequencing. It should also not be stored long-term (no longer than 1-2 months is recommended). Additionally, the concentration of

DNA generated by this method is often lower than for other extraction methods and normally contains suspended impurities that inhibit downstream PCR (Butler 2005; de Lamballerie 1992). Consequently, the method is best suited to sample types containing comparatively high concentrations of *B. pseudomallei*, such as bacterial agar and broth culture samples.

Despite this, Chelex® 100 remains a simple and inexpensive method of bacterial DNA extraction, particularly when used for rapid PCR diagnosis of bacterial culture samples. It may be particularly well-suited to resource-poor diagnostic laboratories that do not have access to more expensive commercial kit-based DNA extraction reagents and equipment. **However, if a lab is going to conduct Chelex® extraction, they must follow standard protocols and adhere to the laboratory guidelines and reagents detailed below.**

See the Chelex® 100 instruction manual for further detail: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT200.pdf.

Chelex® 100 DNA Extraction Procedures for Bacterial Cultures and Enrichment Culture Broth

Equipment and Reagents

- Chelex® 100 Resin- Mesh size 100-200 (Bio-Rad #1422825) (Figure 72)
- 150 mL glass beaker
- Magnetic stir bar
- Stir plate
- Distilled water
- Pipettes and pipette tips

- Heat block or water bath set to 95°C
- 2 mL flat bottom screw-cap microtubes (e.g., Fisherbrand™ Free-Standing Microcentrifuge Tubes with Screw Caps)
- 1.5 mL closable microtubes (e.g., Axygen™ MaxyClear Snaplock Microtubes or Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes)
- Waterproof labelling pen
- Vortex mixer
- Microcentrifuge
- PPE (lab gloves, lab coat, googles)
- BSC
- 10% bleach solution or DNAerase
- 70% ethanol

Figure 72- Chelex® 100 Resin- Mesh size 100-200 from Bio-Rad.

1. Preparing Chelex® 100 Resin solution:

a. To a sterile beaker containing a clean magnetic stir bar on top of a stir plate:
   - Add 5 grams of Chelex® 100 resin to 50 mL of sterile water.
   - NOTE– Ensure the Chelex® mesh size is 100-200 and not 200-400 as specified on the bottle.

b. Aliquot/measure 250 µL into 2 mL flat-bottom screw-cap tubes. Ensure the stir plate is turned so there is adequate mixing and dispersal of resin beads as they are dispensed into each tube.

c. Store tubes at 4°C until needed.

2. Chelex® 100 DNA Extraction Protocol

From Enrichment Broth

1. Culture the clinical or environmental specimen in *B. pseudomallei*-selective enrichment broth (Ashdown or TBSS-C50 media) as described in Chapter 10.
2. Incubate the enrichment broth for 48 hours at 37°C.
3. In a BSC, pipette 1 mL of the broth and place it in a labelled 2 mL microcentrifuge tube.
4. Centrifuge the tube at max speed (20,000xg/~14,000rpm) for 5 minutes.
5. Remove 900 µL of the supernatant.
6. Resuspend the pellet and add it to the previously prepared 250 µL Chelex resin in labelled screw-cap tubes.
7. Vortex the tubes for 15 seconds.
8. Incubate the tubes at 95°C for 20 minutes using a heat block or water bath.
9. Vortex samples for 15 seconds.
10. Centrifuge the tubes at max speed (20,000xg/~14,000rpm) for 10 minutes.
11. Transfer 100 µL DNA solution to a new labelled 1.5 mL closable microtube (such as an Eppendorf tube) and store at 4°C.

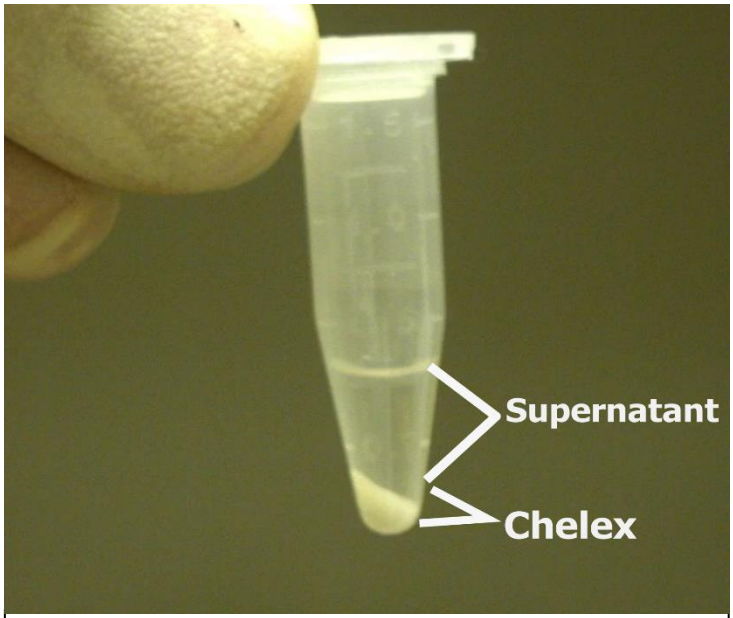


Figure 73- After heating and centrifugation, the DNA is available in the supernatant while Chelex® beads retain the cellular debris.

12. Disinfect all bench tops, centrifuge, vortex, pipettes and Biosafety cabinet with a 10% bleach solution (or DNAerase) and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

The extracted Chelex® 100 DNA is not high quality and PCR screening should be performed within a month. Ensure no Chelex bead resin is present in the final DNA solution, as these can inhibit PCR reactions.

From Agar Culture Plates-

1. Culture the specimen on *B. pseudomallei* selective agar as described in Chapter 10 to achieve single colonies. Incubate plate for 48 hours at 37°C.
2. After 48 hours incubation, transfer some of the agar plate culture (approximately ½ of a loop of a 10 µL disposable loop) to the previously prepared 250 µL Chelex® 100 slurry in 2 mL screw-cap flat bottom tube in a Biosafety cabinet. Use the same loop to mix culture.
3. Vortex the tubes for 15 seconds.
4. Incubate the tubes at 95°C for 20 minutes using a heat block or water bath.
5. Vortex samples for 15 seconds.
6. Centrifuge the tubes at max speed (20,000xg/~14,000rpm) for 10 minutes.
7. Transfer 100 µL DNA solution to a new labelled 1.5 mL closable microtube (such as an Eppendorf tube) and store at 4°C.

8. Disinfect all bench tops, centrifuge, vortex, pipettes and Biosafety cabinet with a 10% bleach solution (or DNAerase) and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

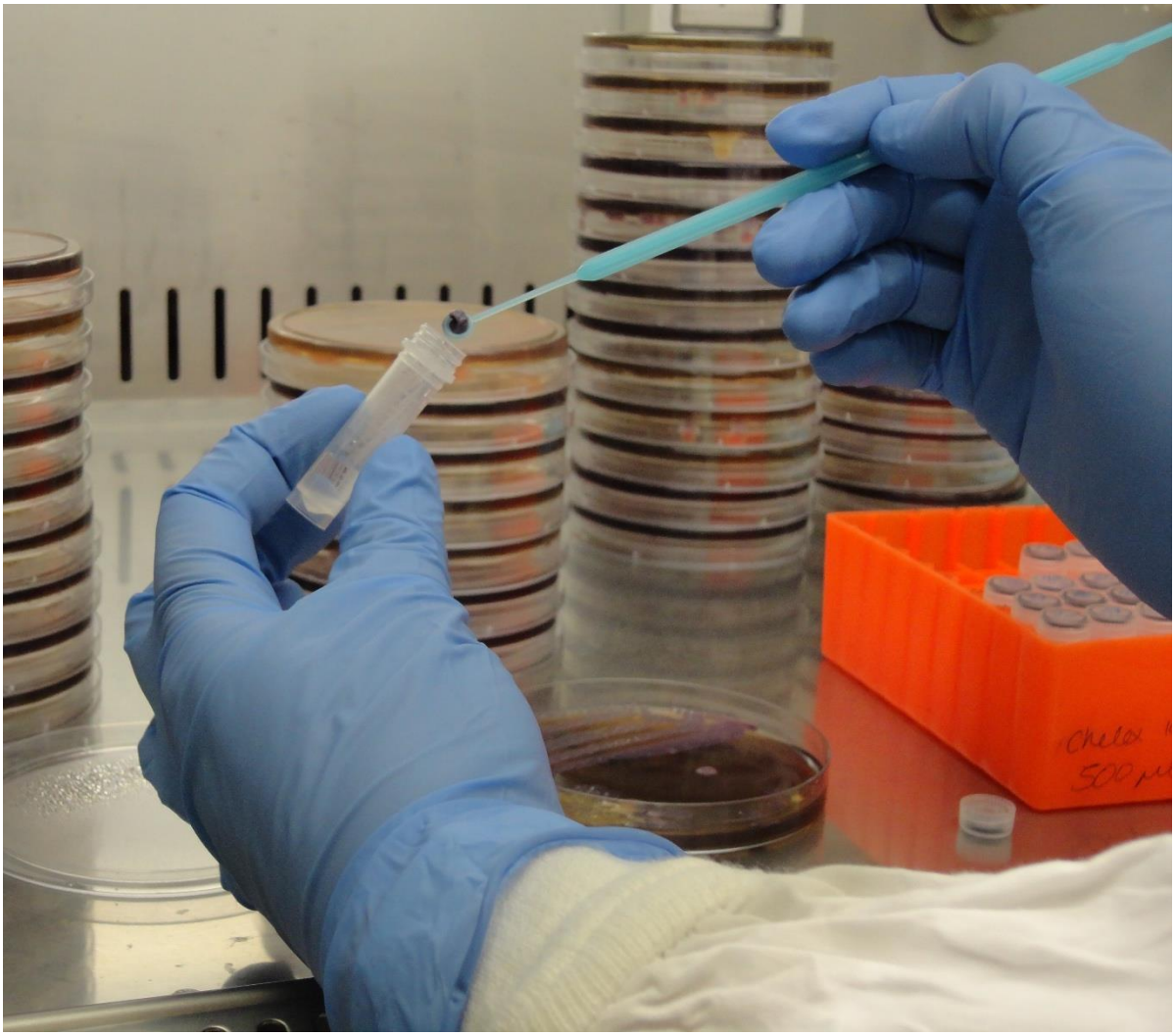


Figure 74- Add a small loopful of culture aseptically to a 2 mL screw-cap tube containing the Chelex® resin bead solution before vortexing and heating the mixture (Image courtesy of Menzies School of Health Research, Australia).

Assessing DNA Sample Purity and Quality/Quantity Control

Examining DNA sample purity, quality, and concentration after it has been extracted is essential to obtaining accurate and reliable results in many downstream molecular biology applications, such as PCR and genetic sequencing. Overestimating the DNA concentration in a sample can lead to too

little input DNA in a molecular reaction or for preparation of a “library” which is DNA prepared in a manner appropriate for whole genome sequencing (WGS). In the case of PCR, too little input DNA can result in weak or no amplification. PCR yield is also reduced if large amounts of RNA are left in the DNA sample, or by inhibitors such as residual EDTA or other negatively charged ions that chelate Mg^{2+}. DNA quality control (QC) usually includes quantitative and qualitative aspects (Bustin 2012; Wahlberg et al. 2012). Parameters monitored typically include:

- Nucleic acid concentration
- Detection of chemical contaminants and other impurities
- Nucleic acid intactness

Measuring DNA Quantity

DNA quantity is normally measured using UV spectroscopy or fluorescence (Brennan et al. 2009). To determine DNA concentrations using the UV-absorbance method, the absorbance of the DNA sample at 260 nm and 280 nm is typically measured using a spectrophotometer. By measuring the amount of light absorbed at a defined wavelength, the concentration of the molecules of interest can be calculated. (Glasel 1995; Olson & Morrow 2012; Wahlberg et al. 2012).

Fluorescence methods determine DNA concentration by using selective DNA binding dyes, such as SYBR Green I (SG), AccuBlue™ or PicoGreen that fluoresce when bound to double-stranded DNA (dsDNA). The fluorescence intensity can then be measured using a fluorimeter or fluorometer (such as the Qubit) (Singer et al. 1997).

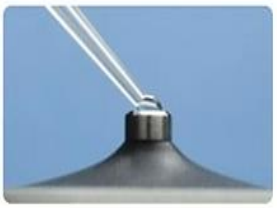
Figure 75- Example of a fluorescence detection methods used to quantify DNA concentration following extraction. The dyes can be used to easily quantify a large number of dsDNA samples (AmiScience 2018).

Measuring DNA Quality (Purity and Intactness)

DNA quality can be assessed in terms of purity, presence of inhibitors and intactness. Purity can be measured using UV spectroscopy, the presence of inhibitors using a PCR inhibition assay, and intactness using gel electrophoresis. UV spectroscopy is used to gauge DNA purity by measuring a sample's absorbance spectrum at 230nm, 260nm and 280 nm, and by measuring the A_{260}/A_{280} and A_{260}/A_{230} ratios (Glasel 1995). These absorbency ratios specify different types of contaminants and the suitability of the DNA for different downstream applications. The A_{260}/A_{280} ratio is a good indicator for PCR suitability. Ratios between 1.8 and 2.0 for A_{260}/A_{280} are used to indicate pure DNA. RNA and protein contamination are denoted by A_{260}/A_{280} ratios that are either above or below 1.8

and 2.0. For the A_{260}/A_{230} ratio the most commonly accepted range is 1.8 to 2.2 and values below this range may be indicative of phenol, salt, protein or polysaccharide contamination (Olson & Morrow 2012). To measure the purity, a specific spectrophotometer, called a NanoDrop, is typically used. These allow scientists to quickly quantify and assess the purity of a small amount (0.5 µL to 2 µL) of DNA or RNA sample (Desjardins et al. 2009).

With the arm open, a sample is pipetted directly onto the pedestal.



After the arm is closed, a sample column is formed.

The pedestal then moves to automatically adjust for an optimal path length (0.05–1mm).

When the measurement is complete, the surfaces are simply wiped with a lint-free lab wipe before going on to the next sample.

Figure 76- A NanoDrop machine is typically used to assess the purity and concentration of extracted nucleic acid. Very small quantities of a sample (0.5-2 µL) are added to the NanoDrop pedestal as shown above, rapidly measured by spectrophotometry and then wiped clean before adding the next sample (ThermoFisher 2021).

PCR inhibitors or impurities can decrease PCR efficiency. These can be examined using an internal inhibition control where known DNA plasmids are added to the extracted DNA and examined using qPCR. An increase in the threshold cycle (C_t) value compared to control reactions typically indicates that PCR inhibitors are present. DNA fragment size and intactness can also negatively affect detection assay results, since the efficiency is reduced if the qPCR target is fragmented, which can prevent amplification from occurring. Gel electrophoresis can be used to evaluate the size of extracted DNA fragments (Olson & Morrow 2012).

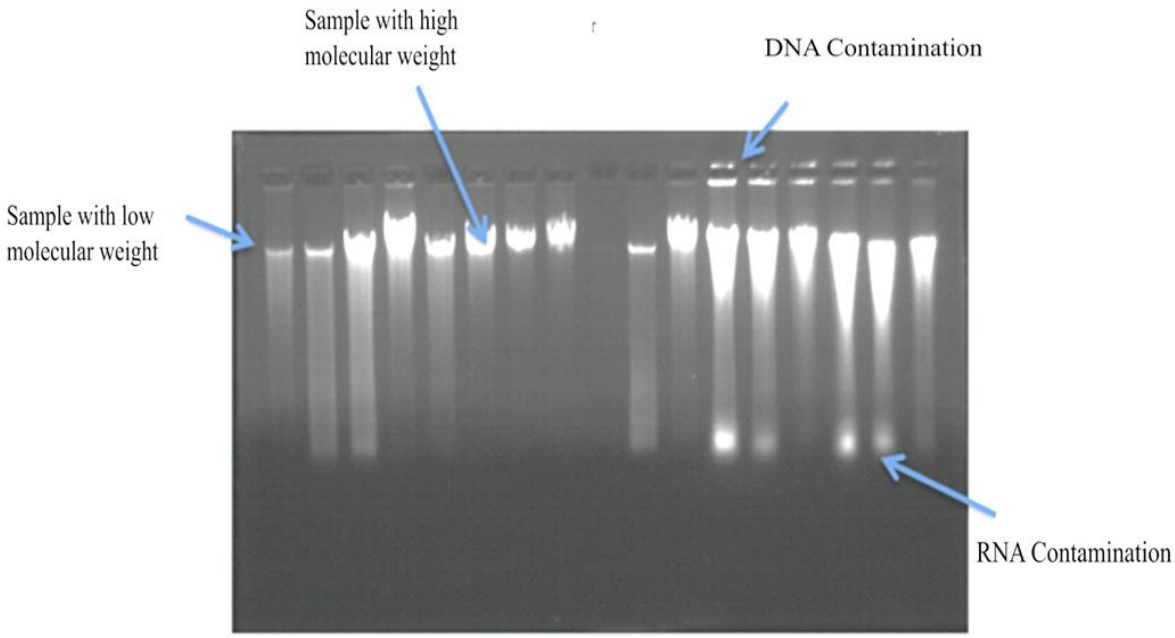


Figure 77- Gel electrophoresis can be used to examine the size of a DNA product and whether there are any PCR inhibitors caused by RNA or DNA contamination.

Chapter Summary

The preceding chapter presents an overview of various DNA extraction processes, DNA extraction quality control, and describes several protocols for DNA extraction from *B. pseudomallei* clinical and environmental specimens. DNA extraction is required for a variety of molecular biology applications and serves as a starting point for PCR diagnosis and detection. Specific protocols describing

conventional and real-time PCR detection of *B. pseudomallei* are discussed in the subsequent chapters.