Genomics Training Academy (GTAC)

Virtual reality resource supporting material: Polymerase chain reaction (PCR)

Model answers document

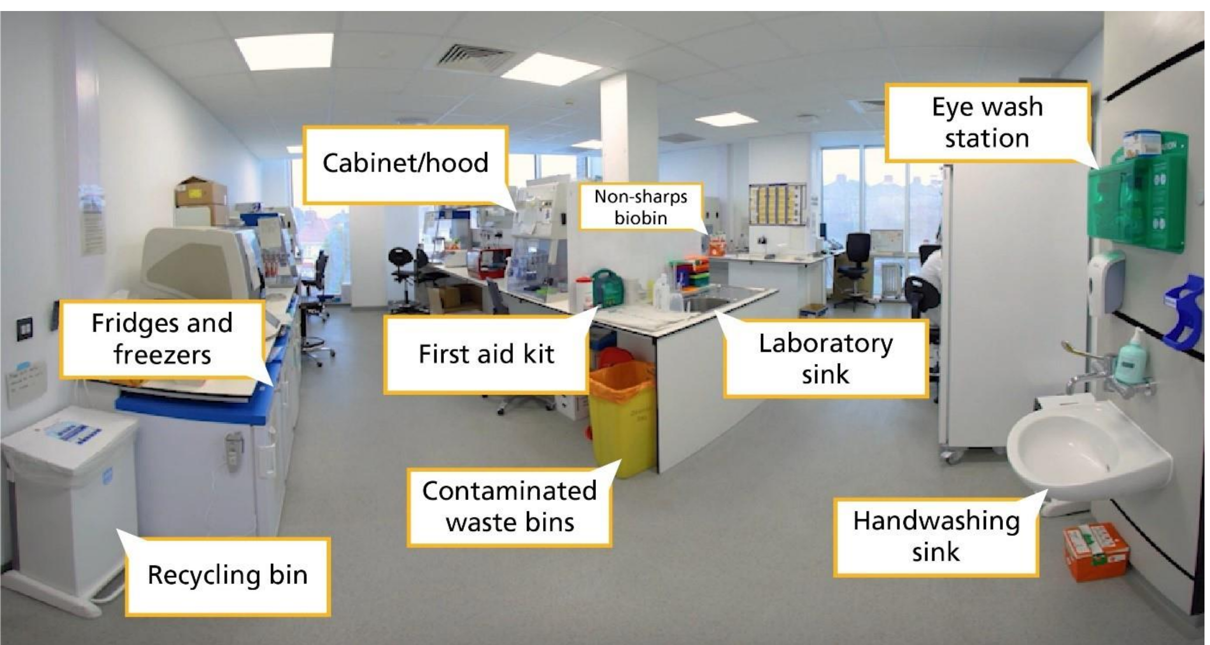
V1 13/09/24

About this document

This document has been designed to support the polymerase chain reaction (PCR) virtual reality resource in training sessions. The questions and model answers below provide a reference point for discussions that will have happened during the session, and can be kept by learners for future reference, or used by trainers to aid in session planning.

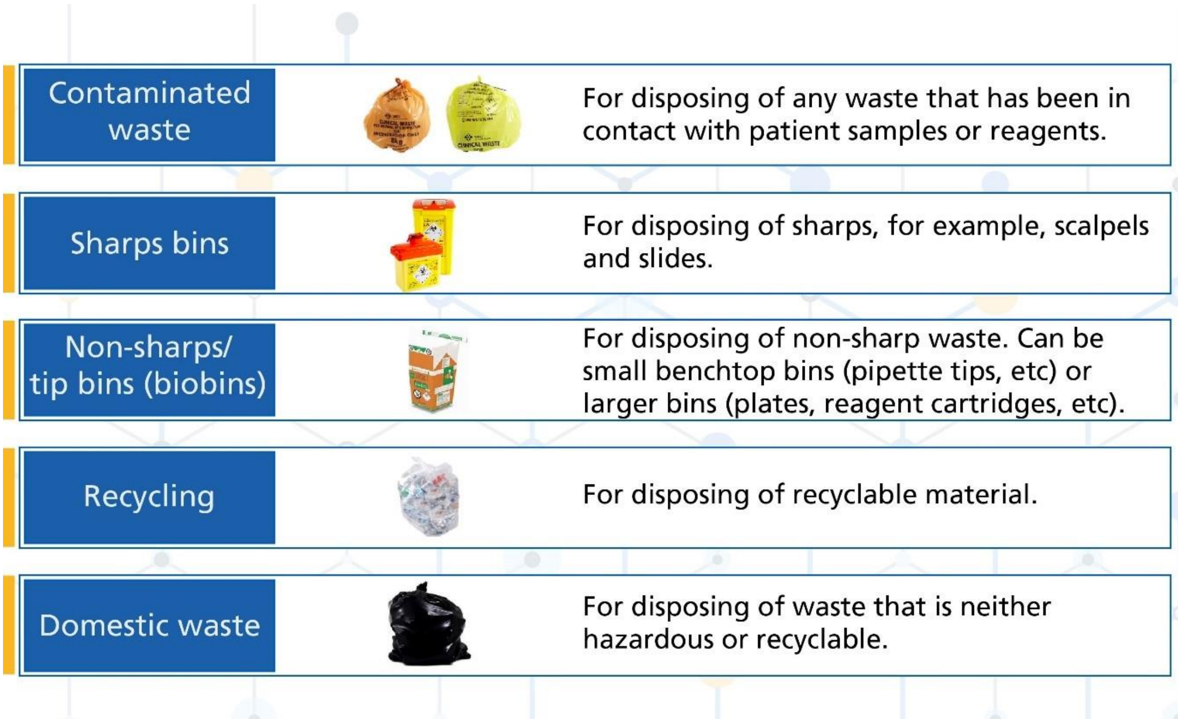
Model answers

Bookmark 1: Laboratory overview (1:59)



1. What items used in the PCR procedure can you see?

- Fridges/freezers – reagents for that room will be kept here. Fridges and freezers should have temperature monitoring. The main DNA bank is likely to be in a separate room, however samples may be stored here temporarily while in use.
- Cabinets/hoods – these are Class II cabinets: open-fronted cabinets that protect the technician from reagents being used in setting up the PCR procedure, but also protect the sample from contamination. There are also Class I cabinets which are designed to protect the technicians, but not the sample, during set-up.
- Bins – for disposing of various kinds of waste. See the figure below for more information on the types of bin and their use.



2. What other laboratory items can you see?

- Handwash station – it is important to have a handwash station in every laboratory.
- Laboratory sink – this is separate to the handwash station and is for washing laboratory equipment.
- First aid kit – for immediate first aid only, follow local procedures.
- Eye wash station – for immediate first aid only, follow local procedures.

3. What other generic laboratory items would you expect to see?

Other generic laboratory items include:

- Centrifuges/microfuge/mini-spin – used to ensure samples/reagents are at the bottom of tubes/plates and, in some procedures, are used to separate constituent components of a mixture.
- Robots – used to automate some techniques.
- Vortex – used to mix reagents or samples.
- Heat block – used to maintain sample/reaction at a specific temperature.
- Pipettes – used to transfer set volumes of liquids.
- Spill kit – used for cleaning up larger reagent spillages.
- Gloves – used to protect user and samples.
- Lab coats – used to protect user and samples; lab coats need to be hung up when not in use and washed frequently to ensure they are clean.

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- Cleaning materials – it is important to keep the laboratory clean to prevent contamination. Cleaning materials may be disinfectants to remove bacteria/viruses and/or nucleases to remove DNA-based contaminants.

Bookmark 2: Preparation (2:40)

4. Why are the reagents and DNA kept refrigerated or frozen?

Manufacturers will provide information regarding how reagents need to be stored, and these guidelines must be followed to ensure that they remain stable.

Reagents that have not been stored correctly are not suitable for use as they may cause a false negative or false positive result, or a run failure. Correct reagent storage is a requirement for UKAS accreditation. DNA samples being kept long-term should be stored in the freezer (-20ºC/-80ºC) to preserve their stability; in the short term, they can be stored in the fridge.

5. Where is the storage information for each reagent kept?

SOPs (standard operating procedures) are the source of information regarding where reagents should be stored – whether this be in a fridge, freezer or at an ambient temperature. In addition, manufacturers will provide the material data sheet for reference.

6. Why is it important to centrifuge samples and reagents after vortexing?

When samples or reagents have been mixed, they are centrifuged to ensure all of the liquid is at the bottom of the tube. This is done for two reasons: first, it is safer as liquid is much less likely to splash, but it also ensures that no material is lost.

Bookmark 3: PCR set-up (5:09)

7. Why do we need to dilute the patient's DNA?

DNA is diluted to ensure that the input concentration, which will be specific to the procedure, is suitable for the PCR reaction. If too little DNA is added to the PCR reaction, it may not be possible to determine an accurate result as the signal intensity on the analyser will be too low. If too much DNA is added, then the signal intensity on the analyser will be too high, causing saturation, which can also impact on the accuracy of the result. This is particularly the case in quantitative methods, such as real-time quantitative PCR, where an accurate measurement of template DNA is required for analysis.

8. What do we use to dilute the DNA?

DNA is usually diluted with molecular grade water or a buffer such as Tris EDTA (TE). It is important that the diluent is molecular grade to ensure that no contaminants are introduced into the PCR reaction and that there are no nucleases that could degrade the DNA.

9. What reagents go into a PCR master mix and what do they do?

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- Polymerase – this is an enzyme that creates new strands of DNA from the template DNA under the right conditions. It is sourced from thermophilic bacteria. The most commonly used polymerase is Taq.
- Nucleotides/dNTPs – these are monomers of DNA incorporated by the Taq polymerase into the new DNA strands. These are usually supplied as a mix of all four bases (dGTP, dCTP, dTTP, dATP) although they can be added individually.
- Buffer – the buffer creates the ideal reaction environment for the Taq to amplify the DNA and will be specific to the protocol. The buffer will maintain the optimal pH and will also contain additives such as:
    - magnesium which enhances polymerase activity;
    - potassium which promotes primer annealing; and
    - DMSO which promotes primer annealing.
- Primers – single-stranded oligonucleotides that are complementary to the sequence of DNA which is to be amplified. The primers act as the starting position for the Taq polymerase to begin copying the DNA. Each reaction will have a forward and a reverse primer which bookend the region to be amplified and are complimentary to the 3’ ends flanking that region (see image below).

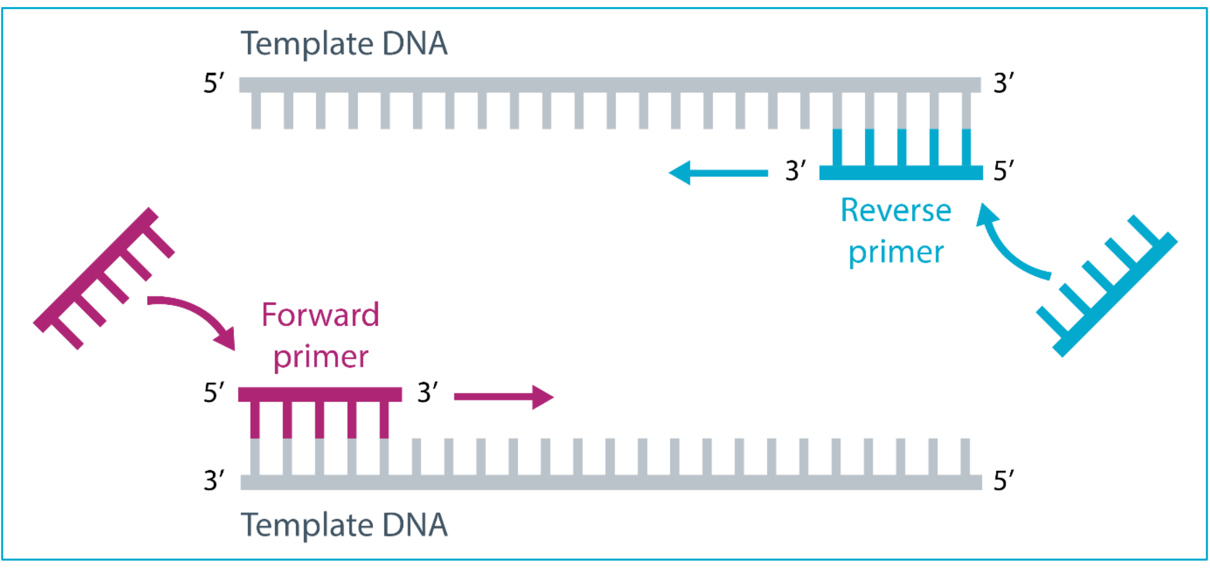

Figure 1 - Reference image of primer annealing process

- Laboratories often use a commercially available mix that contains the reagents required for the PCR reaction. Therefore, depending on the procedure not all constituents will be added individually.

10. What are the key considerations when designing primers?

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Primers need careful design to ensure that the PCR is giving the expected result. They are designed so that they are unique to the sequence to be amplified and to avoid known single nucleotide polymorphisms (SNPs) that could reduce accuracy of primer annealing. They shouldn't have a high GC content and should have an annealing temperature that is standard for your laboratory.

Commercially available assays/kits will come ready with primers specific to the desired sequence, but laboratories will, however, often need to design primers for patient specific reactions – for example, MRD (minimal residual disease) and familial testing. There are several programmes available to assist in primer design, these include Primer Blast and Primer 3 SNP check.

11. What controls would you use in a routine PCR? Why?

Controls are used in PCR to ensure the reliability, consistency and accuracy of the results. They are used to detect contamination within the run and to detect any inter-run variability. Most PCRs will contain the following controls:

- Non-template/water blank negative – this is to ensure there is no amplifiable DNA within the master mix. A run must be failed and repeated if there is evidence of amplification within the non-template control.
- Negative/normal control – this is a DNA sample (either a previous patient or commercial control) that represents a normal or negative result.
- Positive control – this is a DNA sample (either a previous patient or commercial control) that's represents a positive result.

When choosing controls, it is important to ensure that they reflect the intended use of the PCR and that they react as similarly as possible to a patient sample. The type of normal and positive control will be specific to a particular assay. For example:

- QF-PCR – the controls need to ensure that the PCR conditions enable the detection of a trisomic ratio.
- STRs – the controls need to ensure that the PCR has detected the maximum expansion expected.
- Methylation – methylated and unmethylated controls are required.

End of film

12. What factors pertaining to the DNA sample may affect the reaction?

- Contamination; poor quality; suboptimal samples

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13. What steps do we take to ensure that no mistakes are made with the patient samples?

You will see several steps that are taken to ensure there are no mistakes:

Organisation

- The work area, in this case the PCR cabinet, is free from unnecessary items and only the reagents and patient samples used in the assay are taken into the hood.
- As the operator pipettes a reagent or sample, they move it in the rack or to a different rack so that they can keep track of where they are in the process.
- The operator will only have one reagent or sample open at one time to reduce the chance of using it incorrectly.

Checks

- The operator gets a second person to check that they have set up the samples correctly before transferring them. In this case, the actual transfer of samples wasn't witnessed.

Lack of distractions

- It is important that the operator is not disturbed during the PCR set-up.

Training and competence

- The operator must be trained and competent in the procedure. All laboratories will have procedures for training and assessing competency of staff.

SOPs/worksheets

- The operator will be following a SOP to ensure that the PCR is set up in the same way each time. They also have a worksheet that details the samples that are to be used in this PCR and may also contain information about which reagents to use and in what volume. Worksheets are often created by or using the LIMS (Laboratory Information Management System).

13b. (sub question) How is competency assessed in your laboratory?

14. When are witnessed transfer checks used?

Witnessed checks are often used when transferring samples into a plate (as is the case in this PCR) or when the samples are particularly precious (for example, prenatal samples). In this VR film, only one patient was being tested, therefore it was checked but not witnessed. Some laboratories use barcode scanning instead of witnessed checks.

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14b. (sub question) How are checks performed in your laboratory?

15. The person completing the check is described as 'trained' – why is this important?

The person completing the check must understand what they are looking at, so that they notice if there has been a mistake. Who can carry out which checks will vary based on laboratory policy, which can take into account the complexity of the check and/or the consequences of the check being completed incorrectly.

16. What steps have been taken to reduce contamination and why?

PCR cabinet:

- The PCR cabinet circulates air away from the PCR set-up to reduce contamination from airborne particles. It is not always necessary to set up a PCR in a cabinet. This PCR was a real-time quantitative PCR (RQ-PCR/qPCR) and is more sensitive to contamination than a standard PCR so it was used this time.

Organisation/cleanliness:

- The PCR workstation is clean and free from unnecessary items that could contain contaminants. The PCR cabinet has a UV light that can be used to ensure there are no nucleic acids or nucleases on the work surface or equipment – it is important not to use any UV light on any samples or reagents that contain nucleic acids! Laboratories will have procedures for their cleaning,

Keeping items inside the cabinet:

- All items used in the procedure are only opened inside the PCR cabinet. You will notice that when the operator takes out the plate from the box, this is done inside the cabinet.

PPE (personal protective equipment):

- The operator wears gloves and a lab coat throughout the procedure. If gloves are contaminated, they must be changed immediately.

Changing pipette tips:

- The operator uses a fresh pipette tip whenever they are transferring a patient sample or transferring a reagent to a destination that already contains reagents/samples.

- The pipette tips are discarded in a container within the PCR cabinet, ensuring that everything the operator needs is within easy reach.

Pre- and post-PCR:

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- Most laboratories have a separate area for pre- and post-PCR. As described in the video, PCR creates many copies of DNA and therefore it is important that these copies are kept separate from new reactions. If the new reaction is contaminated by previously amplified copies of DNA, the reliability and accuracy of the results will be compromised.

17. What other quality control measures might be taken as part of this procedure?

Other quality control methods that might be used include:

- Reagent records – the operator will make a record, either on the worksheet or in the LIMS/electronic record, of all reagents that have been used in the procedure. This record will include the lot number, opened date and expiry date of the reagents. Reagents should also be acceptance-tested prior to use.

- Equipment records/maintenance – all equipment used in the procedure will have a record as part of the laboratory Quality Management System (QMS). External and internal maintenance will be logged and recorded.

- Risk Assessment/COSHH – all procedures will have a risk assessment and a COSHH (control of substances hazardous to health) assessment to ensure the safety of staff and patients.

- Accurate records – as well as the above records, there will be records pertaining to all aspects of the procedure including operator, checker and dates.

18. Why is it important to set the cycle number and annealing temperature?

The annealing temperature will vary between primers; most laboratories will have a target temperature such as 60ºC or 63ºC. The annealing temperature can be determined by calculating the melting temperature of the primers and then reducing this by a few degrees. These factors will be considered when designing primers. If the temperature is not correct, the primers will remain unbound, causing the PCR to fail.

The number of cycles will depend on the expected yield of the product; each cycle sees an amplification of the product. Generally, 25-35 cycles are expected, but this will vary depending on the amount of DNA input. Large numbers of cycles can result in non-specific PCR products, and therefore it is essential that it is optimised for the service being run.

19. How do you ensure that the PCR machine has run correctly?

When the run is complete, the PCR machine will default to a holding temperature, which should be detailed in the SOP for the PCR being undertaken. If it is a real-time machine, check that the screen indicates the end of the run, and is not displaying any errors. Any errors must be investigated as they may have an effect on the product and skew results.

20. What happens if the controls fail?

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Controls must pass and be showing the correct result – if they don't, further investigation should be undertaken. In general, the run would be failed. The controls act as quality control, checking that the assay is working as expected, and confirming that there is no contamination (non-template/negative control) and that the assay can pick up positive cases (positive controls).

21. Why was the plate put in a different waste bin to the gloves?

The plate is put in contaminated waste due to reagents being present in the base of the plate. There will also be some residual DNA. These therefore cannot go in the domestic waste bins. Gloves, if they have not come into contact with any DNA or contaminated waste, can be safely discarded into the domestic waste. This helps reduce costs as clinical waste is more expensive to dispose of.

22. When would you put the gloves into the contaminated waste bin?

Where gloves have been contaminated, they need to be changed immediately and disposed of in the contaminated waste bin.

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