PCR Process Overview

Polymerase chain reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment. PCR enables a small amount (as low as a single copy) of DNA of a particular target/test sample to be exponentially amplified to generate thousands to millions more copies of that particular DNA segment. A precursor reverse transcript process (RT-PCR) can also be added if the target sample is RNA. The RT process first converts the RNA to DNA, and then the PCR process follows with DNA amplification.

These are the typical reagents and components involved in a typical PCR method and the role that they each play:

1. **Nucleotides** (example: Nucleoside Triphosphate, dNTP) – these are the molecular building block "raw material" for creating new DNA

2. **Enzymes**
   - I. **(Taq) DNA Polymerase** - enzyme required to drive the DNA replication process
   - II. **Reverse Transcriptase** – enzyme required to first convert target RNA into DNA

3. **Primers** – synthetic DNA oligonucleotides. Short, single-strand DNA/RNA fragments, which are designed specifically to flank the target DNA region of interest; they provide the starting point for the DNA synthesis; two primers are needed, one for each side of the region of interest.

4. **Detection Probes or Dyes**
   - I. **Probes** – fluorescently-labelled oligonucleotides which bind downstream of each primer; two different probes are needed, and they fluoresce when both are attached to a specific DNA segment to provide a detectable indicator signal as amplification progresses
   - II. **Dyes** - which will fluoresce in the presence of DNA to provide a detectable indicator signal as amplification progresses

5. **Other excipients**, such as MgCl₂ cofactor, pH buffer, mannitol, trehalose, BSA, PEG20000

6. **Target DNA/RNA**
   - I. For diagnostic kits, it is the DNA of interest which the kit is testing for and is replicated for detection (virus, bacteria, etc); obtained from the sample being tested, if present
   - II. For research/lab work – specific DNA to be replicated

There are numerous processing challenges with the PCR method. The various component solutions need to be accurately combined in a plate well or tube "cocktail". The components require refrigeration and they have a relatively short shelf life and cross contamination is a concern. R&D lab bench pipetting errors can also be troublesome.
The standard PCR process requires a custom thermal cycler instrument to repeatedly heat/cool the reaction test tube for 30-40 cycles, to allow the DNA amplification process to multiply. The same instrument may also have a fluorimeter to monitor/measure the amplified target DNA.

Promising newer amplification methods, including Loop-Mediated (LAMP) and Recombinase Polymerase Amplification (RPA) are isothermal processes which to not require thermal cycling and use of special equipment.

Newer technology is also being developed to load reagents onto microfluidic channels/chips. The lower thermal mass of the smaller amount of reagent promises for faster and more precise temperature cycling which then would yield quicker results.

Frozen Polymerase & Reverse Transcriptase enzymes may contain up to 50% glycerol as a cryo-protectant and stabilizer. Glycerol is not conducive to successful freeze-drying as it can interfere with moisture removal, which affects product stability and structure. Glycerol-free reagents are always preferable when freeze-drying.

Freeze-Drying Equipment Considerations

With reagent sample sizes measured in microliters, the total moisture content in freeze-dried PCR reagent batches is very small. Freeze dryer designs do not need to have a 1:1 shelf surface to ice condenser surface area ratio, which is typical for equipment used in high-moisture, bulk liquid, or vial applications.

Product probes are difficult to use when the product sample size is in the microliter range. For process development work, comparison of Pirani & Capacitance Manometer pressure readings is a suggested drying endpoint tool. Pressure rise testing can also be used.

The refrigeration system in the lyophilizer can also be right-sized (as opposed to over-sized), with the smaller compressors resulting in greatly reduced room heat to contend with. This means less impact on HVAC and energy usage. Air-cooled pilot-sized freeze dryer models are commonly used for medium batch size diagnostic kit applications, which avoids the need for cooling water utilities which may not be easily obtained in many PCR kit processing facilities.

The VirTis Ultra Freeze Dryer is ideally suited for diagnostic applications. It provides over 2m² of shelf area in a compact configuration. The design allows for great flexibility in processing.
Freeze-Drying Process Considerations for PCR Diagnostic Test Kits

The obvious advantage of freeze-drying PCR diagnostic kits is eliminating reliance on the cold chain of refrigerated transportation and storage of the kits, and extending their usable shelf life.

The 96-well plate is a standard configuration used for PCR diagnostic test kits. Automated liquid-filling equipment is commercially available, which provides greater process control and repeatability, in addition to faster loading times and more throughput.

Evaporation and the liquid product temperature can be a concern during the batch loading process into the freeze dryer, especially for larger units with larger batch sizes, which will take longer to fully load. Loading onto pre-cooled shelves can help. If loading onto chips or microfluidic channels, then evaporation is even more of an issue.

The lyophilization process depends on adequate heat transfer into the product. Most 96-well formats are plastic tubes with minimal surface area at the bottom to be in contact with the freeze dryer shelf. Aluminum cooling-blocks can be used during freeze-drying to increase conductive heat transfer.

For signal calibration purposes, diagnostic test kits will often contain both a Negative Control mix with no target DNA/RNA, and a Positive Control mix containing the target DNA/RNA. The control samples are analyzed in addition to the mix containing the patient test sample, and the fluorescent signals are compared to determine if the patient sample is positive or negative. One issue with processing positive control samples is that there is high risk of cross-contamination inside a freeze dryer. The use of multiple freeze dryers can help, with the positive control always processed in the same unit.

Freeze-Drying of Individual PCR Reagent Source Materials

In addition to extending their shelf life and avoiding the need for a supply cold chain, the other benefits of freeze-drying PCR reagents for laboratory use include:

- More robust process with improved reliability and less batch variation, as freeze-drying greatly reduces the potential for lab bench pipetting errors and contamination
- Avoids the damaging effects of multiple freeze/thaw cycles
- Reduced set-up and processing time
- Less waste from expired product, and lower shipping and storage costs

For convenience purposes, pre-formulated freeze-dried PCR “Master-Mix” reagents are also commercially available, which can improve throughput by eliminating some of the mixing steps at the lab bench. The master mix would typically contain the nucleotides, enzymes, cofactor, and buffer.

Final moisture content in the range of 1-3% are desirable. This means that the freeze-drying process will need to include a secondary drying phase once all the ice has sublimed. Moisture measurement via Karl-Fisher titration is recommended.

Freeze-Dried Reagent Beads

A common approach employed by some PCR reagent suppliers for laboratory experiment use is to provide freeze-dried reagent master-mix beads, which are frozen using specialized equipment with liquid nitrogen before they are then freeze-dried.
There are trade-offs with using liquid nitrogen to freeze the reagent before it is to be freeze-dried. The very fast freezing rate does minimize the freeze concentration effect such as pH shift, common with slower freezing rates. However, it does result in very small ice crystal sizes, which result in more resistance to vapor flow during drying and slower drying times. The small sample volumes typical with PCR mitigates this issue, but colder shelf temperature and lower vacuum settings will be needed to keep the beads from melting during primary drying.

In addition, the beads must be kept frozen as they are prepared and accumulated for a freeze-drying batch. A refrigerated cold plate can be used to assist. The freeze dryer shelves also need to be pre-cooled to avoid product melting before vacuum is pulled. Pre-freezing the shelves can lead to frost build up from ambient conditions before the product chamber door is sealed under vacuum.

The freeze-dried pellets or beads are often placed in PCR tubes and then sealed in a moisture-proof pouch.

For more information on the SP VirTis range of freeze dryers, please visit https://www.sp-scientificproducts.com/virtis