The **Polymerase Chain Reaction (PCR)**, developed in the **1980s** by Dr. **Kary Mullis**, is often referred to as a "molecular photocopier" due to its ability to efficiently and accurately amplify specific sequences of DNA. This technique allows for the rapid synthesis of large quantities of a particular DNA fragment, transforming molecular biology research and applications. PCR has had a profound impact on fields like **genetic manipulation**, the **diagnosis of genetic and infectious diseases**, **genotyping**, and **DNA forensics**.

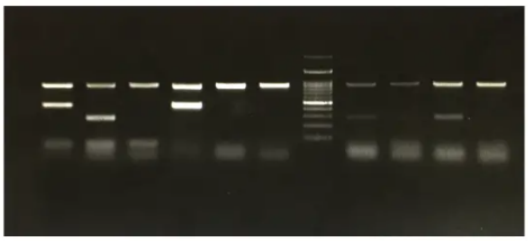
PCR's versatility and accuracy have made it one of the most important scientific advancements of the 20th century. Since its inception, many derivative techniques have been developed, such as:

1. **Real-Time PCR** (also known as **Quantitative PCR (qPCR)**): This variation allows for both PCR amplification and the detection of the DNA sequence in a single step, providing **real-time monitoring of the amplification process**. It is widely used for gene expression analysis and quantification of DNA or RNA.

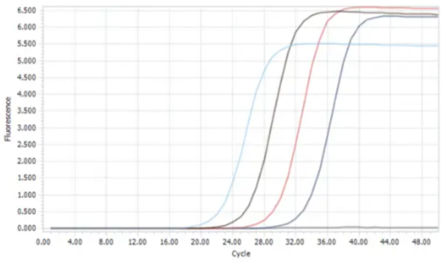
2. **Reverse Transcription PCR (RT-PCR)**: This technique works by converting **RNA** into complementary DNA (cDNA) using the enzyme **reverse transcriptase**. RT-PCR is particularly useful for **analyzing gene expression and studying RNA viruses.**

# Similarities between PCR and qPCR:

Both methods are used to amplify or synthesize the DNA. Both the techniques are based on the temperature-based amplification. The machine used for both techniques is known as a thermocycler. Both RNA and DNA can be amplified in PCR as well as qPCR however, to amplify the RNA a different type of DNA polymerase is used.



Results of PCR



Results of qPCR

**PCR vs. qPCR: Key Differences and Applications**

1. **Primers and Probes**:
    - **PCR**: Uses **simple, non-labeled primers**.
    - **qPCR**: Utilizes **labeled probes** with both a **quencher dye** and a **reporter dye** for fluorescence detection.
2. **Amplification Process**:

- **PCR**: Involves three main steps—**denaturation**, **annealing**, and **extension**. The results are typically analyzed after the reaction is completed, often using **gel electrophoresis** to visualize the DNA fragments.
- **qPCR**: Also includes the basic three steps, but the key difference is **quantification**. It measures **fluorescence** during the **exponential phase** of amplification, offering real-time data about the amount of DNA.

3. **Results Detection**:
   - **PCR**: Results are visible after the reaction, typically as distinct **DNA bands** or **amplicon bands** on an **agarose gel electrophoresis**.
   - **qPCR**: Results are recorded as **fluorescence emissions**, which are converted into **peaks** on a graph. These peaks indicate positive amplification and can be monitored during the reaction.
4. **Resolution**:
   - **PCR**: A **low-resolution technique**, producing discrete bands after the reaction.
   - **qPCR**: A **high-resolution technique** that provides more detailed, quantitative data during the amplification process.
5. **Time and Expertise**:
   - **PCR**: A more **time-consuming** process, taking **3 to 4 hours** for preparation and execution. Analyzing results requires **high expertise**, especially in interpreting gel electrophoresis data.
   - **qPCR**: **Faster**, typically completed in **1 to 1.5 hours**, and requires less technical expertise to interpret results, as the machine provides the data in real-time.
6. **Type of Data**:
   - **PCR**: **Qualitative**—detects the presence or absence of a specific sequence or mutation.
   - **qPCR**: **Quantitative**—measures the **amount** of DNA or RNA during the amplification process.
7. **Applications**:
   - **PCR**: Primarily used for **mutation detection**, **genetic analysis**, and **amplifying DNA templates** for further analysis (e.g., sequencing).
   - **qPCR**: Used for **quantifying DNA/RNA**, **gene expression studies**, **microbial identification**, **quantification of ancient DNA**, and **detecting pathogens** in various fields such as clinical diagnostics, food safety, and forensic analysis.
8. **Technical Principle**:
   - **PCR**: A **basic amplification technique** suitable for detecting mutations and confirming the presence of specific alleles.
   - **qPCR**: A more **advanced method** that quantifies the DNA or RNA based on the **hydrolysis** of the **probe** during amplification. This provides both qualitative and quantitative information about gene presence and abundance.

9. **Specific Variations**:

    ○ **RT-PCR (Reverse Transcription PCR)**: A specialized form of **qPCR** that **reverse transcribes RNA into complementary DNA (cDNA)** before quantifying it. This is used specifically for **gene expression studies**.

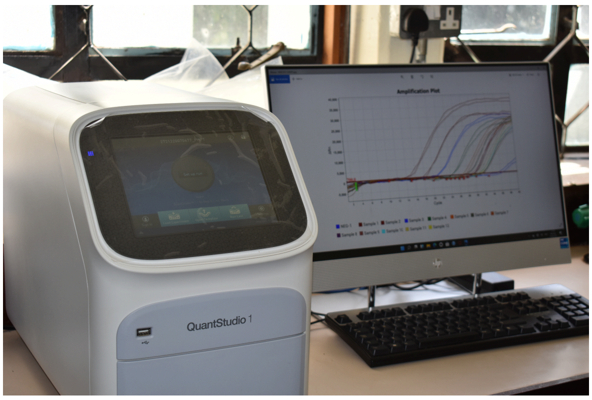
10. **Limitations and Advantages**:

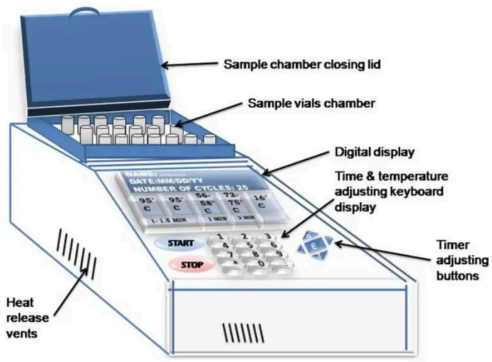
    ○ **PCR**: Primarily limited to qualitative detection of **monogenic mutations**, and used to prepare samples for **DNA sequencing** or **microarray analysis**.
    ○ **qPCR/RT-PCR**: More versatile and can be applied to **gene expression**, **mutation quantification**, **microbial identification**, and various diagnostics in medical and research settings.

In conclusion, while **PCR** is a foundational technique that provides qualitative data for gene detection, **qPCR** expands on PCR's capabilities by providing **quantitative** data, enabling it to be used in more complex analyses like **gene expression profiling** and **pathogen quantification**.

Here's the simplified comparison in a table format:Here's the updated table with DNA-based techniques and RNA-based techniques grouped together:

| Technique | Process |
|---|---|
| **Normal PCR** | DNA is isolated → PCR amplifies the target area |
| **RT-PCR** | RNA is isolated → cDNA is made using reverse transcription (RT) → PCR amplifies the target area |




**Post-PCR Analysis**:

○ PCR reactions often generate specific bands that correspond to the amplicon of interest. The size of the band can confirm whether the PCR amplified the correct target sequence.

- PCR products, or **amplicons**, can be visualized using **agarose gel electrophoresis**, a method that separates DNA fragments based on their **size** and **charge**.
- However, **primer dimers**—undesired by-products of PCR—may appear as **smudgy bands** near the bottom of the gel. These result from primers binding to each other rather than the target DNA, potentially interfering with the desired PCR product.

**qPCR vs. Gel Electrophoresis**:
Unlike traditional PCR, **quantitative PCR (qPCR)** or **real-time PCR** does not require post-PCR analysis like gel electrophoresis. Instead, qPCR analyzes the DNA product **in real-time** as the amplification occurs, allowing for the monitoring of the quantity of DNA during each cycle of amplification.

| | PCR | qPCR |
|---|---|---|
| Full form | Polymerase chain reaction | Quantitative polymerase chain reaction |
| Principle | Primer amplification | Either probe hydrolysis or fluorescence through intercalating dye |
| Chemistry | Non-fluorescence | Fluorescence |
| Ingredients | PCR primers, Taq DNA polymerase, PCR buffer and template DNA | Set of probes, dye, primer set, PCR buffer, template DNA, taq or reverse transcriptase enzyme |
| Assay set up | Reaction preparation, amplification and agarose gel electrophoresis. | Reaction preparation, amplification and real time detection. |
| End results | DNA bands on gel | Peak or graph of amplicons |
| Resolution | Low resolution amplification | High resolution |
| Applications | Amplification, detection of mutation | Amplification and quantification |

**qPCR and Fluorescent Chemistries**

1. **Fluorescent Dyes**:

    - qPCR uses fluorescent chemistries to measure PCR product concentration.
    - **SYBR Green I** is the most common dye, emitting fluorescence when bound to double-stranded DNA. The fluorescence intensity increases with the PCR product concentration.
2. **Challenges with SYBR Green I**:

- SYBR Green I binds to all double-stranded DNA, including nonspecific products like **primer dimers**.
- Careful primer design is necessary to avoid nonspecific binding.

3. **Melt Curve**:
    - To ensure specificity, a **melt curve** is used after PCR.
    - The reaction is exposed to a temperature gradient (60°C to 95°C) to melt the DNA.
    - Fluorescence decreases as the double-stranded DNA dissociates. Specific products melt at a unique temperature, while nonspecific products like primer dimers melt at lower temperatures.
4. **Amplification Graph**:
    - qPCR data can be plotted on an **amplification graph** with cycle number (X-axis) and fluorescence (Y-axis).
    - The **threshold cycle (CT value)** is the cycle when fluorescence exceeds the background.
    - The higher the target DNA amount, the lower the CT value (detected earlier).
5. **Quantification Methods**:
    - **Absolute Quantification** measures the exact number of target molecules (e.g., viral particles in blood).
    - **Relative Quantification** compares gene expression between samples and calculates fold changes.

In short, **SYBR Green I** helps quantify PCR products, and the **melt curve** ensures specificity. **Absolute** and **relative quantification** methods are used based on the experimental need.

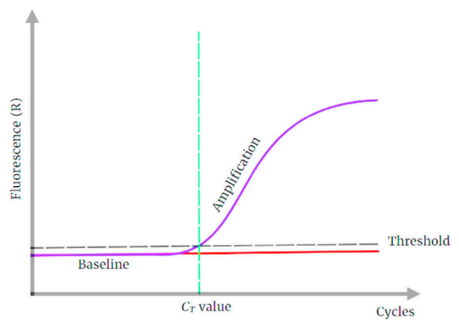
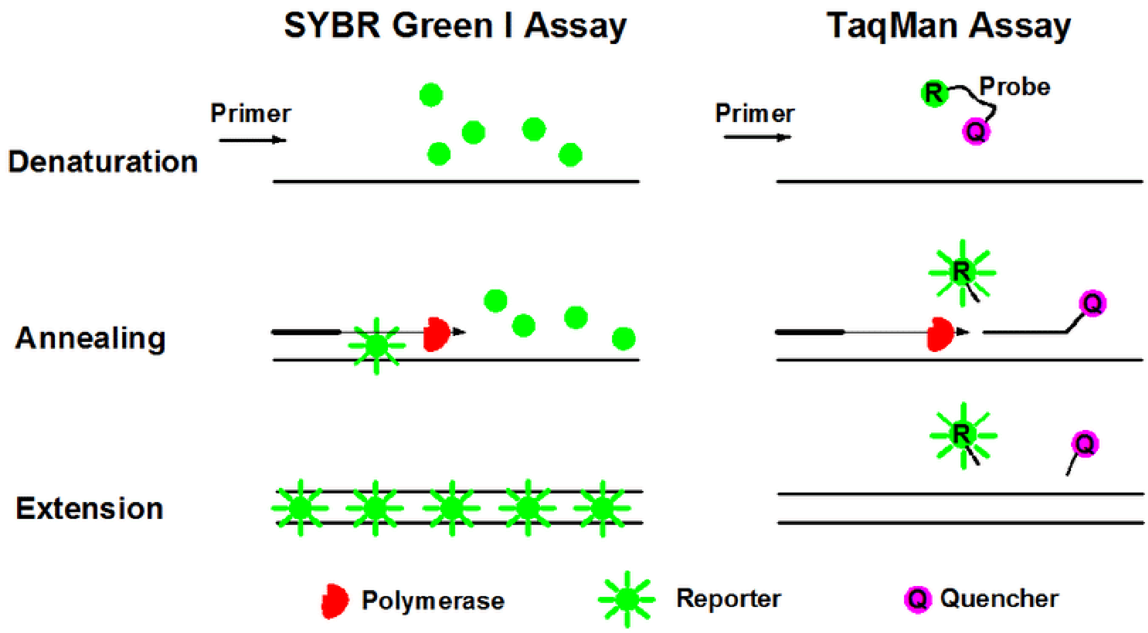



**FIGURE 1.7** qPCR amplification plot. Baseline-subtracted fluorescence versus number of PCR cycles. The threshold cycle ($C_T$) is the cycle number at which the fluorescent signal of the reaction crosses the established threshold line.




| SYBR Green vs Taqman | |
|---|---|
| SYBR Green is based on DNA binding dye. | Taqman depend on hybridization probes and 5' to 3' exonuclease activity of Taq polymerase. |
| **Fluorescently Labeled Probes** | |
| No fluorescently labeled probes are not required. | Dual-labeled probes are required. |
| **Multiplex Gene Analysis** | |
| It can not be used for multiplex gene targets. | It can be used for multiplex gene targets. |
| **Cost** | |
| This is less expensive. | This is more expensive. |
| **Specificity** | |
| This is less specific and binds with any double strand DNA | These are highly specific since probes detect the specific amplification products. |
| **Effectiveness** | |
| This is less effective. | This is highly effective. |
| **Application** | |
| This is used in real-time PCR, agarose gel visualization, DNA labeling etc. | This is used in real time PCR, quantification of gene expression, detection of genetic polymorphisms, etc. |

**PRIMER DESIGN**

https://youtu.be/mcOwIFVEino?si=vTQwnC7bMIuACRQw

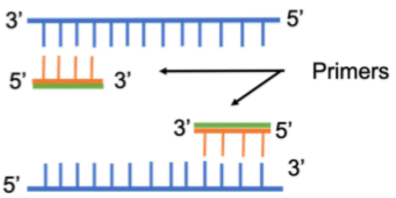
1. **Importance of Primer Design**: Proper primer design is essential for a successful PCR experiment, especially for real-time PCR (qPCR), RT-qPCR, bisulfite PCR, and methylation-specific PCR (MSP). Key factors include primer specificity, size, and amplicon length.

2. **PCR Process**: PCR amplifies a target DNA sequence by cycling through heating and cooling steps. Primers bind to the DNA and help DNA polymerase replicate the target region, doubling the DNA amount in each cycle.

3. **Challenges in PCR**: Low primer efficiency can hinder PCR. To ensure good results, the PCR product must be concentrated and pure.

4. **Solution for Purity**: Zymo Research's DNA Clean & Concentrator Kits help concentrate and clean PCR products, making them suitable for sensitive downstream applications.

5. **Primer Design Tips**: Proper primer design is crucial for effective PCR, qPCR, RT-qPCR, bisulfite PCR, and MSP.




| | Normal Target Sequence |
|---|---|
| Normal Primer | 3' CGTTAGACGAT...5'<br>.......G CAATCTGCTA... |

PCR workflows are sensitive to various factors that can affect the results. Some variables, like the sample source or the need for reverse transcription, are unavoidable. Assay design is crucial and can determine PCR success, reproducibility, and sensitivity. Here's how the process flows:

1. **Target Location**: First, choose the target sequence. This may depend on the application, like SNP detection or gene copy number.
2. **Primer Selection:** Choose the most suitable primers, and make necessary modifications. When multiplexing, consider potential primer interactions and target abundance.
3. **Difficult Cases:** For detecting low-copy targets or small differences, test multiple primer combinations with appropriate probes.

Using software like OligoArchitect simplifies assay design. The online tool offers a range of options, and more specialized designs can be requested from expert molecular biologists.

**Amplicon Selection:**

- The amplicon is the DNA region analyzed, defined by the forward and reverse primers.
- The size of the amplicon depends on the analysis method:
    - Gel electrophoresis: The fragment should be large enough to stain and fit within size markers.
    - Capillary electrophoresis: PCR products should range from 100 bp to 2 kb.

- qPCR: Smaller amplicons (75–200 bp) provide accurate quantification.

**Amplicon Sequence Design Guidelines**:

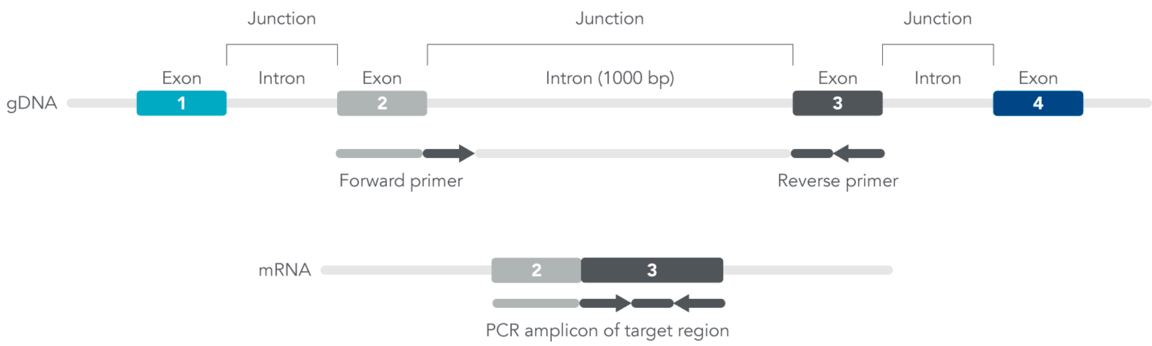
1. **Target Region Assessment**:
   - Check for unexpected SNPs, as mismatches can affect PCR efficiency.
   - Ensure the sequence doesn't match other regions in the genome (or transcriptome) of the target species, especially in multi-organism systems (e.g., pathogen detection).
2. **Secondary Structure**:
   - Use software (like OligoArchitect or mfold) to check if the target sequence forms secondary structures (like stem loops) at the primer's annealing temperature. Avoid regions with strong secondary structures.
3. **Sequence Composition**:
   - Avoid repetitive, palindromic sequences and regions rich in G/C content. Aim for about 50% GC content for better results.
4. **Multiplex Assays**:
   - Keep amplicons similar in length and GC content to avoid uneven amplification in multiplex assays.
5. **Gene Family Assays**:
   - Align sequences and choose regions with suitable consensus sequences for gene families.
6. **Transcript-Specific Design**:
   - For RT-qPCR, choose primers that span exon-exon junctions or flank large introns to avoid amplifying genomic DNA.
   - Use resources like EnsemblGenomes or BLAST for identifying introns and designing primers accordingly.

By following these guidelines, you can optimize primer design and ensure accurate and efficient PCR results.

**Design Considerations:**

- Exon–Exon Junctions: For mRNA quantification, choose primers across exon-exon junctions to avoid gDNA contamination. However, this may affect assay quality.
- Low-Abundant Transcripts: If the mRNA is scarce, gDNA contamination is more problematic, so the assay should account for this.
- SNP Detection: The probe or primer should be near the SNP site.

**DNA methylation** regulates gene expression and is important for development and inheritance. It involves adding a methyl group to cytosine or adenine bases. In most adult tissues, methylation occurs at CpG sites, while in embryonic stem cells, non-CpG methylation is common. Methylation-specific assays typically target CpG islands, especially in gene promoter regions. Tools like Beacon Designer help locate these islands.




**Splice Variant Analysis:**

- To detect all splice variants, select an exon boundary common to all.
- To investigate specific variants, design assays targeting exon junctions or a combination of exons.
- For example, design primers spanning exon 1 and exon 4, with the resulting amplicon length indicating the expressed transcript. A SYBR Green I dye melt curve can be used for post-reaction analysis.

**SNP assays** require precise primer design since the primer sequence must match the target SNP. SNPs can be challenging to detect due to differences in how mismatches destabilize primer binding. For sensitive SNP detection, systems like ARMS (Amplification-Refractory Mutation System) are often used. These primers are typically 30-60 bases long, with a specific SNP-related base at the 3' end, and an additional mismatch near the penultimate position.

**Key Primer Design Guidelines:**

1. **GC Content**: Aim for 40-60% GC content. Include a GC-rich 3' end (GC Clamp) to improve binding stability. Avoid excessive repeating G or C to prevent primer-dimer formation.
2. **High-Quality DNA**: Use pure, high-quality DNA to ensure reliable PCR results.
3. **Primer Length**: Keep primers between 18-30 bases long for effective binding and specificity. Shorter primers generally bind more efficiently.
4. **Melting Temperature (Tm)**: Target a Tm between 65°C and 75°C, and ensure both primers (forward and reverse) have Tms within 5°C of each other. More GC content increases Tm.
5. **Annealing Temperature (Ta)**: Set the Ta 3-5°C below the Tm for efficient primer binding.
6. **Secondary Structure**: Avoid regions that could form secondary structures (like hairpins) and ensure a balanced distribution of GC and AT regions.
7. **No Repeats**: Avoid long runs of the same base (e.g., AAAA or GGGG) and dinucleotide repeats (e.g., ATATAT). Avoid G/C repeats at the 3' end of the primer to prevent off-target binding.
8. **Avoid Homology**: Do not have complementary sequences within the primer (intra-primer homology) or between the forward and reverse primers (inter-primer homology) to prevent primer-dimers.
9. **Restriction Enzyme Sites**: Add 3-4 nucleotides 5' to the restriction enzyme site in the primer for efficient cutting.
10. **Purification for Cloning**: For cloning purposes, cartridge purification is recommended for primers.
11. **Mutagenesis Primers**: Place mismatched bases toward the middle of the primer for optimal mutagenesis.
12. **TOPO Cloning**: For TOPO cloning, avoid phosphate modifications on the primers.

13. **Amplicon Length**: Keep the amplicon length between 70-140 bp for efficient primers and a probe (for qPCR).
14. **Exon/Exon Junctions:** Users can specify that primers span exon-exon junctions with adjustable bases on either side.
15. **Intron Spanning:** Primers can also span introns, with options to specify intron sizes.
16. **RefSeq Requirement:** These features require a RefSeq accession since it offers accurate exon/intron boundary annotations.

These guidelines will help ensure your primers are effective, specific, and free from issues that can affect your PCR results.

**TaqMan® Probe Design Tips:**

1. **Probe Tm**: Keep probe Tm 4-8°C higher than the primers for the best specificity.
2. **Probe Length**: Make probes 20-25 base pairs long for stability.
3. **No Overlap**: Ensure the primer and probe binding sites don't overlap.
4. **Avoid Guanine at 5' End**: Guanine can reduce probe signal.

**General Primer/Probe Considerations:**

1. **Avoid SNPs**: Check for common SNPs that could interfere with primer or probe binding.
2. **Avoid Hairpins/Dimers**: Ensure primers and probes don't form hairpins or dimers, especially at the 3' end.
3. **Specificity Check**: Use tools like NCBI BLAST to check primer specificity against the genome.

**RT-qPCR Primer Tips:**

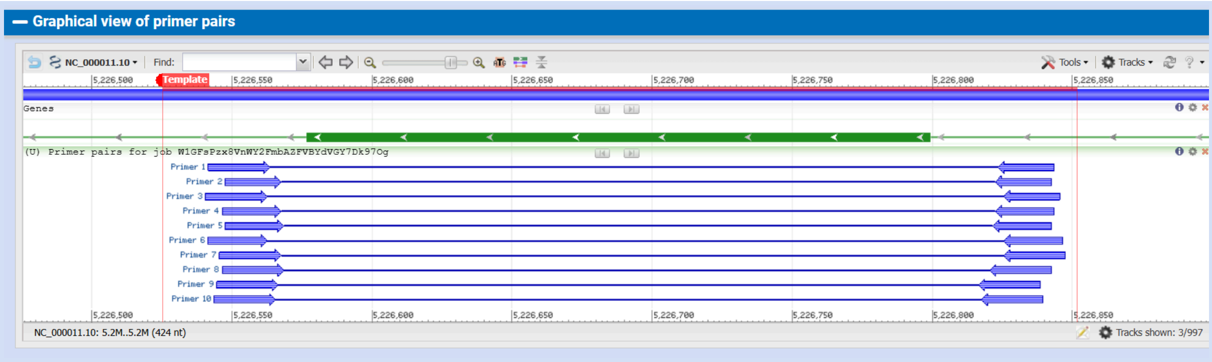
1. **Design Over Exon-Exon Junctions**: To avoid genomic DNA interference, design primers over exon-exon junctions to ensure specificity for mRNA.

**https://www.zymoresearch.com/blogs/blog/how-to-design-primers-for-pcr-experiments?srsltid=AfmBOorAtd2bNlgJgawJAgtHkTS2blykZ6vXMMBljVwWBRqYGTDUCcN4**

**How to design a primer**

1. **https://www.ncbi.nlm.nih.gov/**
2. **The HBB gene in humans** codes for the beta-globin protein, which is a key component of hemoglobin, the protein responsible for carrying oxygen in red blood cells; essentially, mutations in the HBB gene can lead to genetic disorders like sickle cell anemia and beta-thalassemia as it affects the structure of the hemoglobin molecule by altering the beta-globin chain.
3. Gene> HBB (https://www.ncbi.nlm.nih.gov/gene/?term=HBB)
4. 

5. https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi
6. https://www.bioinformatics.org/sms2/pcr_primer_stats.html


Graphical view of primer pairs
NC_000011.10
Find:
Tools
Tracks
Template
5,226,500
5,226,550
5,226,600
5,226,650
5,226,700
5,226,750
5,226,800
5,226,850
Genes
(U) Primer pairs for job W1GFsPzx8VnWY2FmbAZFVBYdVGY7Dk97Og
Primer 1
Primer 2
Primer 3
Primer 4
Primer 5
Primer 6
Primer 7
Primer 8
Primer 9
Primer 10
NC_000011.10: 5.2M..5.2M (424 nt)
Tracks shown: 3/997