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Review Article

# Primer Optimization for SNP Based PCR And Quantitative Real Time PCR Assays

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## ABSTRACT

Primer designing is critical for efficient polymerase chain reaction (PCR) and real time PCR, especially for quantification of real time PCR assays and single nucleotide polymorphism (SNP) for genotype analysis. Careful selection and optimization of primers is necessary for specific, reliable and efficient results in these methodologies. While detecting SNP, it is important for primers to amplify specifically the target region with minimal risk of non-specific amplification or primer dimer formation. In real-time PCR or quantitative PCR (qPCR), primer designing is more complex as primers must be compatible with both the target sequence as well as fluorescent reporter system used to monitor the amplification in real-time. Therefore, knowledge and understanding of primer designing and principles of optimization are necessary for researchers and analysts who are dependent on PCR-based methods for required results. This review provides a comprehensive overview of the key considerations to design primers in SNP-based PCR and real-time PCR assays, covering the important aspects such as primer length, melting temperature, GC content, and specificity. It also elaborates the use of specialized software tools for primer designing and the importance of experimental validation to ensure optimal assay performance.

## INTRODUCTION

Considering a Microsoft word document of 30,000 words titled "Manuscript of life" and you have given a task to find a word "DNA" in a specific context within the document; you may end up with having more than 5000 meaningless results. The case with the deoxyribonucleic acid (DNA), present in our cells, is somewhat same. It is a string of As, Ts, Gs and Cs. Complex human DNA is about 3.2 billion base pairs long, searching a sequence "ATTGCAC" might make you land up on thousands of results. This is the rationale behind Invention of Polymerase Chain Reaction (PCR) by Kary B. Mullis. *"DNA before PCR was long and stringy, not really molecular at all,"* said Kary Mullis [1]. PCR is a chain reaction that amplifies the region of interest using three main steps: denaturation, primer annealing and extension.

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Frame of reference to the gene of interest is provided by the primers. Primer is a short stretch of nucleotides that provides a starting point for the synthesis of DNA. The 3' end of the primer acts as a hook for DNA polymerase where it hangs the successive bases complementary to the template strand [2].

DNA polymerase cannot initiate replication by itself as it needs a free 3'-OH group to add nucleotides. DNA polymerase recognizes the primer template junction and begins to add the nucleotides to the exposed 3'-OH terminal of primer. Thus, primer is an essential component of both *in vivo* and *in vitro* nucleic acid amplification process. *In vitro* DNA amplification is performed for many diagnostic and research purposes like viral quantification and detection, gene expression analysis, cloning and Mutation detection. For all these amplification techniques, a primer is needed [3].

### Qualities of a Good Primer

**Specificity:** A primer must be specific to its target sequence to carry its amplification effectively. Poor specificity of primer can result in undesirable PCR products. Specificity of a primer can be determined

from Primer BLAST software [4]. For non-specific dyes such as SYBR Green, melt curve analysis is used to achieve the specificity of primers [5].

**Primer Length:** Specificity of a primer is associated with its length. Generally, a good primer pair has optimal length of about 18-24 base pairs (bp). Primers within this range have substantial specificity to bind its target sequence and adequate annealing capacity. *In vivo*, Primers have relatively shorter length while primers used *in vitro* are longer to accomplish the specificity [6].

**GC Content and $T_m$:** Primers should have (Guanine Cytosine) GC content of about 40 to 60%. Melting temperature ($T_m$) of a primer is related to its GC content. $T_m$ is the temperature at which half of the double stranded DNA (dsDNA) is denatured into single stranded DNA (ssDNA). Primers with about 50% of GC content have 55-62°C Tm which is considered reasonable for annealing process. A primer pair (forward and reverse) should have minimum difference in their annealing temperatures. Low $T_m$ of a primer can jeopardize its specificity but at high $T_m$ there are chances of mispriming. So, the GC content and $T_m$ are very critical for proper primer functioning and specificity [7].

**GC Clamp:** Presence of Guanine (G) or Cytosine (C) bases at 3' end of primer is essential for specific binding of primer to target sequence because G and C bases have stronger hydrogen bonding as compared to A and T bases. The presence of one or two G or C bases among last 5 bases at 3'end provides specificity of primer because of the stronger bond but more than three GC bases at 3' end can make the annealing process difficult, therefore the quantity of GC bases should not exceed 3 in last 5 bases at 3' end [6].

**Secondary Structures:** Intramolecular and intermolecular forces among primer bases can produce secondary structures which can hinder substantial and efficient yield by affecting annealing process. Three types of secondary structures can be formed among primers given as:

**Hairpin:** A hairpin loop is formed within a primer because of intramolecular interaction within primer. This happens when bases of a single stranded primer form bonds [7].

**Self-Dimer:** Intermolecular forces among two (same sense) primers result in self-dimer formation. This happens mostly when excess primers are used in amplification process. Rather annealing to the template, they hybridize with each other and form primer dimer that reduce the yield of reaction [6].

**Cross-Dimer:** Intermolecular interaction between sense and antisense (forward and reverse) primer result in cross dimer formation [6].

**Repeats:** A repeat is repeated sequence of two different nucleotides, and it must be avoided as it can lead to mispriming. For example: ACACACACACACAC. Maximum number of repeats allowed in a primer sequence are four [6].

**Runs:** A run is reputation of single nucleotide within primer sequence and it should not be encouraged [6].

## Primer Designing Steps and Online Designing Tools

DNA primers are designed based on the sequence of interest, needed to be copied and amplified. A variety of DNA sequences need to be amplified in order to study polymorphism, mutations, methylation and gene sequence. The process of primer designing can be divided into three steps: sequence retrieval, sequence annotation and primer validation. A plethora of programs are available both freely and commercially to design primers. Several software and programs are increasing day by day. Usually, failure to achieve optimal PCR results is due to the improperly designed primers and demand for the software that determine all the parameters effectively. Table 1 describes commonly used online available tools and software along their websites for primer designing [8].

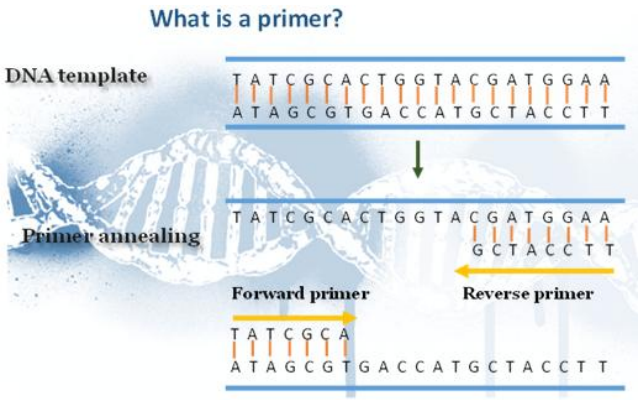


**Figure 1:** Annealing of hypothetical primers to the template strand.

**Table 1:** List of online tools for Primer Design [9]

| Name of Software | URL |
|---|---|
| Primer3 | https://bioinfo.ut.ee/primer3-0.4.0/ |
| Primer3plus | http://primer3plus.com/ |
| NCBI Primer BLAST | https://www.ncbi.nlm.nih.gov/tools/primer-blast/ |
| Primer3web | https://bioinfo.ut.ee/primer3/ |
| Oligocalc | http://oligocalc.eu/ |
| Multiple Primer Analyzer | https://www.thermofisher.com/pk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html |
| Java web Tools | http://primerdigital.com/tools/ |
| Integrated DNA Technologies (IDT) | https://sg.idtdna.com/page/products/qpcr-and-pcr |
| GETPrime | http://updepla1srv1.epfl .ch/getprime/ |
| PrimerBank | https://pga.mgh.harvard.edu/primerbank/ |

**Primer Designing Procedure for SNP detection**

To start with primer designing, the 1st requirement is the sequence of interest. Each gene or sequence of DNA is provided with an accession number in the very same manner as a person who holds a National Identity Number. National Centre of Biotechnology Information (NCBI) calls this accession number "Refseq" while for single nucleotide polymorphism it is termed as "refSNP" or rs number. University of California, Santa Cruz (UCSC) has hosted an online genome browser that can be used to retrieve sequences using reference numbers. This software provides the users with a wide variety of analysis tools including BLAST-like alignment tool (BLAT), variant annotation integrator and In-silico PCR. Ensembl is also a well-known genome browser used by researchers, molecular biologists and geneticists to retrieve genomic information. Ensembl is an outstanding software that provides flanking sequence against the SNP of interest along with its population genetics. One can also go through the literature and citations regarding the SNP under study using the citation feature of Ensembl. It is always facile to copy the retrieved sequence of interest and paste it on a separate word file before proceeding to the next step [10]. For example, genomic properties like allelic changes, genomic location of SNP rs1799983 is retrieved via Ensembl and shown in Figure 2a. To obtain SNP nucleotide sequence along with flanking regions, simply click on "Flanking sequence" option as shown in Figure 2a. This will provide nucleotide sequence of SNP along with upstream and downstream flanking areas sequence as shown in Figure 2b.

**Sequence Annotation**

At this point, the sequence seems meaningless merely a string of four letters. Sequence annotation is a process of giving meaning and identity to the bases present in the sequence by using the BLAT tool of Ensembl or UCSC genome browser. This tool works on the principle on which the world works, gives input and gets output. Yes, enter the sequence of interest in the respective region and submit it. The result would be more than one sequence but only one result will be 100% homology. Select the browser option for that result, and you are good to go with annotation by clicking get DNA option. Extended case/color option can be utilized to bold, italicize or to color the common SNPs, repeats, OMIM Alleles and haplotypes. The result will show the sequence with each letter representing itself and yelling I'm a repeat or I'm a common SNP [11, 12]. In the case of SNP of interest, one wishes to follow up PCR with restriction digestion of the product. NEBcutter is a software that is solely available to look up for type II and type III restriction enzymes that can cut the sequence of interest. After selecting the suitable enzyme, one can proceed with primer designing [13]. Figure 3 shows BLAT results for the sequence presented in Figure 2b. Among BLAT results, sequence with highest number of nucleotides matches corresponds to query sequence (shown in Figure 2b).


**Figure 2a:** Properties of SNP re1799983 via ensembl.org

```
>7 dna:chromosome chromosome:GRCh38:7:150998623:150999423:1
CTACCGGCAGCAGGATGGCTCTGTGCGGGGGGACCCAGCCAACGTGGAGATCACCGAGGT
GGGCACCGAGGGCCACCCATGAGGGTGTCCCCAAGGTGGAGAATGAGGAAACCAGTGGGA
GAAGGCTCGGGGGATCCAGGCAGGAAGAGGGGAGCCTCGGTGAGATAAAGGATGAAAAAC
ACCAAAGGAGGGGTGCCTGGGTGGTCACGGAGACCCAGCCAATGAGGGACCCTGGAGATG
AAGGCAGGAGACAGTGGATGGAGGGGTCCCTGAGGAGGGCATGAGGCTCAGCCCCAGAAC
CCCCTCTGGCCCACTCCCCACAGCTCTGCATTCAGCACGGCTGGACCCCAGGAAACGGTC
GCTTCGACGTGCTGCCCCTGCTGCTGCAGGCCCCAGATGATCCCCCAGAACTCTTCCTTC
TGCCCCCCGAGCTGGTCCTTGAGGTGCCCCTGGAGCACCCCACGTGAGCACCAAAGGGAT
TGACTGGGTGGGATGGAGGGGGCCATCCCTGAGCCTCTCAAGAAGGGCCTGCAAGGGGGT
GCTGATCCCACACCCCAACACCCCCAGGCTGGAGTGGTTTGCAGCCCTGGGCCTGCGCTG
GTACGCCCTCCCGGCAGTGTCCAACATGCTGCTGGAAATTGGGGGCCTGGAGTTCCCCGC
AGCCCCCTTCAGTGGCTGGTACATGAGCACTGAGATCGGCACGAGGAACCTGTGTGACCC
TCACCGCTACAACATCCTGGAGGTGAGGTGCGGGATGGGGCTCGGGCACCGAATGCACCT
GTCCAAGGCAGGAGTCTGGCT
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**Figure 2b:** Sequence retrieved for analysis of SNP via ensembl.org

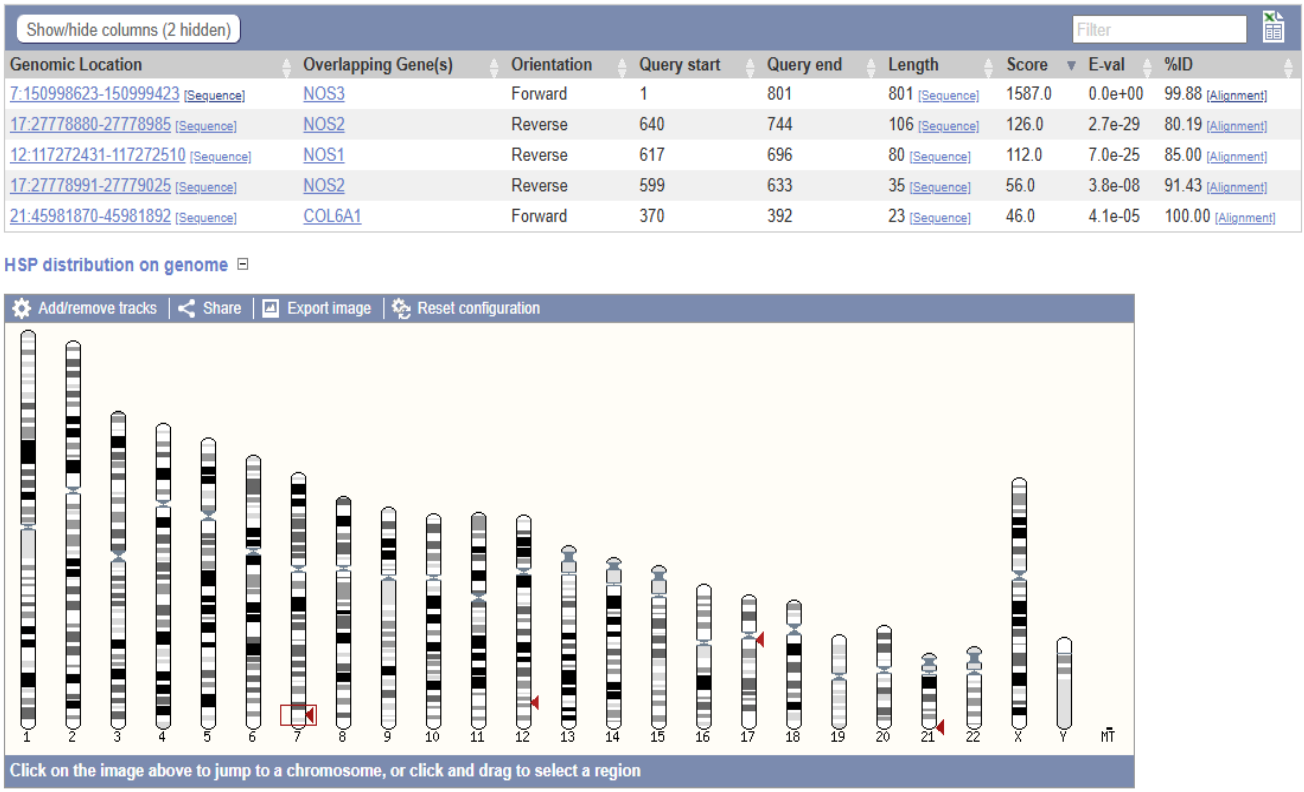



**Figure 3:** BLAT Alignment Results Showing High Nucleotide Identity of Query Sequence with Human Genome at rs1799983 Locus

## Primer Designing and Validation

The sequence can now be used to design primers. Each base of the sequence now has an identity. Select the region of interest to be amplified and leave 20-30 bases on both sides of the region of interest where primers can anneal. This region is enclosed using square brackets. Different online tools are available for primer designing (Table 1). One has to copy target sequence retrieved from any database and paste this sequence into the tool. Primer3Plus is brilliant software that provides primers on one click of the computer within the region specified by user [14]. Primer3Plus generates primer pairs along with detailed properties such as melting temperature, GC content, and potential secondary structures, ensuring optimal primer selection for downstream applications (Figure 4b). Users can specify desired primer qualities such as $T_m$, length, GC content, and target regions. This flexibility enables the design of primers tailored to specific experimental needs ensuring optimal performance in PCR applications (Figure 4a).

Based on experience, a sort of criteria has been set for ideal primers. Primers should be of length between 18-24 nucleotides, and their GC content should be slightly more than 50%. The melting temperature for both the primers should ideally have $T_m$ difference of no more than 2°C [15]. To check a few of these parameters for designed primers, there is an eminent software named OligoCalc. Warren A. Kibbe, the mastermind behind oligoCalc, states *"OligoCalc has a familiar 'calculator' look and feel, making it readily understandable and usable."* This software enables the validation of designed primers. Primers encounter various salts in the PCR tube that might affect the $T_m$ of the primers. OligoCalc is the vital player here and provides salt adjusted reliable $T_m$ for the primers (Figure 5a). This software also provides information about whether the selected primers have any 3' self-complementarity or do they form any secondary structures. If the software says none for all the complementarity parameters, then the primers are successfully validated [16, 17]. Users still get confused about how the primers will perform during PCR. Keeping in view of this problem, UCSC genome browser has introduced the In-silico PCR operation which predicts what kind of product is going to be formed during the reaction (Figure 5b). The successfully designed primers can be ordered online from commercial companies. Cost of primer depends on the number of nucleotides in the design. Soon the cute little white or blue tops containing primers might be sitting in the Biological Safety Cabinet ready to be diluted and used in a Polymerase Chain Reaction [18].

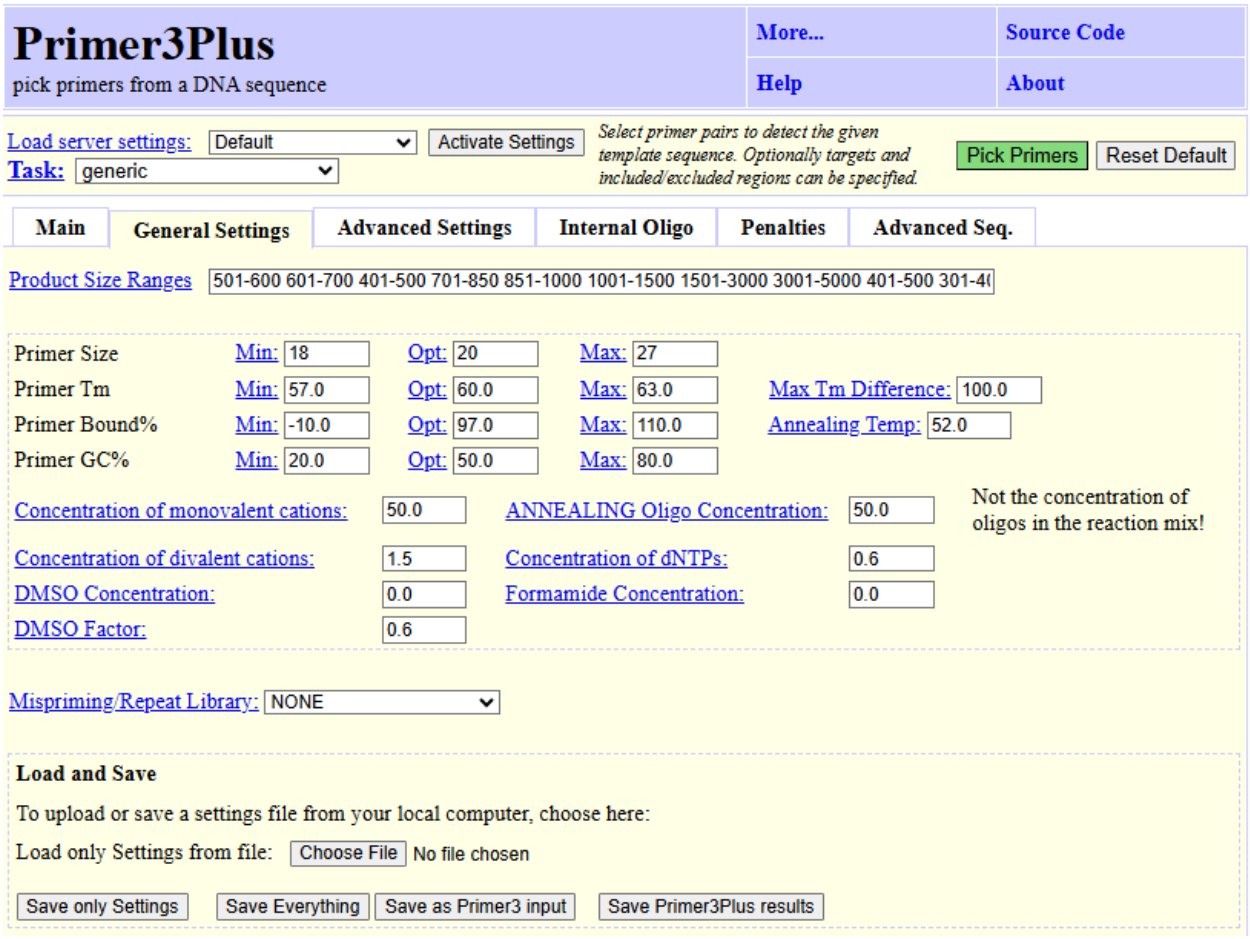



**Figure 4a:** Primer3Plus Interface Displaying General Settings for Primer Design

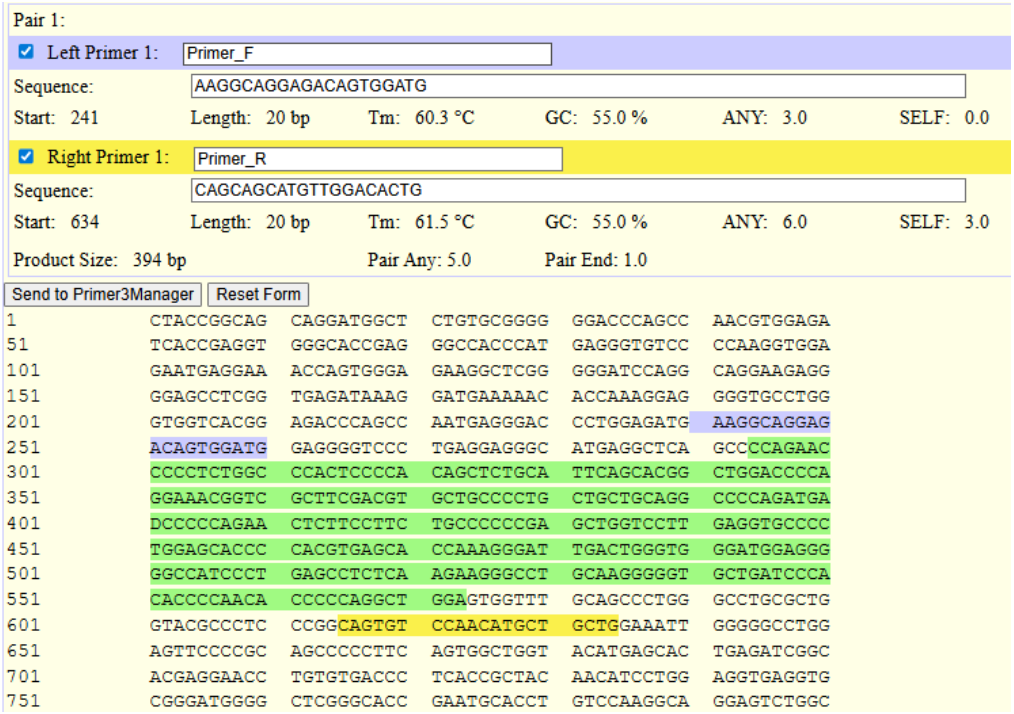



**Figure 4b:** Primer3Plus Output Displaying Primer Pair Binding Sites on the Target Sequence

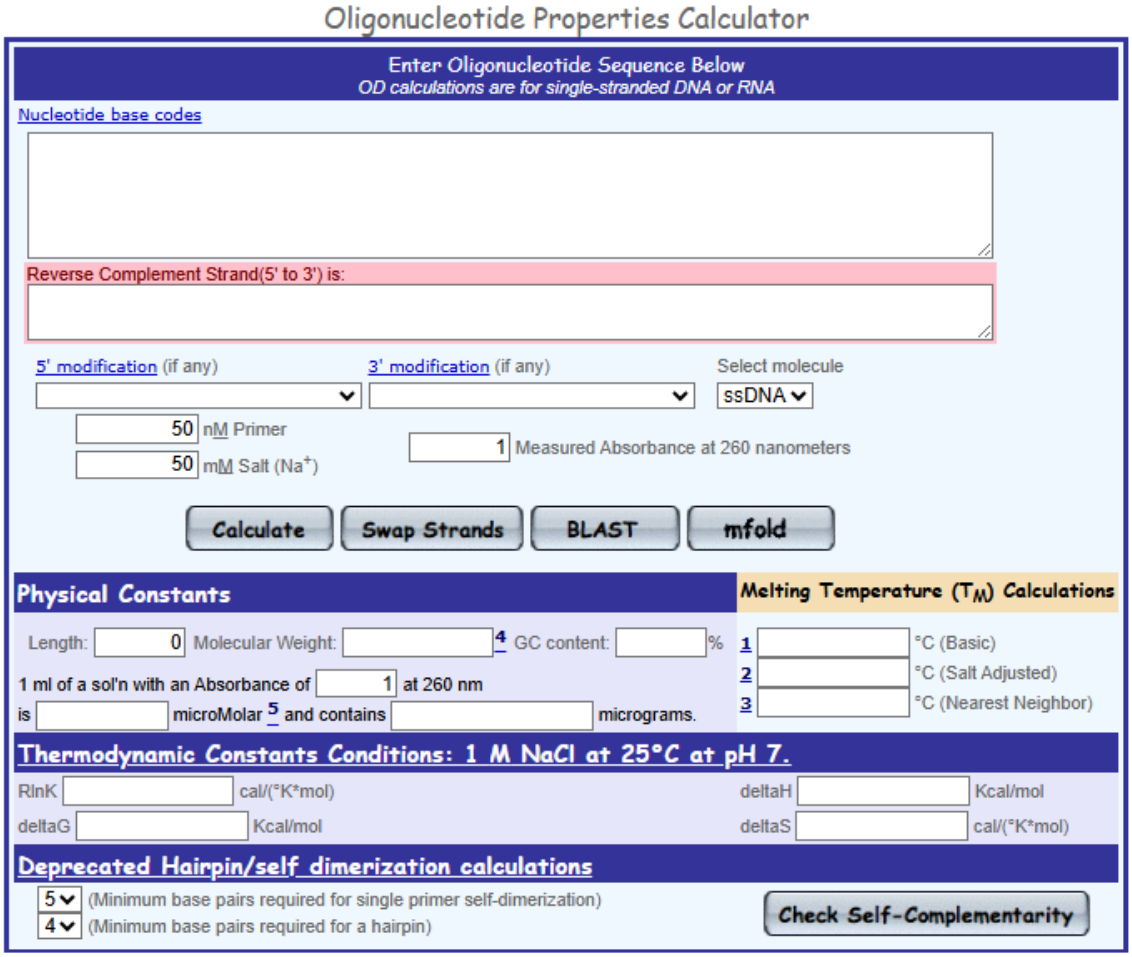



**Figure 5a:** OligoCalc interface displaying various physical and chemical properties of primers

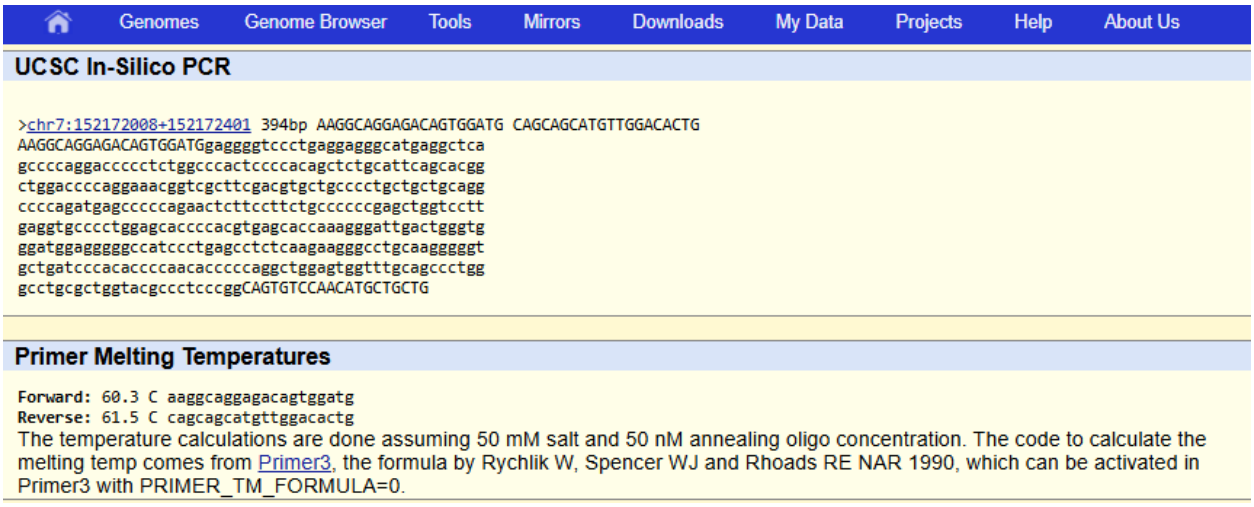



**Figure 5b:** In Silico Analysis Showing Predicted Primer Binding Site

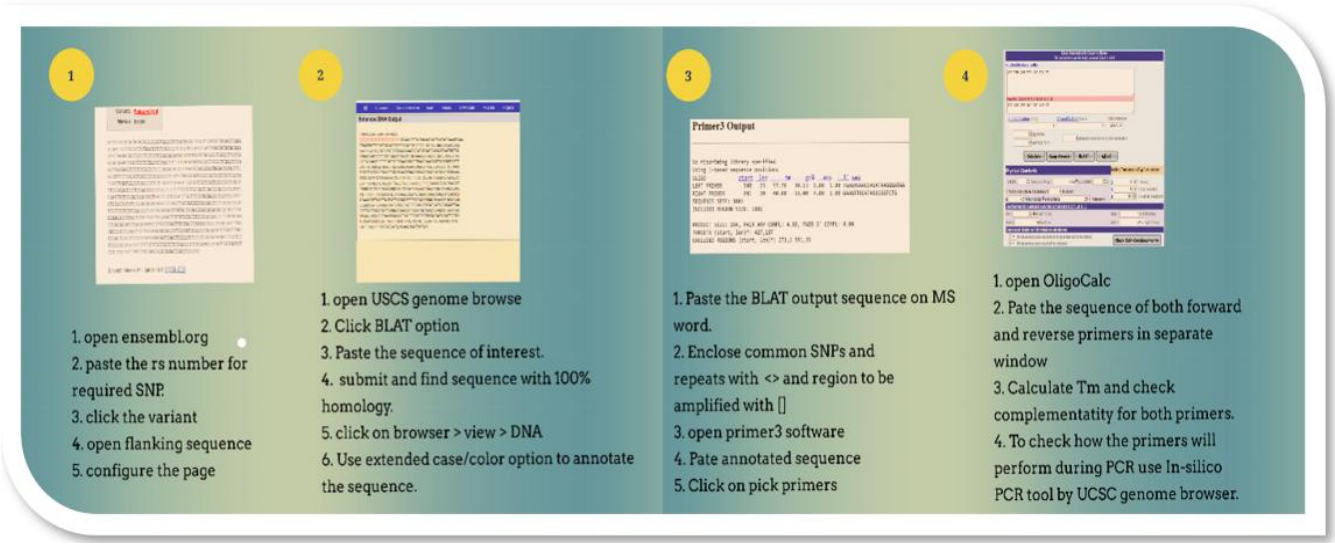



**Figure 6:** Instructions that are to be followed while using ENSEMBL, UCSC genome browser, Primer3 and OligoCalc respectively.

**Primer Design for Quantitative Real Time PCR**

Quantitative PCR (qPCR) is a technique used in clinical or biological fields for quantification of various nucleic acid (DNA or RNA) sequences. qPCR relies on accurate and precise primer and probe design to provide efficiency and specificity. Target sequence is quantified using either sequence specific probes coupled to fluorescent dyes (TaqMan® Probe) or by using nonspecific DNA binding dyes (SYBR Green). In most qPCR protocols either SYBR Green dye or TaqMan® Probe is used [19, 20]. For effective and ideal qPCR designed primers, it must avoid secondary structure and primer dimer formation. While using fluorescent probes like TaqMan® it is necessary that designed probes must be target specific so it could hybridize on target region and generate fluorescent signal during assay [21]. There are various software and programs available online that assist in designing primer/probe combination. Through these programs one can also verify specificity of primer/probe pair and determination of possible cross-reactivity, especially in multiplex qPCR where various primers/probe pairs for different target regions are combined and amplified altogether [22].

An effective and proper designing of primer and probe for qPCR involves multiple steps in sequential order that start from choosing target sequence to be amplified. To amplify microbial genome detection, conserved regions are preferred and for human or other species/strain detection some unique region is selected for specificity [23]. This target sequence is selected from various online databases like NCBI and Ensembl where genome sequence of these species is available in different formats (FASTA and GenBank). After target sequence is finalized, selected primer and probe pairs are optimized and validated using online tools, applications and experimental techniques (gradient PCR, melt curve analysis). This strategic choice ensures assay specificity, whether targeting monophyletic microbial groups or functional genes encoding enzymes [24, 25]. Sequential flowchart of primer designing and validation for quantitative real time PCR is described in Figure 7.

After identification of target sequence, primers and probes are designed either using software tools or by manual alignment. In both cases the designed primer and probes must meet certain criteria like length of amplicon, GC content of primer, melting temperature ($T_m$) and secondary structure formation [26]. All the properties of primer/probe pair are summarized in Table 2. For ideal primers, the length of target region or amplicon should be less than 150 base pairs. Although it is recommended that length should be less than 80 bases pairs, it has been experimentally observed that amplicons having length up to 400 base pairs can amplify efficiently. Efficiency of qPCR is inversely proportional to amplicon length i.e., shorter products amplify more efficiently as compared to longer products and are less prone to secondary structure formation. This is because of the fact that shorter products denature more easily and effectively at (92-95 °C) during denaturation thereby allowing primers to anneal more effectively [27, 28]. It is also important that GC rich regions should be avoided as they can cause difficulty in amplification and efficiency of qPCR [29]. Melting temperature ($T_m$) of primers and probes should be between 55–60°C and there should be no more difference of 1°C between forward and reverse primer. Moreover, $T_m$ of probe should be 8–10°C higher than primers. Optimal length of both primers and probe is between 15-30 nucleotides and recommended GC content should be 30–80% to achieve a balance between probe hybridization and

denaturation [30]. To increase annealing, designed primers should have G or C nucleotides at 3′ and for probes presence of G/C nucleotides should be excluded at 5′ end as it can cause continuous quenching that leads to reduced fluorescence [31]. Additionally, it has been observed that probes that contain relatively more C nucleotides than G because it increases change in fluorescence signal ($\Delta R_n$) due to which weak positive signals can be distinguished easily from background noise [32]. It has also been observed that Primers and probes should avoid self-complementarity, especially near the 3′ end, to prevent secondary structures and primer-dimers that interfere with qPCR. Degenerate nucleotides are generally avoided to ensure consistent amplification, though limited degeneracy may be needed for variable target regions [33].

The alignment of available public database sequences to conserved gene regions to design primer/probe sets from invariant regions is a time-consuming activity, which has been widely improved by automated bioinformatics tools. Although hand-tuned designs seem to work, something with an architecture, which makes them less specific, less efficient, less reproducible. Commercial and online tools (e.g., PrimerQuest™, RealTimeDesign™, Primer3) automate parameter checks—GC content, Tm, 3′ clamp, secondary structures—and prioritize linguistic complexity (nucleotide arrangement) to optimize specificity and sensitivity [22, 36]. The use of such tools leads to validated primers/probes, ensuring reproducibility and limiting off-target binding, which is particularly important in cases of qPCR assays where good accuracy is necessary [8, 25].

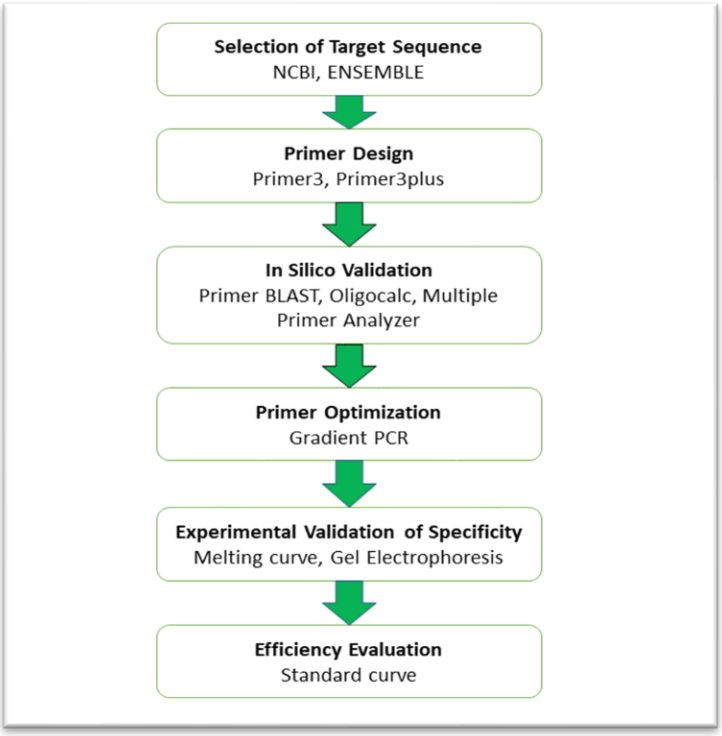



**Figure 7:** Flowchart describing steps for primer designing and validation for qRT-PCR

**Table 2:** Properties for optimal primers for qRT-PCR [34, 35]

| **Properties** | **Primer** | **Probe** |
|---|---|---|
| GC Content | 40-60% | 40-80% |
| $T_m$ | 55-62 °C, $T_m$ of Forward and Reverse primer should not differ > 3 | 68-70<br>Above $T_m$ of Primers |
| Runs of identical nucleotides | Maximum 3 | Maximum 3 |
| 3′ end rule (3′ instability) | Maximum three G or C bases at 3′ end | --- |
| Degeneracy of bases | Avoid | Avoid |
| Hairpin | Avoid | Avoid |
| Exclusion of G at 5′ end | Avoid G at 5′ end | --- |
| Self-Dimer, Cross-Dimer | Avoid | Avoid |

**Table 3:** Bioinformatic tools for optimizing and designing qPCR primers and probes

| Software/Tool Name | URL and Function | |
|---|---|---|
| ABI PRISM Primer Express | URL | http://www.lifetechnologies.com/order/catalog/product/4363993?ICID=search-product |
| | Role/Function | Primer and probe design software, likely optimized for Applied Biosystems (ABI) instruments. |
| AlignMiner | URL | http://www.scbi.uma.es/alignminer/ |
| | Role/Function | Likely a tool for identifying conserved regions in sequence alignments, useful for designing primers targeting multiple species. |
| OLIGO 7 | URL | http://www.oligo.net/ |
| | Role/Function | Comprehensive primer and probe design software. |
| ConservedPrimers 2.0 | URL | http://probes.pw.usda.gov/ConservedPrimers/ |
| | Role/Function | Designs primers targeting conserved regions across multiple sequences. |
| PrimerBank | URL | http://pga.mgh.harvard.edu/primerbank/ |
| | Role/Function | A database of pre-designed primers for various genes. |
| EasyExonPrimer | URL | http://129.43.22.27/~primer/EasyExonPrimer.html |
| | Role/Function | Designs primers that span exon junctions, useful for avoiding amplification of genomic DNA when working with RNA. |
| DFold | URL | http://dfold.cgb.ki.se/ |
| | Role/Function | Predicts secondary structures in nucleic acid sequences, important for avoiding primer self-dimerization and hairpin formation. |
| QPrimerDepot | URL | http://primerdepot.nci.nih.gov/andhttp://mouseprimerdepot.nci.nih.gov/ |
| | Role/Function | A database of qPCR primers, potentially focused on specific organisms (e.g., mouse). |

**Validation of Primers**

Validation of primers is very critical and important for efficient and reliable qPCR assays. After designing primers, both in-silico validation and experimental validation is performed to evaluate efficiency, specificity and reproducibility of amplification. In silico validation is performed using online software programs such as Primer BLAST and In Silico genome browsers which confirms that primers and probe can only bind specifically to target region. After this computational validation of primers wet lab or empirical validation of primers also accessed to access experimental properties of primers such as optimized reaction conditions, annealing temperature and to exclude nonspecific product and primer dimer formation [35, 37].

Optimal concentrations of primers and probes are established by empirical testing to strike a balance between specificity and signal clarity. Primer concentration typically ranges between 50–300 nanomolar (nM) because excessive concentration favor mispriming and low concentration level triggers premature depletion/exhaustion. Sometimes, it is necessary to use different concentrations for forward and reverse primers. Probes work optimally at around 250 nM concentration. Lower concentrations result in poor fluorescence and higher concentrations add background noise. Optimization involves choosing concentrations that yield the lowest quantification cycle (Cq) and the strongest fluorescence for a specific target [34, 38]. Despite predictive software calculations, experimental confirmation is necessary for annealing temperature optimization. Measurement of temperature 5°C lower than the primer $T_m$ helps to find the optimal conditions for efficient amplification. Accurate cycling parameters guarantee that qPCR efficiency meets established standards as theoretical calculations might not include reaction-specific variables [39].

Assessment of specificity fuses several analytical techniques to identify or detect nonspecific products. Melting curve analysis detects primer-dimers by distinct $T_m$ peaks when using DNA-binding dyes, while gel electrophoresis provides better resolution for confirming amplicon size [40, 41]. Sequencing PCR products against reference databases provides

absolute specificity confirmation. Techniques such as hot-start polymerases, preparing reactions on ice, and fluorescence acquisition above $T_m$ of primer-dimers reduce nonspecific binding. qPCR efficiency assessment is based on standard curves obtained through serial template dilutions [41-43]

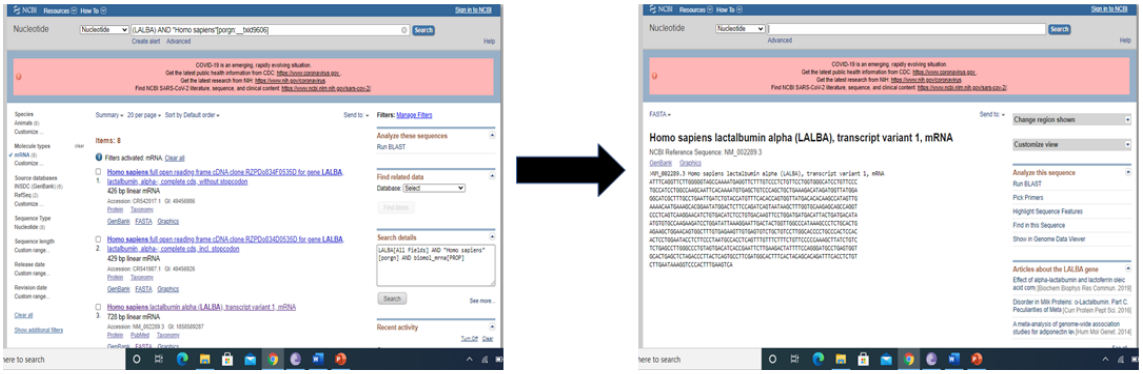
Optimization of multiplex qPCR responds to issues resulting from simultaneous target amplification. Elevated magnesium ($Mg^{2+}$) level or using more hot-start enzyme improves the specificity of the reactions. Primer-limiting assays equilibrate amplification efficiency among targets of different abundances. It is important to validate each primer or probe before combining them to ensure their efficient activity together. If there are significant differences in efficiency, more optimization is needed [5, 44, 45].

**Primers for Gene Expression Analysis**

Genes regulate their product through a phenomenon known as gene expression in which information coded in gene sequence is transcribed via process called transcription. This transcribed information is translated into functional product (protein). In research and diagnostic field Oligo dT (qRT-PCR) technique is utilized for nucleic acid (DNA and RNA) quantification for several diagnostic and research purposes [46, 47].

Numerous specific and non-specific fluorescent dyes are used for nucleic acid quantification which generate fluorescence that is directly proportional to amplified product. There are two ways to perform qRT-PCR, single step and two step. For most diagnostic purposes single step qRT-PCR is used in which transcription of target RNA (mostly viral) and amplification is performed in a single reaction. In two step reactions, which are mostly performed for research purposes, cDNA is obtained from reverse transcription of RNA using oligo dT primers. cDNA obtained from reverse transcription is used for amplification [48, 49]. It is important to keep in mind that choice of RT-qPCR analysis (absolute or relative quantification) affects primer and probe design. Absolute quantification needs only target-specific primers with a standard curve, while relative quantification requires both target and endogenous control primers [43].

Schematic steps for mRNA data retrieval are summarized in Figure 8 and flow chart of primer validation is shown in Figure 7. It is important to note that designed primer must specifically bind to desired gene or sequence otherwise results will be non-satisfactory. To observe specificity of a primer pair we run online primer-BLAST from NCBI database. BLAST results will show specificity of a primer to target sequence. If a primer is target specific, then it can be used for gene expression studies as non-specific primers will produce false positive results by non-specifically. After a primer is designed, its optimal annealing temperature is obtained through gradient PCR [50].




1. Open NCBI Nucleotide database and type the name of gene. NCBI database will show various nucleotide sequences of different species.

2. Select mRNA sequence of desired specie.

3. To analyze gene expression select complete cds or variant 1 of transcript

4. Click on FASTA format.

**Figure 8:** Instructions to be followed for mRNA sequence retrieval from NCBI database

Specific primer designing is extremely important for qRT-PCR because the primer pair can bind to genomic DNA in case of any contamination in template mixture and co-amplify genomic DNA thus jeopardizing the efficiency of reaction. To overcome this problem, primer can be designed on exon-exon junction. Primer designed on exon-exon junction cannot bind to genomic DNA as they will not anneal because of presence of intron. Another way to design primers is to design primer on same exon but this may lead to false results if there is any contamination in sample as shown in Figure 9 [51]. For RT-qPCR (RNA quantitation), primers must cross exon-exon junctions to prevent amplification of genomic DNA, and absolute/relative quantitation strategies determine primer pair choice. To prevent genomic DNA amplification, treat samples with DNase and use $Mn^{2+}$ instead of $Mg^{2+}$ to reduce reannealed DNA amplification. Optimal design reduces nonspecific amplification, providing consistent results in clinical, diagnostic, and gene-expression research (52, 53).

Optimizing primer and probe concentrations (50–200 nM for primers, 100 nM for probes) is essential in RT-qPCR. Methods used for RT-qPCR and type of data analysis (relative or absolute) determine specific optimization needed for primer, probe, and template interactions.

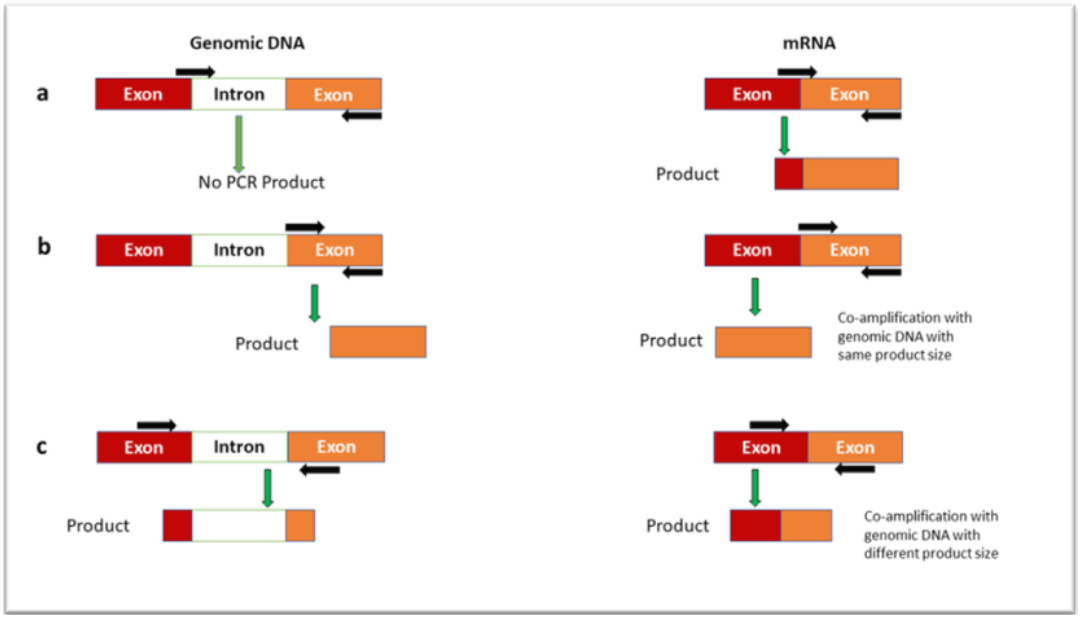



**Figure 9:** Approach to avoid genomic DNA contamination and possible genomic DNA amplification scenarios during qRT--PCR (a) Primer design on exon-exon junction (b) Primer design (Forward and Reverse) within same exon can result in genomic DNA amplification if sample is contaminated (c) Forward and reverse primer design on different exons [25].

## CONCLUSION

For efficient and reliable PCR and qPCR assays, primer designing is very critical and important especially for SNP detection and gene expression analysis. Effective primer designing requires optimization of multiple factors like length of primer, GC content and specificity. Utilization of bioinformatic tools along with experimental validation authenticates optimal assay performance and accurate quantification, making these considerations critical for reliable molecular analysis.

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