Chapter 3

Design of Primers and Probes for Quantitative Real-Time PCR Methods

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Abstract

Design of primers and probes is one of the most crucial factors affecting the success and quality of quantitative real-time PCR (qPCR) analyses, since an accurate and reliable quantification depends on using efficient primers and probes. Design of primers and probes should meet several criteria to find potential primers and probes for specific qPCR assays. The formation of primer-dimers and other non-specific products should be avoided or reduced. This factor is especially important when designing primers for SYBR® Green protocols but also in designing probes to ensure specificity of the developed qPCR protocol. To design primers and probes for qPCR, multiple software programs and websites are available being numerous of them free. These tools often consider the default requirements for primers and probes, although new research advances in primer and probe design should be progressively added to different algorithm programs. After a proper design, a precise validation of the primers and probes is necessary. Specific consideration should be taken into account when designing primers and probes for multiplex qPCR and reverse transcription qPCR (RT-qPCR).

This chapter provides guidelines for the design of suitable primers and probes and their subsequent validation through the development of singlex qPCR, multiplex qPCR, and RT-qPCR protocols.

Key words Quantitative real-time PCR, Primers, Probes, Software and databases, Validation, Reverse transcription real-time PCR

1 Introduction

Quantitative real-time PCR (qPCR) is widely and successfully used in clinical and biological fields for quantification of nucleic acid sequences (DNA or RNA). This is a sensitive and specific technique in which the DNA amount is monitored during the reaction by using fluorescent dyes that are incorporated into the PCR product. The increase in the fluorescent signal is directly proportional to the number of PCR product molecules generated [1]. The fluorescence monitoring through a qPCR reaction can be detected by nonspecific dyes, such as SYBR® Green, or by sequence-specific primers or probes coupled to fluorescent dyes, including hydrolysis probes, molecular beacons, fluorescence resonance energy transfer (FRET)

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probes, and Scorpions primers [2]. SYBR® Green chemistry and TaqMan® hydrolysis probes are the most frequently used methodologies for developing qPCR protocols.

The success in any of the developed qPCR protocol depends on the suitability of the designed primers and probes, since the specificity of the technique is closely related to the annealing of primers to their complementary targets [3] and, afterwards, to the probe hybridization into newly synthesized DNA. In SYBR® Green assays, the proper design of primers is especially critical because the dye intercalates into double-stranded DNA without distinguishing between specific and nonspecific qPCR products [4–6]. In TaqMan® qPCR, the optimal design of probes is essential for their hybridization to the amplified target sequence to increases the specificity of the assay [4, 5].

Although the design of primers for qPCR is not substantially different from those for standard PCR, they need to meet special criteria for the reaction success [7]. Thus, they should allow strictly the synthesis of a single amplicon with good efficiency (ideally two copies of template after every PCR cycle) and without formation of primer-dimers. This is necessary for an accurate and reliable quantification of the target sequence.

To design primers and probes for qPCR, multiple software programs and websites are available being numerous of them free. These tools can be used to design primers and probes, test for nonspecific priming, and assess the formation of secondary structures which might form between primers, probes, templates, or the amplification product [6]. Some of them are described in Subheading 3 of this chapter.

A proper design of primers and probes for qPCR requires sequential steps involved in this process including the selection of target sequences and primer and probe candidates followed by a validation process. The overall procedure of the qPCR primer and probe design is shown in Fig. 1 and is described in this chapter.

The design of primers and probes is especially critical for multiplex qPCR assays since more than one primer pair and probe set is included in the same reaction for amplifying two or more target sequences and consequently the probability of mispriming is higher [1].

On the other hand a special attention to primer and probe design is required for reverse transcription qPCR (RT-qPCR) which is used for gene expression analysis. In this method, RNA is transcribed into cDNA prior to its quantification using qPCR [8]. RT-qPCR can take place in one or two steps. The data can be quantified by absolute or relative methods which determine the selection of the proper primers as described in Subheading 5 of this chapter.

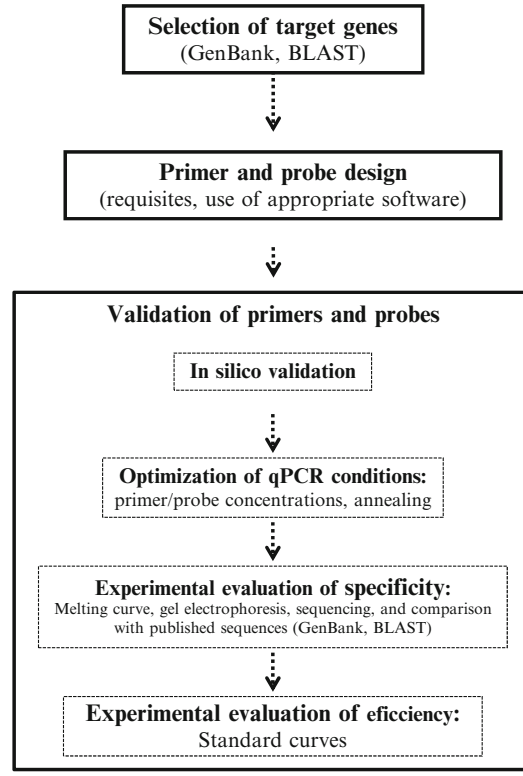

Fig. 1 Flowchart schematizing the procedure used for designing and validating primers and probes for quantitative real-time PCR protocols

2 Parameters to Be Considered When Designing Primers and Probes for Quantitative Real-Time PCR

The use of optimal primer and probe sequences is one of the critical steps for a successful qPCR assay. The criteria for primer and probe design are extensively described in the literature [2, 9–12]. A preliminary and key step when designing primers and probes for qPCR is the selection of target sequences of nucleic acid where they can hybridize. This is particularly important in the area of microbiology where targeting a gene highly conserved among different species can be used in broad-based detection strategies, while targeting a DNA sequence unique for a particular species or even strain can provide a highly specific test [13]. In this way, the target could be a sequence unique for a monophyletic group of microorganisms or could be based on a group of functional genes encoding a specific enzyme.

Once the selection of the target sequences has been done, the next step is to find potential primers or probes targeting regions on the corresponding gene sequences. This can be done manually

Table 1
Default requisites for designing primers and probes for quantitative real-time PCR assays (adapted from refs. 2, 10, 86)

Requisites	Primers	Probes
GC content	30–80 %	
Calculated primer/probe T_m	50–60 °C (always >55 °C) T_m of primers should not differ >2 °C	68–70 °C (8–10 °C above T_m primer)
Runs of identical nucleotides	Maximum 3 (No G bases)	
Primer/probe length	15–30 bp	
PCR product length	50–150 bp (optimum <80 bp)	
Distance forward primer to probe	50 bp	
Primer-dimers, hairpins	Avoid	Avoid
3′ end rule (3′ instability)	Maximum two G or C in the last 5 bp	–
Autoquenching	–	No G on the 5′ end
GC ratio	–	C > G
Degree of degeneracy of bases	Avoid	Avoid

using sequence alignment program or automatically using primer design software. In both techniques it should be checked that the suggested primer and probe set achieves the following criteria: amplicon length, melting temperature (T_m), primer and probe length, GC content, self-complementary, primer-dimer and hairpin formation, degree of degeneracy, 5′ end stability, and 3′ end specificity. An overview of these criteria is given in Table 1 and they are described below.

Amplification products smaller than 150 bp are highly recommended for high efficiency of qPCR [11], although lengths below 80 bp are advisable. However, amplicons up to 400 bp may amplify efficiently. Shorter qPCR products amplify more efficiently than longer ones and are less susceptible to potential secondary structure within them [14]. This is because they are more likely to be denature during the extension step (at 92–95 °C), allowing the primers and probe to complete more effectively the binding to their complementary target sequences. Concretely, the length of the amplification products should be similar in multiplex qPCR protocols [6]. In addition, GC rich regions in the target sequence should be avoided since they are more difficult to amplify [15].

The T_m of the primers and probes consisting of the temperature at which 50 % of them and its complement are hybridized [6] is also an important parameter in the development of qPCR assays. A T_m value of the primers ranging from 55 to 60 °C is recommended for qPCR [12]. In addition, the T_m of sense and antisense

primers should be similar to avoid hairpins [16]. In particular, the T_m of the primer pair should not be more than 1 °C different from each other [6]. Since amplification primers are extended as soon as they bind to their complementary sequences, probe needs a T_m greater than primers to ensure strong binding of itself during the annealing phase [4, 17]. Concretely, the T_m of the probe should be 8–10 °C higher than those of the primers. The T_m of the primers and probe directly depends upon their length and their percentage of GC content [18].

The optimal size of qPCR primers and probes usually ranges from 15 to 30 nucleotides [19]. Shorter primers may decrease the specificity [16]. Despite longer primers could be match better to the target sequence the PCR amplification efficiency could be lower [11]. This reduction in the efficiency of the reaction, especially when using environmental DNA samples, may lead to a significant reduction in the yield and quality of the qPCR product [20]. In addition, the use of longer probes allows more mismatches, does not improve the sensitivity, can exhibit less efficient quenching, and produces lower fluorescence yields whilst use of shorter probes decrease the specificity [10, 11].

Regarding the GC content in the primers and probe sequences, a GC percentage between 30 and 80 % is recommended. In spite of the fact that primers with higher GC content should stabilize probe hybridization [10], they may not denature easily during PCR provoking a decrease of the amplification efficiency. Furthermore, poly-C and poly-G regions in the primers should be avoided since they can make up a tetraplex structure, which is very stable and cannot be transcribed by the polymerase [21]. Non-specific priming can be minimized by selecting primers that have only one or two G/C within 3′ end last five nucleotides [2], since a higher GC content at this end of the primer may prevent the complete annealing of the remainder of the primer sequence and reduce the specificity of the reaction [16]. However, it is recommended that primers have a G or C as nucleotide on the 3′ end to ensure their correct and strong binding to the template [11]. The presence of a G as nucleotide at the 5′ end of the probe should be excluded to avoid a continuous quenching even after probe cleavage, which resulting in reduced normalized fluorescence values [22]. Furthermore, the probe should contain more C than G because of high change of normalization fluorescence (ΔR_n). It allows low positive signals which can be more easily differentiated from the background signal [23].

Primers and probes with a high possibility of self-complementarity, particularly close to the 3′ end, should be avoided because secondary structures, such as hairpins, can be formed and interfere the extension step. Moreover, intramolecular and intermolecular interactions between the primers can generate primer-dimers which should be considered in the design process [24, 25]. This is a common artifact in qPCR reactions which occurs when two primers

bind to each other instead of to the template. In addition, the probe should never overlap with or be complementary to either of the primers. On the other hand, it is advisable to avoid more than five interactions between the primers, especially at the 3′ end position.

The presence of degenerate nucleotides in primers and probes should be excluded in the design process. Differences in the GC content at degenerate positions in the primer target regions of the template DNA could affect the amplification [26, 27]. However, sometimes a certain degree of degeneracy is necessary in order to prevent some under-estimation of target sequences when that has non-conserved regions.

Concerning multiplex qPCR assays, the above mentioned criteria must be considered. However it should be taken into account that in this type of reactions multiple templates and several primer and probe sets are in the same reaction. The presence of multiple primers and probes may lead to interactions with each other and the possibility of mispriming with other templates. For this reason, it is important to ensure that the different primer and probe sets do not exhibit complementarity to one another [28]. Thus a special care must be taken to design proper primers and probes and to select appropriate reporter dyes and quenchers for the probes. Regarding the last concern, three criteria should be considered: (a) the probes should be labelled with reporter dyes whose fluorescence spectra are well separated or show only minimal overlap, (b) selected combinations of reporter dyes and quenchers should be compatible with the detection abilities of the real-time cycler, and (c) non-fluorescent quenchers should be used [8, 28].

3 Software and Other Bioinformatics Tools to Design Primers and Probes for qPCR

In the design of primer and probe from a common gene-specific region, all known sequences in the public databases should be first selected and then aligned to find conserved regions. This may suppose a high time-consuming activity if it is not automated. In addition, although primers and probes seem to generate acceptable results at first, many home-made or "do-it-yourself" primers often come up short in their specificity, qPCR amplification efficiency, reproducibility, and sensitivity. Therefore, there are currently many online and commercial bioinformatics tools for routine use. Software automatically checks for the best primers and probes for considered parameters and provides a list of them. They often control most of the default requirements for primers and probes previously described in Subheading 2 (CG content, primer and probe length, primer and probe T_m, CG 3′ end terminal enforcement, etc.). Additionally several programs take into account other main parameters for a more accurate and comprehensive selection of primers and probes, such as the general nucleotide structure of primers such as linguistic complexity (nucleotide arrangement

and composition), specificity, the melting temperature of the whole primers and the melting temperature at the 3′ and 5′ termini, self-complementarity, and secondary binding [29].

There are multiple primer and probe design tools available on the net that allow producing high quality primers (Table 2). Though most of them are freely available, they have variable quality and some of them are not well-maintained. This often results in missing links and sites that may have been useful previously but they may not be functional at a later date [30]. These programs can be used to generate potential primers and probes, check for non-specific hybridization, and evaluate the formation of secondary structures. On the other hand, it has to be taken into account that the use of these online programs requires practice since online guides may not be available to support novice users in designing primers and probes [6]. In general, these tools are very effective, yielding success rates well in excess of 95 % in the hands of experienced users [31].

Several companies supplying primers and probes offer Web-based tools for their design and free applications are also available on the net [30]. Despite the fact that most of the algorithms considered by them have been conceived for standard PCR, they are also helpful for qPCR primer and probe design [32]. According to Gubelman et al. [33] an ideal qPCR primer and probe design program should at least include the following features: (a) all annotated splice variants of each gene to enable either gene or transcript specific expression profiling should be considered, (b) for RT-qPCR assays at least one primer needs to span exons to avoid amplification of contaminating genomic DNA, (c) the specificity of primers and probes needs to be automatically assessed by similarity search, (d) no cumbersome post-processing should be required to retrieve the best primer combination, and (e) the location of primer pairs within their genomic context should be visualized for easy and final evaluation by the end user.

3.1 Software and Programs for Designing of Primers and Probes for qPCR

Next some of the most suitable software for supporting in the design of primers and probes for qPCR are going to be briefly described.

OLIGO software is the first computer application that performed on the market for designing primers and probes. However OLIGO went through many transformations to latest software in 2010, OLIGO 7. Based on nearest-neighbor thermodynamics, OLIGO's search algorithms find optimal primers for PCR, qPCR (TaqMan® probes), and sequencing. OLIGO 7 searches also for hybridization, ligase chain reaction probes and molecular beacons and even siRNAs [34].

Primer3 is one of the most commonly used primer design software [35]. It is a frequently updated, open-source project and used by many Web-based applications to develop useful functions for primer and probe design [12]. Its popularity is likely due to several factors that include the availability of a relatively easy-to-use Web

Table 2
Some of the available commercial and online software for quantitative real-time PCR primer and probe design and their websites

Name	URL	References
ABI PRISM Primer Express	http://www.lifetechnologies.com/order/catalog/product/4363993?ICID=search-product	[48]
AlignMiner	http://www.scbi.uma.es/alignminer/	[59]
ConservedPrimers 2.0	http://probes.pw.usda.gov/ConservedPrimers/	[50]
EasyExonPrimer	http://129.43.22.27/~primer/EasyExonPrimer.html	[53]
EcoPrimer	http://www.grenoble.prabi.fr/trac/ecoPrimers	[52]
DATFAP	http://cgi-www.daimi.au.dk/cgi-chili/datfap/frontdoor.py	[56]
DFold	http://dfold.cgb.ki.se/	[31]
Gemi	http://sourceforge.net/projects/gemi/	[46]
GETPrime	http://updepla1srv1.epfl.ch/getprime/	[33]
Java Web Tools	http://primerdigital.com/tools/	[58]
MultiPriDe (Multiple Primer Design)	Available upon request to aziesel@emory.edu	[43]
OLIGO 7	http://www.oligo.net/	[34]
PerlPrimer	http://perlprimer.sourceforge.net/	[38]
PRaTo	http://prato.daapv.unipd.it/	[32]
Primer3	http://biotools.umassmed.edu/bioapps/primer3_www.cgi	[35]
Primer3Plus	http://primer3plus.com	[37]
Primer3web	http://primer3.wi.mit.edu http://bioinfo.ut.ee/primer3/	[36]
PrimerBank	http://pga.mgh.harvard.edu/primerbank/	[57]
Primer-Blast	http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHomeAd	[87]
PrimerCE	http://tch.hebau.edu.cn/shengm/download/down.html	[54]
PrimerDesign	http://www.hiv.lanl.gov/tools/primer/main	[47]
PUNS (Primer-UniGene Selectivity)	http://okeylabimac.med.utoronto.ca/PUNS	[40]
RTPrimerDB	http://medgen.ugent.be/rtprimerdb/	[55]
QPrimerDepot	http://primerdepot.nci.nih.gov/ http://mouseprimerdepot.nci.nih.gov/	[88]
QuantPrime	http://www.quantprime.de/	[42]
RASE	http://designs.lgfus.ca/cgi-bin/bsp_designs/index.pl	[45]
TOPSI	http://www.bhsai.org/downloads/topsi.tar.gz	[44]

service, robust engineering, open access to the program source code, suitability for use in high-throughput pipelines for genome-scale research, and the simplicity for incorporating into or interoperating with other software [36]. This software has been recently enhanced with new Web interfaces, *Primer3Plus* and *Primer3Web* [36, 37]. The most notable enhancements incorporate accurate thermodynamic models in the primer design process, both to improve melting temperature prediction and reduce the likelihood that primers will form hairpins or dimers [36].

PerlPrimer is a cross-platform graphical user interface application for the design of primers for qPCR as well as standard PCR, bisulfite PCR, and sequencing. This program combines accurate primer-dimer prediction algorithms with powerful tools such as sequence retrieval from Ensembl genome database and the ability to BLAST search primer pairs [38, 39]. Using the default settings, PerlPrimer searches for small amplicons (100–300 bp) which span an intron–exon boundary and possess at least one primer hybridizing across an intron–exon boundary.

PUNS (Primer-UniGene Selectivity) software is a CGI/Perl-based Web server to perform in silico PCR on PCR primer sequences. PUNS server simulates PCR reactions by running BLAST analysis on user-entered primer pairs against both the transcriptome and the genome to assess primer specificity. PUNS is particularly suited for the identification of highly selective primers for microarray experiments which are usually carried out either by semiquantitative or by RT-qPCR [40]. The use of PUNS in primer design follows a three-step process. Firstly users enter primers into the database. Users then submit their primer sequences for a BLAST analysis [41]. Finally, the information in a primer pair is combined by an in silico PCR which identifies potential amplicons by both identity and size. The in silico PCR report allows deciding if potential primer pairs are accepted or rejected for experimental use [40].

QuantPrime is an intuitive and user-friendly, fully automated for using in primer pair and probe design for qPCR analyses. QuantPrime can be used online or on a local computer after downloading. QuantPrime specifically tests primer pairs for qPCR, developed to satisfy needs of advanced users in low to high-throughput transcript profiling experiments, while keeping the user interface very simple providing important features missing in other available software and Web services. The public QuantPrime server is currently set up with publicly available transcriptome and genome annotations from 295 different eukaryotic species [42]. The parameter flexibility for designing and specificity testing offered in QuantPrime makes it straightforward to be used in the design of oligonucleotides for additional quantification applications, such as qPCR with hydrolysis probes (e.g., TaqMan® probes, Scorpion primers) or quantitative in situ hybridization of mRNA. Such protocols are added to QuantPrime as program gather experimental data and feedback from users.

MultiPriDe (Multiple Primer Design), a Perl tool that accepts batch lists of Gene database identifiers, collects available intron and exon position data critical to RT-qPCR primer development and supplies these sites as identified targets for the Primer3 utility to maximize successful primer design [43].

TOPSI (Tool for PCR Signature Identification) is a computationally efficient, fully integrated tool for the design of qPCR-based pathogen diagnostic assays. The TOPSI pipeline efficiently designs qPCR primers and probe sets common to multiple bacterial genomes by obtaining the shared regions through pairwise alignments between the input genomes [44]. TOPSI uses pairwise alignments to identify sequences that are common to multiple genomes and compares these sequences with non-target genomes to identify unique segments suitable for designing signatures.

RASE (Real-Time PCR Annotation of Splicing Events) is a pipeline that allows accurate identification of a large number of splicing isoforms in human cell lines and tissues [45]. The RASE automatically designs specific primer pairs for 81 % of all alternative splicing events in the NCBI build 36 database. With this program a quick identification of splicing isoform signatures can be obtained in different types of human tissues. However, this program does not enable the design of gene-specific primers. In addition, its associated Web interface only supports low-throughput experiments [33].

GETPrime is a primer database supported by a novel platform that uniquely combines and automates several features critical for optimal qPCR primer and probe design. These include the consideration of all gene splice variants to enable either gene-specific or transcript-specific expression profiling, primer specificity validation, automated best primer pair selection according to strict criteria, and graphical visualization of the latter primer pairs within their genomic context [33]. This program is very useful due to the fact that it combines and automates all of the important features required to address the increasing demands in qPCR primer design for high-throughput qPCR experiments, especially those requirement to target genes in gene- or transcript-specific fashion without post-processing [33].

Gemi is an automated, fast, and easy-to-use bioinformatics tool with a user-friendly interface to design primers and probes based on multiple aligned sequences. This tool can be used for the purpose of both conventional and qPCR and can deal efficiently with great number of large size sequences [46]. The main criterion used by Gemi to identify primers and probes is the nucleotide sequence belonging to conserved DNA, but it provides the dissociation temperature, length, and GC percentage in the final output file for each to select primers or probes. The application executes directly on a computer and provides a simple and user-friendly interface allowing an easily and quickly primer design. This tool can be particularly useful in the microbiology field [46].

PrimerDesign is a novel computer program for designing primers and probes for highly variable DNA targets. The design takes into account genetic variation and several user-specified as well as automatic design features related to the aim of a particular study and the intended experimental setting. It has been reported as useful tool for designing primers and probes for biological systems with high levels of genetic variation [47]. The overall software procedure proceeds through interconnected steps: (a) the target locations for primers and probes are determined guided by sequence entropy estimates and complexity, (b) primer melting temperatures are optimized, (c) bio-barcodes and adaptors are added, and (d) risks of dimerization are estimated. Each interconnected step informs the subsequent steps. In addition, if previous steps have to be reoptimized, the information to next steps occurs automatically [47].

ABI PRISM Primer Express 3.0 is a primer and probe design tool. It allows designing oligonucleotides for qPCR applications using a customized application specific document for absolute/relative quantification and allelic discrimination assays. Besides the primers, ABI PRISM Primer Express 3.0 helps in designing the labelled probes, selection of the appropriate reagents, use of universal thermal cycling parameters, and use of default primer and probe concentrations (or optimizing if necessary). The Primer Express software includes a Primer Test document that allows evaluating primers for their T_m, secondary structure, and primer-dimer formation [48].

DFold is a software that creates PCR primers without stable secondary structures [31]. DFold combines the use of Primer3 [35] for assessing of PCR primers and the MFold package [49] for predicting secondary structures.

ConservedPrimers 2.0 was developed as application able to design large numbers of PCR primers in exons flanking one or several introns on the basis of orthologous gene sequences in genetically closed species. This program has been developed for designing intron-flanking primers for large-scale single nucleotide polymorphism (SNP) discovery and marker development [50]. This tool uses non-redundant expressed sequence tags (EST) and related genomic sequences as inputs. Intron-flanking primers are then designed based on the intron–exon information using the Primer3 core program [35] or BatchPrimer3 [51].

EcoPrimer is a software which fulfills all the requirements for designing new barcode regions suitable for metabarcoding studies. This software has the ability to scan large training databases, since it is used to design highly conserved primers to amplify variable DNA regions [52].

EasyExonPrimer is a Web-based software that automates the design of PCR primers to amplify exon sequences from genomic DNA. It uses Primer3 [35] to design PCR primers based on the genome builds and annotation databases available at the University of

California, Santa Cruz (UCSC) Genome Browser database (http://genome.ucsc.edu/). It masks repeats and known SNP sites in the genome and designs standardized primers using optimized conditions [53].

PrimerCE is a reliable primer design program that specifically fulfills the need for gene cloning aimed at produce proteins. The main applications of PrimerCE include inspection of restriction enzyme recognition sequence, open reading frame verification, stop codon inspection, base adjustment, primer optimization, sequence assembly, and protein analysis [54].

3.2 Other Commercial Bioinformatics Tools for Designing Primers and Probes for qPCR

Besides software for designing qPCR primers and probes, there are additional bioinformatics tools and also databases gathering a huge amount of validated primers and probes which prevent to spend time in their design and experimental optimization and validation. Several of these additional tools are described below.

RTPrimerDB is a public database for primer and probe sequences used in qPCR assays engaging popular chemistries (SYBR® Green, TaqMan®, and Molecular Beacon) to reduce time-consuming primer and probe design and experimental optimization. In addition, this program introduces a certain level of uniformity and standardization among different laboratories [55]. RTPrimerDB includes records with user submitted assays that are linked to genome information from reference databases and quality controlled using an in silico assay evaluation system. The primer evaluation tools intended to assess the specificity and detect features that could negatively affect the amplification efficiency are combined into a pipeline to test custom designed primer and probe sequences. An improved feedback system guides users and submitters to enter practical remarks and details about experimental evaluation analyses [55].

DATFAP (Database of Transcription Factors with Alignments and Primers) is a free, Web-based, and very user-friendly browsing tool based on a new database of more than 55,000 EST (expressed sequence tag) sequences from 13 plant species, classified as transcription factors. Further, the database offers primers and probes designed for qPCR as well as homology alignments and phylogenies for the sequence analysis [56]. DATFAP is equipped with a sophisticated search facility and specific primers for almost all sequences, DATFAP constitutes a valuable tool to researchers in all areas of plant molecular biology working with transcription factors. No other multi-species transcription factor database offers such easy interspecies and intraspecies navigation in the network of related transcription factors [56].

PrimerBank is a robust bioinformatics process for primer design. The algorithm has been used to design many qPCR primers to cover the most known human and mouse genes, all of which

are freely accessible via the PrimerBank website. PrimerBank primers have been designed and validated to perform at an invariant annealing temperature of 60 °C. The expression profiles of thousands of genes can be simultaneously determined, making the primers useful for high-throughput nanoliter-scale qPCR platforms. In addition, PrimerBank contains a high number of experimentally validated primers, comprising the largest collection of its kind in the public domain [57].

qPrimerDepot is a qPCR primer database which provides optimized primers for all genes in the human and mouse Reference Sequence collection (RefSeq). The primers are designed to amplify desired targets under unified annealing temperature in order to facilitate their application in large-scale high-throughput assays. In addition, qPrimerDepot allows designing specific primers to perform gene expression studies using RT-qPCR.

PRaTo is a simple to use and easy interpret Web-based tool that enables checking and ranking of primer pairs because of their attitude for an optimal and reliable performance when used in qPCR experiments. It can be used as a stand-alone tool or in association with software for primer and probe design or for calculating oligonucleotide properties [32].

Java Web tool (jPCR) is based on the FastPCR software for Windows [58] and provides a more flexible approach for designing primers and probes for many applications. It checks if either primers or probes have secondary binding sites in the input sequences that may give rise to an additional PCR product. The jPCR tool eliminates intraoligonucleotide and interoligonucleotide reactions before generating a list with primer pair candidates. This is very important for qPCR efficiency since production of stable and inhibitory primer-dimers is predicted and can be avoided, particularly the complementarity in the 3′ end of primers whence the DNA polymerase will extend [29]. Primer-dimer prediction is based on the analysis of non-gap local alignment and the stability of both the 3′ end and the central part of the primers.

AlignMiner is a Web-based application to detect matching (convergent) and divergent regions in alignments of conserved sequences focusing particularly on divergence. Virtually without exception, all available tools focus on conserved segments or residues. Though small divergent regions are biologically important for specific qPCR, genotyping, etc., they have received little attention. As a consequence, they must be selected empirically by the researcher [59]. This software tries to cover the gap in bioinformatics function by evaluating divergence, rather than similarity, in alignments of closely related sequences. Hence, it is expected that its usage will ensure an objective selection of the best-possible divergent region when closely related sequences are analyzed, saving researchers' time of analysis [59].

3.3 Advances in the Selection of Software for Designing Primers and Probes for qPCR

The optimal design of primers and probes for qPCR using some of the above programs is essential to ensure specific and efficient amplification of the amplification products. Thus, the advantages and disadvantages of the above described software should be carefully checked before selection, with special emphasis in avoiding primer containing secondary structures. Taylor et al. [60] recommend the use of Primer-Blast, a NCBI's program that uses the algorithm Primer3 [35]. The program MFold has been reported as appropriate to be used to analyze amplicons for potential secondary structures in RT-qPCR primer design [60].

Furthermore, new advances in research about primer and probe design should be added progressively to different algorithm programs. In this sense, these programs should consider annealing failure caused by single nucleotide variant (SNV) situated inside the primer sequences. Novel allele dropout mechanism causing genotyping errors originated by a non-primer-binding-site SNV has been recently reported by Lam and Mak [61]. These authors emphasize the need of the next generation of primer and probe design software to be able to analyze the secondary structure of primers, probes, and template sequence taking SNV in all the sequences to avoid secondary hairpin structure formation of the PCR products and amplification failure.

4 Validation of Primers and Probes Designed for qPCR

After designing qPCR primers and probes by using the available tools, in silico validation (BLAST specificity analysis, http://blast.ncbi.nlm.nih.gov/Blast.cgi) has to be conducted to confirm the specificity of targeted gene sequences. BLAST algorithm allows carrying out sequence-similarity searches against several databases, returning a set of gapped alignments with links to full database records [10]. The query coverage and the maximum identity should be 100 %. The BLAST program reports a statistical significance, called the "expectation value" (*E*-value) for each alignment. This is an indicator of the probability for finding the match by chance. *E*-values ≤ 0.01 normally suggest homologous sequences [41, 62]. The *E*-value is a widely accepted measure for assessing potential biological relationship [10]. In addition to BLAST, other in silico tools could be used for validating the designed primers and probes as previously mentioned in Subheadings 3.1 and 3.2.

Furthermore, in silico PCR tools could be used for predicting the potential PCR products and searching of possible mispriming of the designed primers or probes as it has been described in the previous section. Despite the fact that in silico tools provide valuable feedback, the specificity of the qPCR assay using the designed primers and probes has to be validated empirically with direct experimental evidence as described below [63].

The next factor to take into consideration for optimal qPCR results should be reagent optimization including primer and probe concentration. To select the optimal concentrations of them for qPCR it is necessary to check the obtained amplification plots and select the combination showing the lowest value of quantification cycle (C_q), the cycle in which fluorescence reaches a defined threshold [64], and the highest fluorescent signal for a fixed target concentration [65]. Primer concentrations are normally between 50 and 300 nM owing to the fact that higher concentrations could promote mispriming, nonspecific amplification product accumulation, and lower concentrations primer exhaustion [2]. The optimal concentration for both primers could be different in a qPCR protocol [65]. The optimal probe concentration should be estimated after optimizing primer concentration. Probe concentrations normally vary between 50 and 250 nM [66], 250 nM being the optimal one. When the concentration is too low, no fluorescent signal will be observed and if it is too high a high fluorescent background could be detected [65].

After optimizing primer and probe concentration in the qPCR protocol, the optimal cycling conditions must be determined. Although the optimal annealing temperature is determined by the primer design software, it can differ greatly from the experimental annealing temperature [65]. Thus, an optimization could be necessary. It is recommended testing several annealing temperatures, starting around 5 °C below the T_m, to determine the optimal experimental annealing conditions [67] with which the efficiency of the qPCR method meets the criteria listed below.

The specificity of a qPCR protocol can be affected by the presence of nonspecific amplification products produced by primers binding to apparently random sites in the sample DNA other than the intended target or sometimes to themselves forming primer-dimers. Specificity of amplification products can be checked by analyzing the melting curves, also called dissociation curves, generated in those qPCR protocols based on double-stranded DNA-binding dyes including SYBR® Green, since they can bind to primer-dimers and other reaction artifacts producing a fluorescent signal [4, 5]. The melting curves can be carried out in all reported software programs for performing qPCR reactions immediately after amplification [68]. The specific amplicon in absence of primer-dimers appears as one single and narrow peak in the obtained melting curve (Fig. 2a) [10, 68]. If unintended amplification products are present they show a relatively lower T_m value than that expected for the amplicon or broader peaks are visualized in the melting curve (Fig. 2a) [1, 18]. Nonspecific PCR products melt at lower temperature values than the desired products mainly because of GC content and length [1, 18, 69]. The presence of primer-dimers can be experimentally demonstrated by comparing the T_m value of the checked template with that of no template

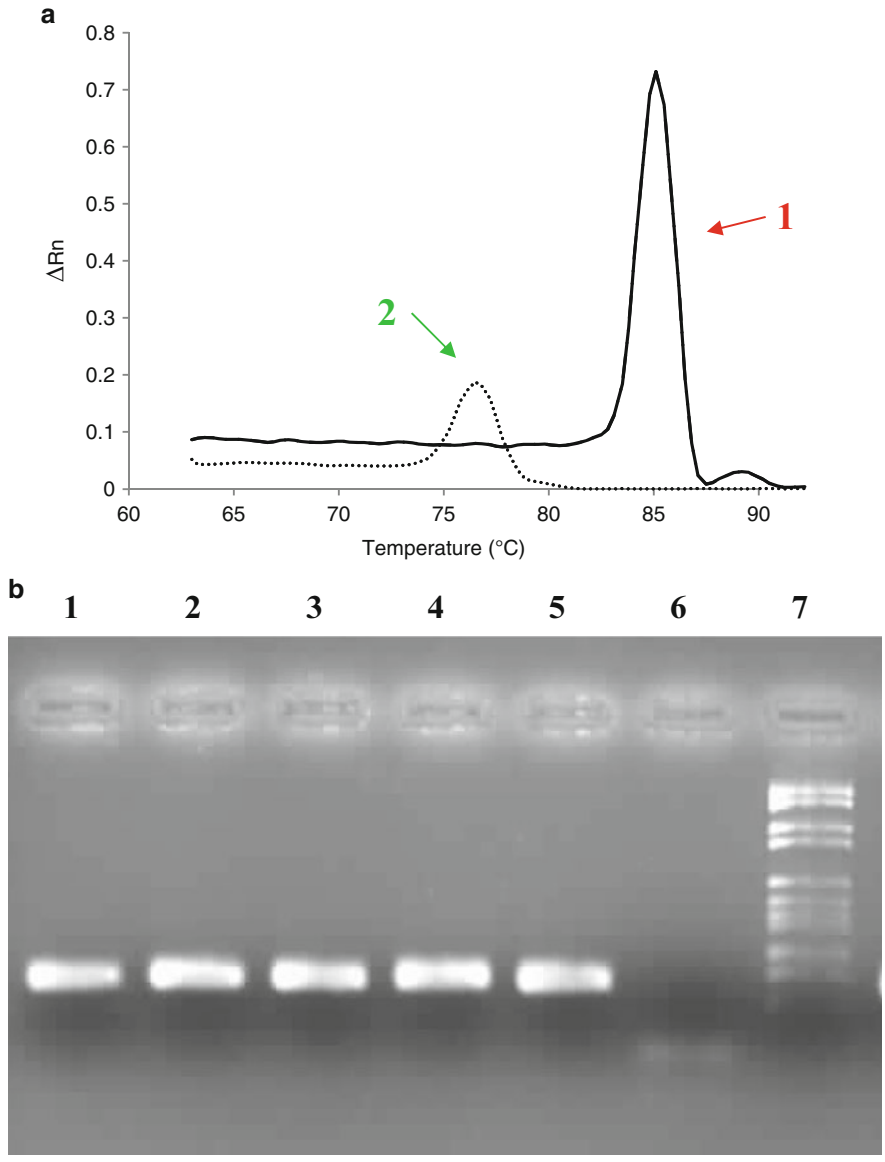

Fig. 2 (**a**) Melting curves showing specific amplification of the target sequence and primer-dimer formation in the no template control (NTC) sample. *1*: Sample containing DNA; *2*: NTC sample. (**b**) Agarose gel analysis to investigate primer-dimer formation. *Lines 1–5*: qPCR products obtained by using SYBR® Green methodology; *Line 6*: NTC sample with the presence of primer-dimer observed as diffuse band at the bottom of the gel; *Line 7*: DNA molecular size marker of 2.1–0.15 kbp (Roche Farma S.A.)

control (NTC) [1], because this artifact is much more common when template is not present. When using SYBR® Green chemistry or other double-stranded DNA binding dyes the absence of reaction artifacts has to be confirmed by using gel electrophoresis analysis since it has a higher resolution than melting curve analysis (Fig. 2b) [69]. Only a PCR product of the expected size must be

visualized in the gel when nonspecific products are detected. In spite of the fact that nonspecific PCR products do not affect to the fluorescent signal in probe-based assays the analysis of PCR product using gels is necessary since the PCR results will still be affected by the presence of nonspecific amplification. Furthermore, for a precise verification of the amplification, the specific PCR product must be sequenced, followed by a comparison of the obtained sequence with published sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/; [10]) using some of the bioinformatics tools detailed in Subheading 3.

Nonetheless, despite efforts for designing proper primers, primer-dimers or other nonspecific amplification products could be generated [1]. In this case, reaction conditions could be modified for reducing this kind of artifacts. Thus, a PCR protocol incorporating a hot-start, where an inactive DNA polymerase is activated at the start of qPCR by incubation at high temperature, could be used when performing a SYBR® Green protocol [8, 70]. This allows avoiding an early extension of primer complexes by the DNA polymerase. When this kind of enzyme is not used, reactions could be prepared on ice and the thermal cycler preheated to 95 °C before adding the reaction tubes or plates [67]. Another possibility for reducing the primer-dimer presence consisting of performing the fluorescence acquisition at a temperature higher than primer-dimer T_m, but lower than the T_m of the expected amplicon could solve this problem [71].

After that, the efficiency of the qPCR protocol has to be evaluated because an unsuitable measurement of selected target sequence fully invalidates the assay. The efficiency of a qPCR reaction should be 100 %, meaning during the exponential amplification two copies from every available templates are generated with each cycle [64]. Under experimental conditions efficiency may be as close to this value as possible. However several factors including primer characteristics may influence on it. Thus, one of the factors affecting the ability of qPCR for quantification is the efficiency of the designed primers and probes. When they are inefficient they should provoke imprecise qPCR efficiency.

Estimation of the efficiency of a qPCR method is based on constructing standard curves. Most qPCR instruments have software able to elaborate automatically a standard curve and calculate the efficiency of the reaction. If it is not available, the standard curve can be constructed by plotting the C_q values against a series of increasing and known concentrations of the template (tenfold serial dilutions of nucleic acid). For this at least four but preferably six or more points should be included [72]. The amplification efficiency can then be calculated from the formula $E=[10^{(-1/S)}]-1$ where S is the slope of the standard curve [63, 73]. Generally slopes between −3.1 and −3.6 with PCR efficiency values in the range of 90–110 % are considered satisfactory (Fig. 3) [10, 72].

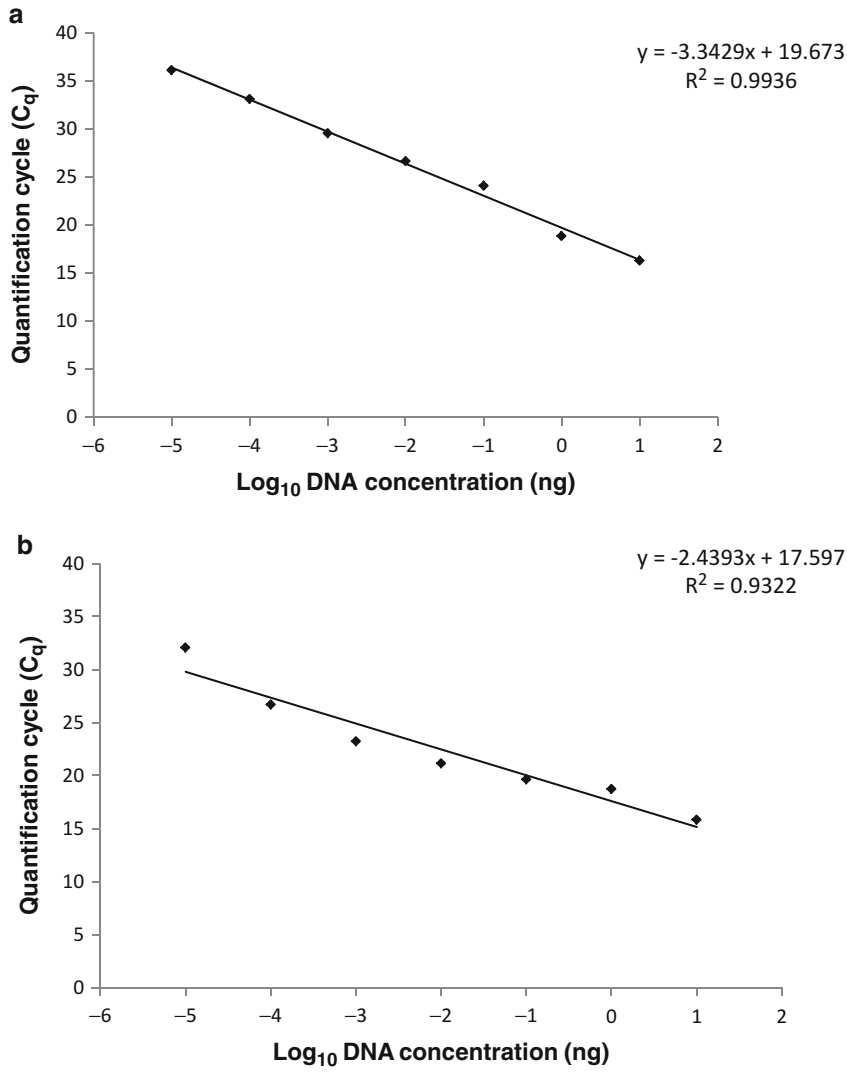

Fig. 3 Examples of an optimized standard curve (**a**) and an inappropriate standard curve (**b**) constructed with DNA standards of know concentration. Quantification cycle (C_q) values are plotted against the logarithm of seven 10-fold dilutions of standard DNA. Standards are measured in triplicate for each concentration

Furthermore, the optimal correlation coefficient (R^2) derived from the standard curve has to be between 0.99 and 0.999 [10].

A special consideration should be made for multiplex qPCR, since more problems related to primers and probes could occur such as a higher formation of nonspecific products [2]. Thus, when optimizing the protocol additional steps, such as including a higher amount of magnesium or of the hot-star enzyme if it is used, could be necessary [1], since they become limiting in later cycles and the amplification of the less efficient or less abundant target is compromised. Consequently, it is advisable to perform a primer-limiting assay to find the primer concentration giving the lowest possible C_q

value for the more abundant target without distorting the C_q value of the less abundant target [66]. Regarding the validation of the designed primers and probe for multiplex qPCR assays, before their combination in a multiplex PCR assay primers and probes for each target should be validated in single runs and their individual efficiencies determined. After that the efficiency of the overall multiplex qPCR assay should be performed [1]. The values obtained for a given target in the individual and multiplex assays should not differ significantly. If the C_q values from the individual and multiplex assays are significantly different, reactions need to be optimized [28].

5 Design of Primers and Probes for Gene Expression Studies Using Real-Time Reverse Transcription PCR (RT-qPCR)

RT-qPCR has proven to be a powerful method to gene expression analysis [25, 74] which is increasingly important in a variety of clinical and biological research fields [75]. To avoid missing any gene expression, primers must detect every alternative transcript and splicing variant of the target genes [76].

RT-qPCR can be performed in one-step or in two-step. In a one-step procedure the transcription and the amplification of the target sequences are carried out in one reaction. However in the two-step protocols, cDNA synthesis is firstly obtained by RT of RNA and a cDNA aliquot is then used for amplifying the specific target [77].

Priming of the cDNA can be performed using oligo-dT, random primers, or target-specific primers depending on whether one-step or two-step is used and the choice of primer can provoke marked variation in calculated mRNA copy number [77, 78]. Therefore, one-step RT-qPCR is always performed with gene-specific primers [8]. In two-step RT-qPCR, the three types of primers or their mixtures could be used in the RT step prior to cDNA amplification using gene specific primers by means of qPCR. Gene-specific primers yield the most specific cDNA and provide the most sensitive quantification method [77, 79].

The method selected for analyzing the data derived from RT-qPCR will also influence upon the design of the primers and probes. The data analyses can be either of absolute levels to determine the absolute transcript copy number or relative levels to measure differences in the expression level of a specific target between different samples [80]. For absolute quantification, only target gene specific primers and/or probes are necessary since a RNA standard curve of the gene of interest is required. However, for relative quantification, target and endogenous specific primers and/or probes must be designed [81].

The design of gene-specific primers and/or probes to be used in the RT-qPCR should fulfil certain requirements apart from those

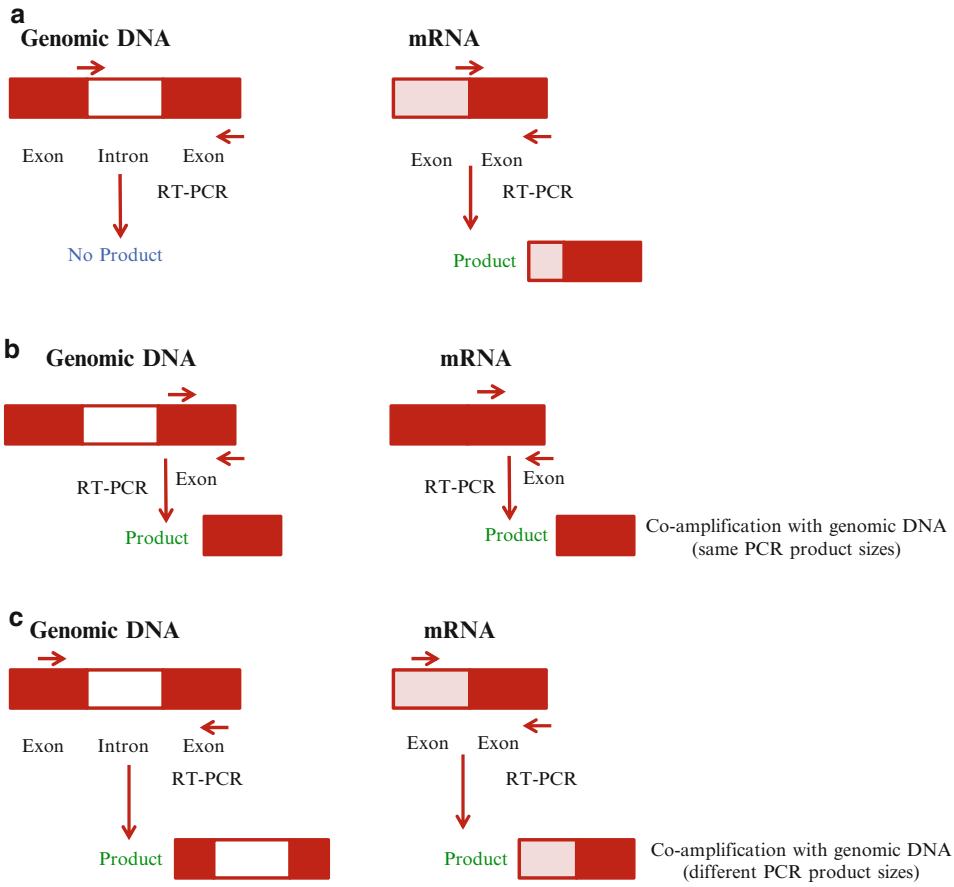

Fig. 4 Primer design to remove amplification of potential contaminating genomic DNA when using reverse transcription real-time PCR (adapted from ref. 8). (**a**) Forward primer crosses an intron–exon boundary. (**b**) Forward and reverse primers span within the same exon. (**c**) Forward and reverse primers span two different exons containing an intron

detailed in the above sections. These primers and/or probes could be designed using the specific software for them detailed in Subheading 3 and then validated as described in Subheading 4. For this kind of qPCR a proper primer and probe design is even more important, since the specific target (and the reference one when it is required) sequences should be unique, the length of the amplification product should be between 75 and 150 bp with a GC content of 50–60 %, and not containing secondary structures [60]. In addition, the primers and probes either should span an exon–exon splice junction enabling amplification and detection of RNA sequences only or they should be designed within the same exon (Fig. 4) [82]. In the first kind of primer and probe design, genomic DNA can be excluded as a template in a RT-qPCR reaction because primers or probe will bind to cDNA synthesized from sliced mRNAs but not to genomic DNA [8]. Nevertheless in the second one

contaminating genomic DNA could serve as a template resulting in a co-amplification with the cDNA (Fig. 4) and it is necessary to decide if genomic DNA is sufficiently negligible being necessary to treat the template RNA with RNAse-free DNAse [83]. Alternatively, primers and probes for RT-qPCR protocols can be designed to flank a region containing at least one intron (Fig. 4) [8]. Products amplified from cDNA without introns will be smaller than those amplified from genomic DNA which contains introns. If possible, a target with very long introns should be selected. Therefore, the RNA target may then be preferentially amplified because of the higher PCR efficiency of this shorter PCR product without introns. As previously described, if genomic DNA contamination is detected a treatment of the RNA sample with RNase-free DNase should be performed. Otherwise, the primers and probes should be redesigned to avoid amplification of genomic DNA. In addition, the use of Mn^{2+} rather than Mg^{2+} minimizes any problems caused by amplification of reannealed DNA [84]. Thus, the correct design of primers and/or probes for RT-qPCR assays could prevent co-amplification of genomic DNA avoiding a reduction of the assay sensitivity and specificity by competition of the intended PCR product and the product derived from genomic DNA [10].

The optimization of the concentration of primers and/or probes used in RT-qPCR reactions is crucial for performing gene expression analyses. The optimal primer and probe concentrations are ranging between 50–200 nM and 100 nM, respectively [85]. Depending on the selected method to perform the RT-qPCR (one- or two-step) and analyses of data (absolute or relative quantification) derived from it, different optimization steps taking into account the potential intramolecular and intermolecular interactions between primers, probes, and/or templates would be required. Thus, the one-step RT-qPCR requires the same primer concentration for RT and qPCR, reducing flexibility in primer concentrations optimal for multiplexing. In addition, in this kind of RT-qPCR both gene-specific primers have a higher tendency to dimerize at 42–50 °C RT conditions. This can be especially problematic in reactions using DNA-binding dyes for detection [70]. However, in two-step RT-qPCR, the qPCR primer concentration may be optimized for multiplexing, without having any adverse effect on RT [70]. Concerning the method for data analyses from RT-qPCR in order to analyze unique specific target sequence, the absolute one must be performed using an individual assay, and the relative one could be performed using either individual or multiplex assays, designing specific primers and probes taking into consideration all criteria previously described [83].

The validation process of the RT-qPCR assays for gene expression studies using the primers and/or probes previously designed is influenced by the chosen method for analyzing gene expression data. For validating a RT-qPCR when the absolute quantification is

used, a RNA standard curve plotting C_q values against several concentrations of the obtained cDNA is required to calculate the number of copies. This standard curve should be evaluated according to the criteria described for qPCR in Subheading 4. If the qPCR efficiency obtained is not in the optimal range, either new primers and/or probes should be designed or reagent and thermal conditions should be optimized. However, for an appropriate validation of a RT-qPCR which uses the $2^{-\Delta\Delta C}{}_T$ method for relative quantification two assumptions should be met [81]. If these assumptions are not fulfilled, then new primers and/or probes should be selected and redesigned. The correct selection of the reference gene and the design of primers and probes targeting the above gene are essential for carrying out the relative expression analysis. Thus properly selected reference genes will normalize differences in the amount and quality of starting material as well as in the reaction efficiency. Normalization uses reference genes with the assumptions that their expression is: (a) similar between all samples in a given study, (b) is resistant to experimental conditions, and (c) undergoes all steps of the qPCR with the same kinetics as the target gene [85].

Finally, the RT-qPCR assays for relative expression analyses could be performed using individual or multiplex assays. To undertake a multiplex assay several requirements should be met as follows: (a) the expression level of the reference gene must be greater than that of the target gene and (b) the gene that is more highly expressed (reference gene) should be setup with its primers at a limiting level. This ensures an accurate quantification of both genes since the competition between targets is excluded. In order to test that the reference gene is more abundantly expressed than the target gene, it should be tested that samples span the expected range of target gene expression. If the experiment is not successful, new primers and/or probes from the tested reference gene or other new ones consistently expressed in the sample have to be evaluated or the primers and/or probes designed on the basis of the reference and target genes may be run in separate wells (individual assays) [83].

6 Conclusions

Guidelines for designing primers and probes for qPCR are revised in this chapter, since this is the one of the most critical factor affecting the success and ability for quantifying of this PCR technique. The parameters to be considered when designing primers and probes have been profoundly described, highlighting special criteria which should be met if these primers and probes are used for multiplex qPCR. A brief description of some of the numerous available software programs and bioinformatics tools for designing primers and probes has been given. However new advances in research focused on this subject should be progressively added to

different algorithm programs for a higher suitability of the designed primers and probes. Once primers and probes have been designed, as detailed in this chapter a special attention has to be done to their validation process for obtaining successful results of the qPCR. Finally, a special remark has been done in the design of proper primers and probes for RT-qPCR protocols and their validation.

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