Protocol

Designing Polymerase Chain Reaction Primers Using Primer3Plus

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Designing oligonucleotide primers is a crucial step for successful molecular biology experiments that require the use of the polymerase chain reaction (PCR). PCR involves cycles of three steps: denaturation, annealing, and extension. During denaturation, double-stranded DNA (dsDNA) molecules (templates) are separated into single strands. During annealing, a pair of primers is annealed to the complementary regions of the single-stranded molecules. In the extension step, DNA polymerase extends the primers to produce DNA molecules that correspond to the region bracketed by the primers (the amplicons). All of these steps are temperature sensitive, and the common choice of temperatures is 94°C, 60°C, and 70°C, respectively. Poorly designed primers may lead to no amplification product or additional undesired amplified fragments. The goals of primer design include good primer specificity, high annealing efficiency, appropriate melting temperature, proper GC content, and the prevention of primer hairpins or primer dimers.

MATERIALS

Equipment

Computer equipped with Internet connection and a web browser
Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)

METHOD

1. Go to the NCBI nucleotide database (Pruitt et al. 2007) to download the mRNA sequence of human albumin (http://www.ncbi.nlm.nih.gov/nuccore/215982788?report=fasta).
2. Go to Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).
    i. Set the Task as "Detection," paste the ALB sequence (without the FASTA header), and provide "ALB" as the Sequence ID. Use the mouse to highlight a region and click "< >," "[ ]," or "{ }," to tell Primer3Plus to include, flank, or exclude, respectively, the region for primer design.
3. Primer3Plus has a large number of parameters; however, only a few of them need to be adjusted, whereas the others can be left at their default values.
    i. As shown in Figure 1, click the "General Settings" tab and change the "Min" and "Max" of the "Primer GC%" to 40.0 and 60.0, respectively.

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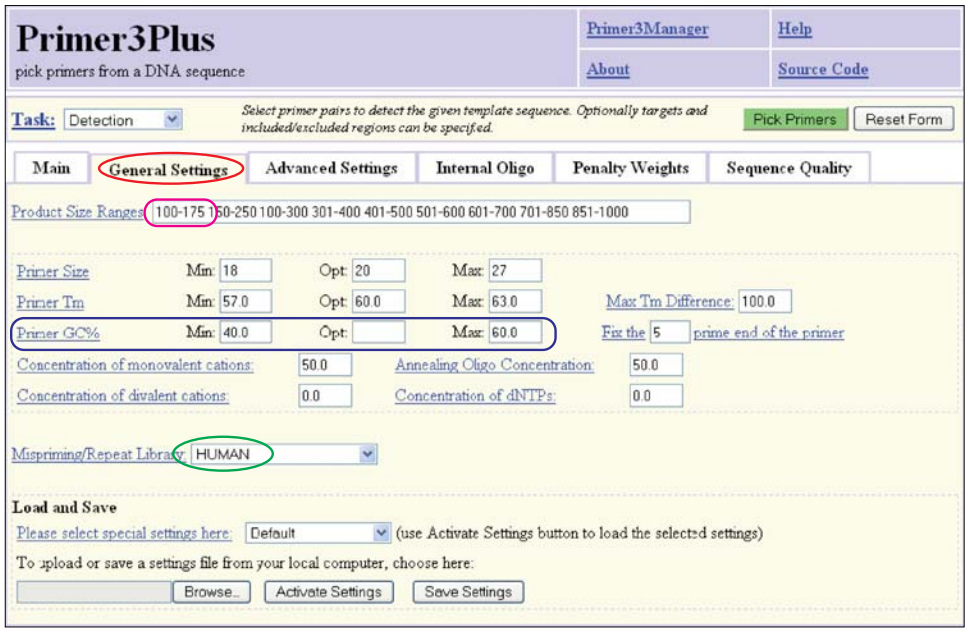

FIGURE 1. Parameters in general settings. Indicate preferred product ranges with decreasing priority in the "Product Size Ranges" field, as marked in the magenta oval. Change the Primer GC% Min and Max values, as marked in the blue oval. Select the Mispriming/Repeat Library corresponding to the species of the target sequence, as shown in the purple oval.

ii. Select "HUMAN" as the Mispriming/Repeat Library. This will tell Primer3Plus to use its human repeat library to filter out regions that can lead to undesired amplification.

At the present time, Primer3Plus only provides human, rodent, and Drosophila *libraries. If you design primers for species other than human, rodent, and fly, you must replace repeats in your sequence with "N"s before submitting it to Primer3Plus.*

iii. If you have a preferred product size range (e.g., 100–175 nt), insert "100–175" in the "Product Size Ranges" window, and Primer3Plus will try to find primer pairs that give rise to products in that specified range.

Multiple size ranges can be specified (in order of decreasing priority) and Primer3Plus will try the subsequent ranges should it fail to find a primer pair that leads to the most desirable size.

4. Additional parameters can be set in "Advanced Settings" (Fig. 2).

i. Set "Max Poly-X" as "3" to limit homopoly-A/C/G/T of maximal length 3.

ii. Choose the "SantaLucia 1998" method for both "Table of thermodynamic parameters" and "Salt correction formula," as marked in purple. Set "CG Clamp" and increase "Max 3′ Stability" (marked in cyan) to prefer primers with more CG in the 3′ ends.

iii. "Max Self Complementarity" and "Max 3′ Self Complementarity" (marked in magenta), when tuned up or down, ease or tighten the restriction of the primers to form hairpins or dimers.

iv. Four additional parameters highlighted in the orange box in Figure 2, when tuned up or down, allow more or less sequence similarity between primers to any repeats. To compute a sequence complementarity score, Primer3Plus assigns 1.00 for a complementary base, −0.25 for a match of any base (or N) with an N, −1.00 for a mismatch, and −2.00 for a gap (only single-base pair gaps are allowed).

Ideally, it is best to completely avoid self-complementarity and similarity to repeats; however, setting these parameters too strictly may yield no primers. In this example, leave these parameters at their default values.

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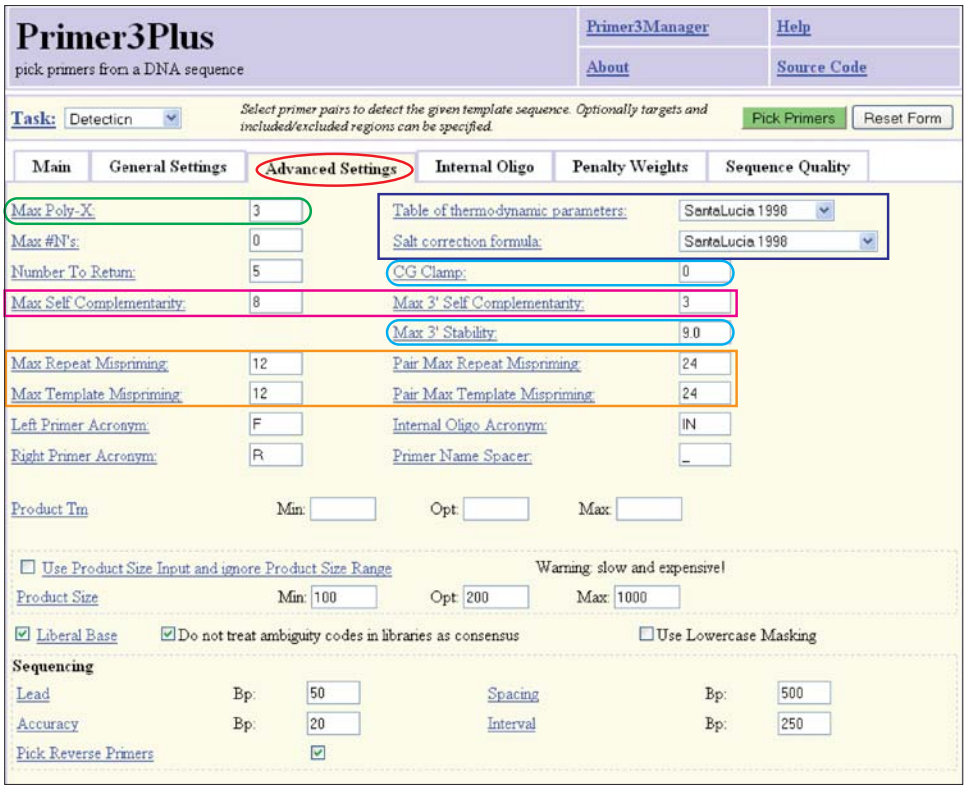

FIGURE 2. Parameters in advanced settings. (Green oval) Allow only poly(A/T/C/G) stretches in maximum lengths of 3. (Purple rectangle) Set the thermodynamic parameters and salt correction formula to SantaLucia 1998. (Cyan oval) Control 3′ GC; left as default. (Magenta rectangle) Adjust the allowable levels of self-complementarities, which control the chance of forming hairpins and dimers; left as default. (Orange rectangle) Tune the degree of sequence similarity to any repeat; left as default.

v. When all parameters have been set, click "Pick Primers."

5. If Primer3Plus can determine any primer pairs that meet all of the input requirements, it reports them as shown in Figure 3A. In this example, the left (forward) primer is "AAGCTGCCTGCCT GTTGCCA," starting from position 666 of the ALB mRNA, 20-nt long, denatured at 59.5°C, etc. A similar report can also be found for the right (reverse) primer. This primer pair gives rise to a 135-nt-long product, and their binding sites are highlighted on the target sequences, as shown in Figure 3A.

   i. Scroll down to the end of the results page and find a Statistics table, as shown in Figure 3B, which reports the amount of candidate primers that were filtered out by each of the parameters; for example, the GC content parameter excluded 8312 primers. This table can provide a useful diagnosis when Primer3Plus reports no primers, and some parameters can be adjusted accordingly.

DISCUSSION

When primers bind to unwanted regions, the results can be disastrously misleading. Checking all possible binding targets for each primer using BLAST is a simple screening method that should be performed before applying the primers. In addition, be very careful if you have to use gene sequences as input to design primers for mRNAs (e.g., finding a hypothetical isoform) and avoid primers that bind to introns and untranscribed regions. In addition, consider the alternative splicing and possible

A

Pair 1:

Left Primer 1: ALB_F

Sequence: AAGCTGCCTGCCTGTTGCCA

Start: 666 Length: 20 bp Tm: 59.5 °C GC: 60.0 % ANY: 4.0 SELF: 3.0

Mispriming: 11.00, MLT2D Interspersed repeat MLT2D - a consensus

Right Primer 1: ALB_R

Sequence: GGCGAGCTACTGCCCATGCTTT

Start: 800 Length: 22 bp Tm: 59.8 °C GC: 59.1 % ANY: 4.0 SELF: 0.0

Mispriming: 12.00, MLT1e (MLT1e subfamily) - consensus sequence

Product Size: 135 bp Pair Any: 5.0 Pair End: 1.0

Pair Mispriming: 21.00, MLT1e (MLT1e subfamily) - consensus sequence

Send to Primer3Manager | Reset Form

```
1    AGTATATTAG  TGCTAATTTC  CCTCCGTTTG  TCCTAGCTTT  TCTCTTCTGT
51   CAACCCCACA  CGCCTTTGGC  ACAATGAAGT  GGGTAACCTT  TATTTCCCTT
101  CTTTTTCTCT  TTAGCTCGGC  TTATTCCAGG  GGTGTGTTTC  GTCGAGATGC
151  ACACAAGAGT  GAGGTTGCTC  ATCGGTTTAA  AGATTTGGGA  GAAGAAAATT
201  TCAAAGCCTT  GGTGTTGATT  GCCTTTGCTC  AGTATCTTCA  GCAGTGTCCA
251  TTTGAAGATC  ATGTAAAATT  AGTGAATGAA  GTAACTGAAT  TTGCAAAAAC
301  ATGTGTTGCT  GATGAGTCAG  CTGAAAATTG  TGACAAATCA  CTTCATACCC
351  TTTTTGGAGA  CAAATTATGC  ACAGTTGCAA  CTCTTCGTGA  AACCTATGGT
401  GAAATGGCTG  ACTGCTGTGC  AAAACAAGAA  CCTGAGAGAA  ATGAATGCTT
451  CTTGCAACAC  AAAGATGACA  ACCCAAACCT  CCCCCGATTG  GTGAGACCAG
501  AGGTTGATGT  GATGTGCACT  GCTTTTCATG  ACAATGAAGA  GACATTTTTG
551  AAAAAATACT  TATATGAAAT  TGCCAGAAGA  CATCCTTACT  TTTATGCCCC
601  GGAACTCCTT  TTCTTTGCTA  AAAGGTATAA  AGCTGCTTTT  ACAGAATGTT
651  GCCAAGCTGC  TGATAAAGCT  GCCTGCCTGT  TGCCAAAGCT  CGATGAACTT
701  CGGGATGAAG  GGAAGGCTTC  GTCTGCCAAA  CAGAGACTCA  AGTGTGCCAG
751  TCTCCAAAAA  TTTGGAGAAA  GAGCTTTCAA  AGCATGGGCA  GTAGCTCGCC
801  TGAGCCAGAG  ATTTCCCAAA  GCTGAGTTTG  CAGAAGTTTC  CAAGTTAGTG
851  ACAGATCTTA  CCAAAGTCCA  CACGGAATGC  TGCCATGGAG  ATCTGCTTGA
901  ATGTGCTGAT  GACAGGGCGG  ACCTTGCCAA  GTATATCTGT  GAAAATCAAG
951  ATTCGATCTC  CAGTAAACTG  AAGGAATGCT  GTGAAAAACC  TCTGTTGGAA
```

B

Statistics:	
Left Primer:	considered 18142, GC content failed 8312, low tm 6757, high tm 101, high end compl 40, high repeat similarity 22, long poly-x seq 568,high template mispriming score 2, ok 2340
Right Primer:	considered 18250, overlap excluded region 28, GC content failed 8504, low tm 6759, high tm 100, high end compl 2, high repeat similarity 6, long poly-x seq 521, ok 2330
Primer Pair:	considered 43474, unacceptable product size 43456, high end compl 1, ok 17

FIGURE 3. Output of Primer3Plus. (*A*) Information and locations of the identified primer pair. (*B*) Table shows how the parameter settings eliminated other primer candidates.

junction sites of mRNAs when designing your primers. Some primer design tools will intentionally find primers targeting to junction sites to increase their specificity (Arvidsson et al. 2008). This trick is especially useful to avoid primers binding to contaminating DNA, which is common in qPCR (quantitative PCR) and real-time PCR library preparation.

Primer Design

The following principles govern primer design.

1. *Specificity*. For the purpose of amplifying a DNA target during a PCR experiment, two primers anneal to the 3′ ends of the sense and antisense strands of the target. In an ideal case, the primers should be long enough so that they are uniquely complementary to the target sequence. Furthermore, the primers should avoid any sequences complementary to repeats (such as short interspersed nuclear elements [SINEs], long interspersed nuclear elements [LINEs], etc.) to prevent amplifying unwanted regions in the genome.
2. *Efficiency*. On average, shorter primers have higher binding efficiency to their target than longer primers; however, shorter primers also have lower specificity. As a trade-off between these two considerations, primers should be 18–24 nt in length.

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3. *Melting temperature*. During a PCR cycle, the primers and the target form a duplex in the annealing step, and the duplex is separated in the denaturation step. The annealing step is performed at a lower temperature than that of the denaturation step. Therefore, a good pair of primers should have appropriate and similar melting temperatures. The following formula, proposed by Marmur and Doty (1962) for estimating a primer's melting temperature, is widely used:

$$\text{Melting temperature} = 2^{\circ}\text{C} \times (N_A + N_T) + 4^{\circ}\text{C} \times (N_G + N_C),$$

where N_A, N_T, N_G, and N_C are number of As, Ts, Gs, and Cs in the primer, respectively. Generally speaking, the melting temperature of a primer should be ~60°C. However, the formula is not applicable for long primers (>13 nt); therefore, it is now recommended to use the nearest-neighbor parameter set proposed by SantaLucia (1998) for designing primers of all lengths.

4. *GC content*. Besides considering GC content when calculating melting temperature, it is also important to design primers of ~50% GC and to avoid long stretches of GC. High GC content can make the primers "sticky" and increase the chance of mispriming to undesired regions in the template. Long stretches of poly(G,C) can lead to nonspecific annealing to long GC stretches in the template. On the other hand, poly(A,T) stretches can lead to unstable pairing and open the primer–template duplex. If possible, control the occurrences of poly(T,C) and poly(A,G) stretches, because they can induce undesirable secondary structures in primer–template duplexes.

BOX 1. SPECIAL CASES FOR PRIMER DESIGN

Designing Primers for Custom Primer-Based (Re-)Sequencing of Clones

When your application requires pairs of primers for generating amplicons covering a long stretch of nucleotides, such as (re-)sequencing a specific region of DNA, change the "Task" in Figure 1 to "Sequencing." Five additional options can be tuned in the "Advanced Settings" (at the bottom of Figure 2) for the "Sequencing" task: Lead, Spacing, Interval, Accuracy, and Pick Reverse Primer. These options are defined as follows.

1. *Lead*. The space from the start of the primer to the point where the trace signals can be generated by the sequencer (default 50 nt).
2. *Spacing*. The space between two consecutive primers on the same strand (default 500 nt).
3. *Interval*. The space between two consecutive primers on the opposite strand (default 250 nt).
4. *Accuracy*. The size of the area in which Primer3Plus searches for the best primer (default 20 nt).
5. *Pick reverse primers*. In addition, pick primers on the reverse strand (selected by default).

When picking these parameters, remember to change the "Product Size Ranges" in "General Settings" to the appropriate size based on the settings of "Spacing" and "Interval."

Cloning Products Starting or Ending at a Specific Position

Change the "Task" in Figure 1 to "Cloning," specify the region that you want to be cloned using the curly braces, and input either 5 or 3 in the "Fix the *x* prime end of the primer" textbox (next to the blue highlighted region in Fig. 1). By doing so, the 5′ or 3′ ends of the primers are fixed to the boundary of the marked region. This is particularly useful if the reading frame of the PCR product must be fixed.

Checking the Specificity of the Selected Primers

One intuitive approach is to BLAST the primers against the corresponding genome and transcriptome to ensure that the number of hits of each primer is as small as possible and encompasses the desired target. Because the primers are relatively short, relax the *E*-value threshold to 200 to detect more potential off-targets. The *E* value that corresponds to a similarity score is defined as the number of sequence matches to the query with a greater, same, or similar score by chance.

TABLE 1. List of other primer design tools

Tool	URL	Type	Traits
Primo Pro	http://www.changbioscience.com/primo/primo.html	PCR	Uses human transcriptome to analyze and reduce the chance of random priming
GeneFisher2	http://bibiserv.techfak.uni-bielefeld.de/genefisher2/	PCR	Allows inputting of multiple sequences from closely related organisms to design primers on the consensus sequences
Primer-BLAST	http://www.ncbi.nlm.nih.gov/tools/primer-blast/	PCR	Performs BLAST for each primer to automatically filter unspecific ones
QuantPrime	http://www.quantprime.de/	Real-time PCR	Uses exon–exon junctions and BLAST search to increase primer specificity
AutoPrime	http://www.autoprime.de/AutoPrimeWeb	Real-time PCR	Uses information of exon boundary to increase primer specificity
RNAi Design	http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx	siRNA	Designs siRNA duplexes

In addition, the 3′ end of a primer should be G or C so that beneficial tight pairing can occur with its target. This is known as the G/C clamp (Lowe et al. 1990).

5. *Hairpins and primer dimers.* It is possible for a primer to form a stable secondary structure within itself, such as a hairpin, interact with another copy of itself to form a homodimer, or interact with the other primer to form a heterodimer (Hillier and Green 1991; Burpo 2001). To prevent these types of mispairings, avoid complementary sequences within a primer or between primers.

As described above, designing good primers often requires extensive analysis. Primer3Plus is a useful web server for primer design. This protocol demonstrates how to design a pair of primers for measuring the expression level of a gene using Primer3Plus (Rozen and Skaletsky 2000; Untergasser et al. 2007). Note that Primer3Plus can also perform other tasks that require primers, such as cloning, sequencing, etc. (see Box 1 and the help page at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plusHelp.cgi).

Many primer design tools are freely accessible on the Internet and some are specific to certain purposes (see Table 1). General PCR primer design tools, such as Primer3Plus (Untergasser et al. 2007), Primo Pro, GeneFisher2 (Giegerich et al. 1996), and Primer-BLAST can be used for real-time PCR experiments too, but using real-time PCR-specific tools such as QuantPrime (Arvidsson et al. 2008), and AutoPrime (Wrobel et al. 2004) can give primers with higher specificity. Two siRNA duplex designing tools are also included in the list for quick reference.

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