Primer design

Imagine you have the 5'-3' sequence of a gene and you wish to amplify a fragment of it.

5'-NNNNNAGAGACA GTGGGACCGT CTG ------ TGGA CTTGAGGATT CTAGAGNNNNNN-3'

A and G are the start and end of the fragment you wish to amplify.

----: **means** the fragment is big enough so can't write the whole sequence.

(SENSE template)

5'-NNNNNAGAGACAGTGGGACCGTCTG------TGGACTTGAGGATTCTAGAGNNNNNN-3'

(ANTISENSE template)

3'-NNNNNTCTCTGTCACCCTGGCAGAC------ACCTGAACTCCTAAGATCTCNNNNNN-5'

(ANTISENSE IN 5'-3' MODE):

5'-NNNNNNCTCTAGAATCCTCAAGTCCA------CAGACGGTCCCACTGTCTCTNNNNNN-3'

Some software like **DNassist** and **WinGene** can do "reverse-complement" in one click.

Thus, complement –reverse means: to have the sequence of the antisense in 5'-3' direction.

Now to design the primers using the 5'-3' (sense) strand:

One of the primers (the forward primer) will be directed from 5'-3' and anneal with the antisense template. This primer is written as same as the sense template nucleotide sequence.

Forward primer: 5'-NNNNNAGAGACAGTGGGACCGTCTG-3'

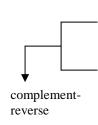
NNNNN: the restriction sequence, GC clamp, ...etc.

The second primer (backward/antisense) will anneal with the sense template and thus it is 3'-5'.

Backward primer: 3'-ACCTGAACTCCTAAGATCTCNNNNNN-5'

Usually both primers are written in 5'-3' formula. Thus, the backward primer can be written as: 5'-NNNNNCTCTAGAATCCTCAAGTCCA-3'

where NNNNN are the restriction sequence, extra nucleotides or GC clamps.



Summary:

If you got the 5'-3' strand sequence of a gene fragment that you wish to design primers to amplify it by PCR, e.g.:

5'- AGAGACAGTGGGACCGTCTG-----TGGACTTGAGGATTCTAGAG-3'

do the following:

(1) Select the nucleotides in the same sequence to the sense template and add at the 5' end any extra sequence of restriction enzyme, GC clamp, ..etc. This serves as a sense (forward) primer.

Thus, a forward primer can be as: 5'-NNNNNNNNNNNNNNNAGAGACAGTGGGACCGTC-3'

For a reverse primer: write the complement sequence of the 3' end of the sense template, reverse it, so it can be read as 5'-3' and add any extra sequence at the 5'end of this primer.

It's easy, isn't it?

<><><><><><><>

- * The 3'end of the primers is critical for the specificity and sensitivity of PCR.
- * Since <u>5'end of the primer is less critical for annealing</u>, add the restriction sequence at this end.

Melting temperature (T_m): the temperature at which half of the primer is annealed to the template. T_m is important for primer specificity.

 T_m for both primers should be (1) similar and (2) >60C.

Rules for primer design:

- (1) Length: 17-30 nt (shorter primers lead to nonspecific amplification of PCR products).
- (2) 50-60% (G+C).
- (3) the 3'end should:
- (a) Should end with C, G, CC or GG (for more efficient priming as this avoid breathing).

- (b) Avoid having ≥ 3 C or G (i.e. GGG) as this <u>stabilizes the nonspecific annealing</u> of the primers.
- (c) This end shouldn't be self-complementarily to avoid secondary structure (hairpin) formation or primer dimmer formation.
- (d) Avoid **thymidine** at this end as it causes mispriming.
- (4) Tm is between 55-80C.