Primer design

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

5'-NNNNNNAGAGACA 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'-NNNNNNAGAGACAGTGGGACCGTCTG---------TGGACTTGAGGATTCTAGAGNNNNNN-3'

(ANTISENSE template)
3'-NNNNNNTCTCTGTCACCCTGGCAGAC---------ACCTGAACTCCTAAGATCTCNNNNNN-5'

complement-reverse

(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'-NNNNNNAGAGACAGTGGGACCGTCTG-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'-NNNNNNCTCTAGAATCCTCAAGTCCA-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'-NNNNNNNNNNNNAGAGACAGTGGGACCGTC-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.

Thus, for the example given above, the 5'-3' mode of the reverse primer will be: 5'- NNNNNNNNNN-CTCTAGAATCCTCAA-3'.

It's easy, isn't it?

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*** The 3'end of the primers is critical for the specificity and sensitivity of PCR.**

*** Since 5'end of the primer is less critical for annealing, 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 stabilizes the nonspecific annealing 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.