DNA Hybridization Reaction

Useful Notes and Observations on Annealing Temperature and Time in PCR

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These notes and observations arise from theoretical and practical experiences in the application of the PCR reaction matured in the recent past with Dr. Riccardo Schiavon and are also the result of the contribution of Dr. Alessandro Vezzi.

Why in PCR you can have an exponential reaction of the entire template even if the annealing temperature is equal to the melting temperature? (at this temperature only half of the template file is hybridized)

Perhaps many have noticed that in the PCR reaction, the exponential amplification of the entire 'template' is obtained at each cycle even if the annealing temperature is set to a temperature higher than the melting temperature.
You realize this especially by doing a realtime-PCR .
It could be due to an incorrect estimate of the Tm, but this is not always the case.
In theory, we should expect that, at the melting temperature, only half of the template is amplified at each cycle.
Instead, often, we obtain the duplication of almost the entire template at each cycle.
Why is this?
One of the reasons should be that the hybridization reaction is a dynamic reaction, due to the thermal movements of the molecules, the duplex between template and primer is continuously formed and undone. It is true that at the Tm you should have half of the template hybridized to the primers, but this is just a statistic. The individual strands of hybridized template change continuously.
What happens during PCR? DNA polymerase has a 'polymerase' activity even at temperatures lower than the elongation temperature. A primer can be 'elongated', even at the annealing temperature, if it anneals to its own strand and the DNA polymerase enzyme also joins. Elongation creates greater stability. This shifts the equilibrium of the ssA + ssB→ dsD reaction to the right, towards the formation of the duplex.
This explains, in part, why the entire template can be copied at each cycle of PCR

Annealing phase in the PCR reaction

This phase is made up of two parameters: temperature and time. Usually only the temperature is considered important (which depends on the primer and the goals you want to achieve), while the annealing time is not considered important and is generally set from 20 to 40 seconds regardless of the other conditions of the PCR reaction. With these notes, I hope to give importance to both parameters. Let's see these two parameters

1) annealing temperature
2) annealing time

1) annealing temperature (Ta):

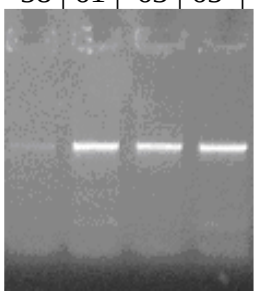
I remind you that the annealing temperature should be set 2-4 °C lower than the primer melting temperature (this is the temperature at which, at equilibrium, 50% of the template should be hybridized to the primers).

Primer + Template → duplex

Since there are at least two primers, these primers should be designed to have a similar Tm and have suitable characteristics for PCR (see our DNA_promix website for primer design). The primer with the lowest Tm should be considered for designing the experimental Ta.
Low annealing temperatures allow primers to hybridize even in non-specific regions and, especially if you use polymerases with 3'→ 5' exonuclease activity such as Fusion, Pfu etc., you can obtain non-specific amplification products.

If you also want to amplify templates containing mismatches, you need to lower the annealing temperature. But be careful not to lower it too much, in fact you are led to think that, if there is no danger of non-specific products, lowering the annealing temperature favors the PCR reaction.
But that's not always true. Sometimes decreasing Ta results in no amplification. This is an example.
Example: PCR reaction in temperature gradient 58 → 65 °C with human genome template (annealing time 40''). The primers had a calculated melting temperature of 64°C

58 | 61 | 63 | 65 |°C



You can see that at 58°C you only get a very small amplification.

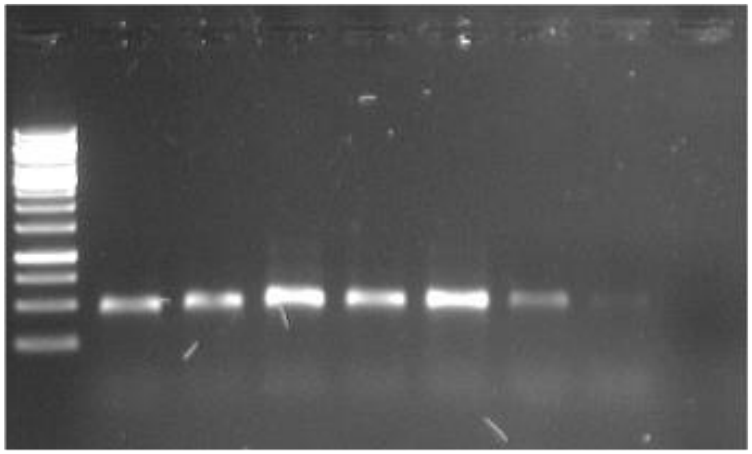
What could be the reasons?
- primers, at low temperatures, can be sequestered by the long template (if many similar regions exist);
- primers form secondary structures (self and hetero dimer or hairpin loop) preferentially at low temperatures;
- the template forms secondary structures in the primer hybridization regions;
- the DNA duplex is reformed between the template strands, which displace the primers;
- anything else ??;

Note that PCR is also successful with 65°C of annealing (higher than the melting temperature of the primers), what could be the reasons?
It could be due to an underestimation of the melting temperature of the primers (unsuitable computer programs and/or incorrect settings in the programs used).
More likely, however, it is due to the polymerase activity of the enzyme that elongates the primers already in the annealing phase (see notes above)

2) Annealing Time:

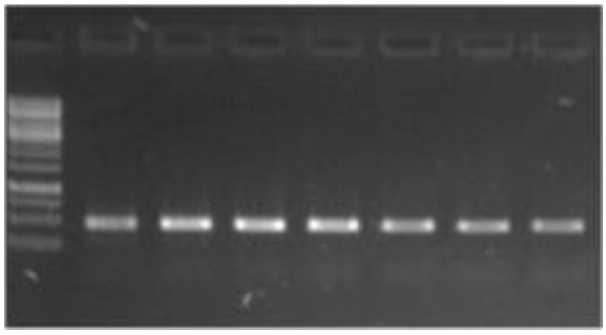
Usually this parameter is not given importance, it is usually set to 20'' - 40''.
From my experiences and from the experiences gained with Dr. Riccardo Schiavon during the HLA project at the Department of Biology of the University of Padua, it turned out that the annealing time should be well evaluated.
If too short, the primers do not have time to hybridize, if too long it could give the possibility of non-specific elongations (especially if polymerases with 3'- 5' exonuclease activity are used such as Fusion, Pfu etc).

Example with too short annealing time.
The primers were designed with a Tm of 63°C, annealing time 25 seconds, in gradient
Ta= 48 50 52 54 58 60 62



As can be seen, significant PCR products are obtained only with annealing temperatures much lower than the melting temperature of the primers.

While increasing the annealing time (40 seconds)
Ta= 55 58 60 62 64 66 68



Amplifications are obtained up to over 68 °C (the product however begins to decrease with Ta > 62 °C) (see also notes written above)

Final considerations:

Anneling temperature:.
It is correct to set this temperature 2-4°C lower than the melting temperature. However, this must be estimated using programs that you can rely on, especially if they use the Nearest Neighbor method. It is advisable, at the beginning, to try several sites and compare the results.
Very important: set the correct parameters such as salt concentrations Na+ and Mg++, many neglect this last cation. Also set the primer concentrations and possibly the dNTPS concentration (these sequester a part of Mg++).

Annealing time:
In my opinion, a short annealing time (30'') should be set only if you use short primers with a low melting temperature (52-58°C) (short primers have a higher mobility and therefore should hybridize faster), while, if you use long primers or primers with a melting temperature higher than 58-60°C, you should use a longer annealing time (40-50 seconds).

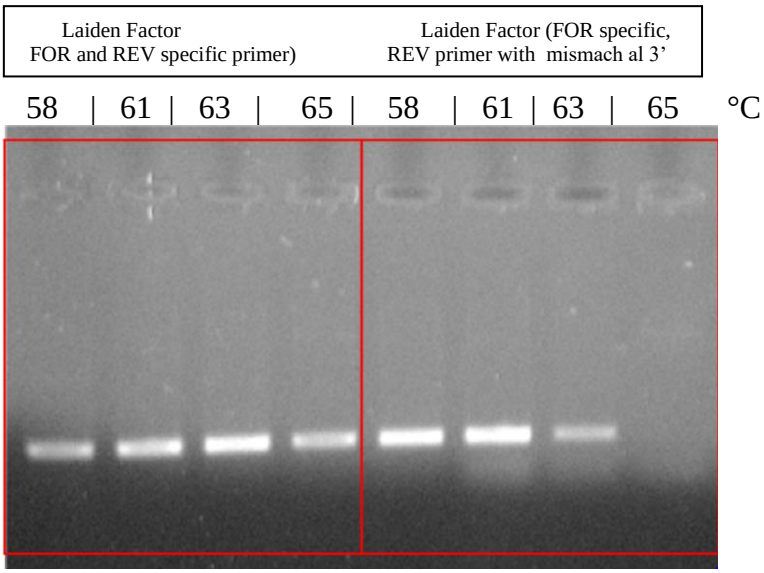
The annealing time to set may also depend on the type of thermal cycler and/or the support used:
The solution inside the well takes a certain amount of time to change temperature and reach equilibrium. This time depends on the speed with which the thermal cycler changes its temperature and the speed of transmission of the temperature from the thermal cycler to the liquid. To optimize your reaction as best as possible, it would be useful to know the characteristics of your thermal cycler and the support used to perform the reaction.

Note:
If you find that you only get an amplification product at temperatures much lower than the (correctly) calculated melting temperature, you should evaluate the existence of a possible problem (error in calculating the Tm, annealing times not long enough, dirty thermal cycler head that does not let the heat pass, etc. etc.)

Primer elongation with 3' mismatch

It may be interesting to analyze this experiment. We wanted to see the ability to discriminate sequences with a point mutation (nucleotide 1691 of the Factor V Leiden gene).
We used a human genome, isolated with the salting-out method and FOR primers specific for a downstream region, two REV primers positioned on the 3' of the mutation, one specific for the reference and the other with the mutation incorporated.
A mismatch at the 3' should block the primer elongation. Instead...

Gradient PCR (4 wells) 58 60 63 65 °C
First quadrant amplification with specific primers
Second quadrant with the reverse primer with a 3' mismatch
All primers were designed with a Tm of approximately 65°C.
Taq polymerase without 3'5' exonuclease activity was used



The first quadrant shows the efficiency of the primers at all temperatures, even if the greatest efficiency is at 60 and 63 °C.

The second quadrant, however, shows that amplification also exists at lower temperatures. This means that the Taq polymerase is able, at low temperatures, to elongate the reverse primer even if it has a 3' mismatch.

This shows that if you want to discriminate sequences with only one 3' mismatch, you need to use high annealing temperatures.