DNA Hybridization Reaction

Details, melting temperature, hybridized fraction, experimental notes

dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

Introduction

The study of thermodynamics in DNA hybridization reactions is important to understand many of the applications (PCR, arrays, etc.) used in biology. To design these reactions, the melting temperature is often used as a parameter. Understanding what it is and how to estimate it is of fundamental importance for the success of the experiments.

I wanted to write these notes to describe in detail the thermodynamic reaction of hybridization of two single strands of DNA using, in particular, the nearest-neighbor method.

Differences in published data

I think that there is a lack of information on this topic, in fact, different sites offer heterogeneous estimation services that are inconsistent with each other.
In addition to adopting different methods and studies, some sites (even those of important companies, see below) make mistakes in applying the theory of DNA hybridization and use incorrect equations. Other sites underestimate the importance of solutions and their salts.

To demonstrate some inconsistencies published on some sites, now anticipate the equation to determine the melting temperature (which will be explored in more detail later) to report some published inconsistencies.
The general formula for determining the melting temperature should be:

$$Tm = \frac{\Delta H}{\Delta S + \mathrm{R}\ln(Cx)}$$ in °Kelvin (see explanation in the next chapters)

I will demonstrate that it is correct to replace Cx with the initial concentrations of reagents 'Ca' and 'Cb' in this way:

- Generic case, always valid: Cx = Ca - ½ Cb;
- In the case of PCR then Cx=Initial primer concentration.
- In case the concentrations of the two reagents are equal Ca=Cb = C then Cx = ½ C

Some website use in PCR reaction Cx= ½ [primer concentration] (e.g. Promega), while others use Cx = ¼ [primer concentration] (e.g. Merck – sigmaaldrich) . Others use our annotation correctly Cx = [primer concentration] (e.g. Premier Biosoft). Link to our Oligo Melting application or PCR Primer Analysis

These work

These notes are the result of a theoretical study of DNA hybridization chemistry and matured from the experience with Prof. Giorgio Valle's group at the Department of Biology of the University of Padua.
They are aimed at people (especially biologists) who already have the basis for understanding the structure of DNA, and who know the fundamentals of chemistry, in particular the basis of chemical reactions.
I have divided the work into several parts:

1) (.pdf) Theoretical bases of the hybridization reaction between two DNA single strands, to arrive at the equation that determines the Gibb free energy as a function of enthalpy, entropy and temperature.
2) (.pdf) Meltig Temeprature: Using the general equation of the first part, determine the melting temperature and its general equation and in particular cases such as PCR.
3) (.pdf) Eraction of the strand template: Determination of the fraction of a strand (template) hybridized as a function of temperature.
4) (.pdf) PCR: notes and observations based mainly on experience.
5) (.pdf) Attachment

DNA Hybridization Reaction

Part 1 **Analysis of the hybridization reaction between two DNA single strands**

dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

Some notes on Thermodynamic Theory:

In chemistry, the Gibbs free energy represents an important parameter and is a state function (like enthalpy (H) and entropy (S)) that depends only on the initial and final states.
The difference in free energy of a reaction between the final state (free energy of the products) and the initial state (free energy of the reactants) defines the change in free energy.

$\Delta G = G\_final - G\_initial$

In chemistry it is an important parameter that describes the spontaneity of reactions. A chemical reaction proceeds from a higher free energy to a lower one:

If $\Delta G < 0$ the reaction is spontaneous towards the product
If $\Delta G > 0$ the reaction is spontaneous towards the reactants
If $\Delta G = 0$ the reaction is in equilibrium

If there is more than one reactant and/or product, the free energy is given by the sum of the individual free energies of each element in the reaction.

$G = \Sigma Gi$ for each element 'i'
$\Delta G = \Sigma Gi(product) - \Sigma Gi(reagent)$.

Free energy is a function that depends on three thermodynamic parameters: enthalpy (H), entropy (S), and temperature (T) and on the concentration of the element.
Usually reference is made to the free energy under standard conditions (components at 1 M concentration and determined in a solution with 1 M Na+)

$G_0=H-TS$

The free energy assiociated to i-th component of chemical reactions, at standard condition and at the temperature T is:

$Go_i = H_i - T\ S_i$

Instead, the free energy (Gi) assiociated to i-th component of chemical reaction at not standard condition is:

$$Gi=Goi+RTln(Ci)$$

Ci is the concentration of i-th component;
Goi is free energy at standard condition of i-th component;
R is the gas constant (1.987 cal/Mol°K or 8.314 J/Mol°K);
T is the experimental temperature in °K.

For example in the hybridization reaction where two single strands (ssA and ssB) of DNA associate to form a duplex (dsD), we can represent the reaction as:

$$ssA + ssB \leftrightarrow dsD$$

$\Delta G = \Sigma Gi(product) - \Sigma Gi(reagent)$.

If C_A, C_B, C_D are the instantaneous concentrations of the individual elements of the reaction,
The instant free energy associated with this reaction is:

$$\Delta G = G_D - (G_A + G_B) = G_{0D} + RT\ ln(C_D) - \left(G_{0A} + RT\ ln(C_A) + G_{0B} + RT\ ln(C_B)\right)$$

$$= \Delta Go + RT\ [\ ln(C_D) - ln(C_A) - ln(C_B)\ ] = \Delta Go + RT\ ln\left(\frac{C_D}{C_A\ C_B}\right)$$

and introducing $\Delta Go = \Delta H - T\ \Delta S$

$$\Delta G = \Delta H - T\ \Delta S + R\ T \ln\left(\frac{C_D}{C_A\ C_B}\right)$$

General Gibbs free energy (ΔG) equation

Remember that the concentrations are the instantaneous ones, not the initial ones
If ΔG is different from zero, then the instantaneous concentrations of the reactants and products change continuously so as to bring the reaction to equilibrium: $\Delta G = 0$

If we know the concentrations of the two initial reagents (C_{Ai} and C_{Bi}), we can rewrite the energy equation:
Hybrid 'D' is formed by one strand of A and one of B. The concentration of D (C_D) is initially zero.
The instantaneous concentrations (C_A and C_B) can be written as a function of the initial ones (C_{Ai} and C_{Bi}).

$C_B = C_Bi - C_D$
$C_A = C_Ai - C_D$

$$\Delta G = \Delta H - T\ \Delta S + R\ T \ln\left(\frac{C_D}{(C_{Ai} - C_D)(\ C_{Bi} - C_D\ )}\right)$$

General Gibbs equation also as a function of the initial concentrations

ΔG in J (or cal), ΔH in J/mol°K (or cal/mol°K), ΔS in J/mol (or cal/mol), concentrations in molar units, R is the gas constant 8.314 J/Mol°K (or 1.987 cal/Mol°K);

NOTE: by setting the free energy to zero, it is possible to determine the temperature as a function of the concentrations, or the concentrations as a function of the temperature.

Influence of the environment on the hybridization reaction

ΔG is influenced by the environment, in particular by the conditions of the solutions.

Generally ΔH and ΔS are measured in 1M Na+ solution. Therefore, in non-standard conditions of the solution, to the ΔG calculated with the previous equation it is necessary to introduce a variation of free energy due to the environment (ΔGa).

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln\left(\frac{C_D}{(C_{Ai} - C_D)(C_{Bi} - C_D)}\right) + \Delta Ga$$

There are various methods to estimate the effect of the environment. Some act by modifying the entropy, others intervene only to modify the melting temperature (see the attachment).

Note: At constant temperature, the reaction tends to equilibrium, so the free energy tends to zero. In this hybridization equation, the only variables in time are the concentrations of the reactants/products, which therefore vary to make the free energy zero.
This concept is used to determine the melting temperature and the fraction of hybridized template (see next chapter)

To apply these equations it is necessary to estimate the thermodynamic parameters of enthalpy and entropy. In our works applied to the DNA_Promix platform, we use the Nearest Neighbor method which assigns to each internal nucleotide pair the experimentally determined thermodynamic values ΔS and ΔH. Furthermore, the initialization variations of ΔH and ΔS are considered

We will see in the next chapter in the determination of the melting temperature.

DNA Hybridization Reaction

Part 2 Melting Temperature (Tm)

dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

In the hybridization reaction seen previously: ssA + ssB ↔ dsD

And starting from the general equation seen previously, depending on the initial concentrations of reagents

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln\left(\frac{C_D}{(C_{Ai} - C_D)(\,C_{Bi} - C_D\,)}\right)$$

In a hybridization reaction the Melting temperature (Tm) parameter is used to estimate the ability of two strands to associate.
This parameter (at equilibrium, i.e. when ΔG=0) corresponds to the temperature at which half of the strand (with the lowest concentration) is associated in the duplex form. This value is used, for example, to set the annealing temperature in the PCR reaction

Let's see all the steps to get to the general equation of Tm

For ease, Placing $Cr = \frac{C_D}{(C_{Ai} - C_D)(\,C_{Bi} - C_D\,)}$

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln(Cr)$$

And breaking down and regrouping
$\Delta H = T\,\Delta S - RT \ln(\,C_r\,)$
$\Delta H = T\,(\,\Delta S - R \ln(\,C_r\,))$

$$T = \frac{\Delta H}{\Delta S - \mathrm{R} \ln(Cr)}$$

or by the property of logarithms

$$T = \frac{\Delta H}{\Delta S + \mathrm{R} \ln\left(\frac{1}{Cr}\right)}$$

As written above, for the melting temperature the following condition must exist:
the concentration of the duplex (dsD) must be equal to half the initial concentration of the less concentrated oligo (ssB) (e.g. the template in the PCR reaction).

Under these conditions $C_D = ½\ C_{Bi}$ Then:

$$Cr = \frac{½\ C_{Bi}}{(C_{Ai} - ½\ C_{Bi})(\,C_{Bi} - ½\ C_{Bi})} = \frac{½\ C_{Bi}}{(C_{Ai} - ½\ C_{Bi})(\,½\ C_{Bi})} = \frac{1}{(C_{Ai} - ½\ C_{Bi})}$$

Or $\frac{1}{Cr} = C_{Ai} - ½\ C_{Bi}$

Thus

$$Tm = \frac{\Delta H}{\Delta S + \mathrm{R}\ln(C_{Ai} - \frac{1}{2}C_{Bi})}$$

general equation to determine the Tm (in °Kelvin)

To convert to degrees Celsius Tm(°C)= Tm-273

ΔH in J/mol°K (or cal/mol°K), ΔS in J/mol (or cal/mol), concentrations in molar units, 'R' is the gas constant 8.314 J/Mol°K (or 1.987 cal/Mol°K);

PARTUCULAR CASES
Tm in PCR reaction

There are cases that simplify this equation.
For example: in the PCR reaction, the template DNA is at very low concentrations (even fM) and primer is 7-10 times higher, therefore, in the Cr parameter, C_{Bi} (which corresponds to the template concentration) can be omitted,
If $C_{Bi} <<< C_{Ai}$

$$\frac{1}{Cr} = C_{Ai} - \frac{1}{2}\ C_{Bi}\ =\ C_{Ai} = primer\ concentration$$

then $$Tm = \frac{\Delta H}{\Delta S + \mathrm{R}\ln(C_{primer})}$$

Another special case: if the initial concentration of the two filaments ssA and ssB were equal

$C_{Ai} = C_{Bi} = C$, then

$$\frac{1}{Cr} = C_{Ai} - \frac{1}{2}\ C_{Bi}\ = \frac{C}{2}$$ then $$Tm = \frac{\Delta H}{\Delta S + \mathrm{R}\ln\left(\frac{C}{2}\right)}$$

IMPORTANT NOTES
The Tm determined with the above equation is valid if the reagents are immersed in a standard solution (Na+ 1M)

In different conditions it is necessary to modify the thermodynamic parameters and therefore the calculated Tm.
Some methods, based on empirical experiences, modify the melting temperature considering only the concentration of the ions, others also consider the length of the oligo, others instead modify the entropy of the oligo.

See in the Attachment the methods commonly used.
In our programs we generally use the methods described in SantaLucia et al.(1998) and von Ahsen et al. (2001):

Another interesting question is the fraction of DNA strands that hybridize as a function of temperature.
This issue will be explained in the next document (.pdf)

DNA Hybridization Reaction

Part 3: Fraction of hybridized template as a function of temperature

dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

In the dynamics of the hybridization reaction of two DNA single strands it is interesting to estimate the amount of hybridized template as a function of temperature.

As explained in the previous chapters:
Hybridization reaction of two strands (ssA and ssB) to form the hybrid (D)
$ssA + ssB \leftrightarrow dsD$

If C_A, C_B, C_D are the instantaneous concentrations of the individual elements of the reaction,
The instant free energy associated with this reaction is:

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln\left(\frac{C_D}{C_A\ C_B}\right)$$ General equation

Using this general equation, we will now see a method to estimate the amount of single strand that hybridizes in the duplex at a given temperature.

Here, for simplicity, I will define A and B as the initial concentrations of the two filaments (called C_{Ai}, C_{Bi} in the previous pages), we will have:

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln\left(\frac{C_D}{(A-C_D)(\mathrm{B}-C_D)}\right)$$
General equation also as a function of the initial concentrations (A and B)

Development of the hybridization equations

In this chapter, we want to see how the concentrations of the components (in particular of the template) vary at equilibrium (ΔG =0) as a function of temperature.
By fixing the temperature, and since entropy and enthalpy are unchangeable parameters for the same reaction, what will change will be only the concentrations of the reagents / products.

Let's start from the equation described above and (explained in the first chapter):

$$\Delta G = \Delta H - T\,\Delta S + R\,T \ln\left(\frac{C_D}{(\mathrm{A}-C_D)(\mathrm{B}-C_D)}\right) = 0$$

to simplify, we put $Cr = \frac{C_D}{(A-C_D)(\mathrm{B}-C_D)}$

then $\Delta G = \Delta H - T\,\Delta S + RT \ln(C_r) = 0$

$$\ln(Cr) = -\frac{\Delta H - T\,\Delta S}{R\,T}$$

$$\ln\left(\frac{1}{Cr}\right) - \frac{\Delta H - T\,\Delta S}{R\,T} = 0$$

$$\frac{1}{Cr} - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)} = 0$$

$$\frac{(A-C_D)(\mathrm{B}-C_D)}{C_D} - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)} = 0$$

If we take the ssB reagent as a reference (the least concentrated, such as the template in the PCR reaction), we can write the other components as a function of the fraction of ssB hybridized in the duplex.
If we define 'f' as the fraction of ssB hybridized in the duplex, we will have:

$f=C_D/B$

Instantaneous concentrations can be written as:
$C_D = f\ B$
$C_A = A - f\ B$
$C_B = B - f\ B$

$$\frac{1}{Cr} = \frac{(A - C_D)(B - C_D)}{C_D} = \frac{(A - f\,B)(B - f\,B)}{f\,B}$$

By developing and simplifying we will have:

$$\frac{1}{Cr} = \frac{A\,B - f\,A\,B - f\,B^2 + f^2\,B^2}{f\,B} = \frac{A - f\,A - f\,B + f^2\,B}{f}$$

But, as written above:

$$\frac{1}{Cr} - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)} = 0$$

$$\frac{A - f\,A - f\,B + f^2\,B}{f} - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)} = 0$$

that is $A - f\,A - f\,B + f^2 B - f e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)} = 0$

We can rearrange this equation as a quadratic equation in standard form:

$$B\,f^2 + \left(-A - B - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}\right) f + A = 0$$

As is known, a quadratic equation has two solutions:
$a\,x^2 + b\,x + c$ and the 2 resolutions are given by $x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$

Referring to the standard quadratic equation:
a = B
b = $-A - B - e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}$
c = A
The relative solutions are obtained:

$$f(B) = \frac{\left(A + B + e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}\right) \pm \sqrt{\left(A + B + e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}\right)^2 - 4\,B\,A}}{2B}$$

As is known, a quadratic equation has two values that satisfy it. In this case only one of the two values is possible (the one obtained with the minus sign). The other, the one obtained with the plus sign, turns out to be greater than 1, which is not possible (the fraction is always less than one).

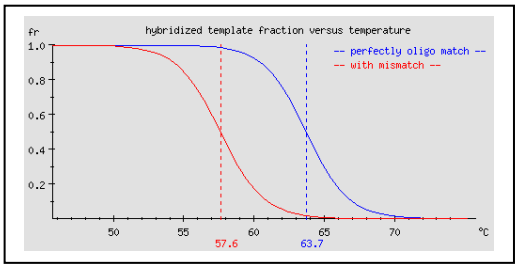
$$f(B) = \frac{\left(A + B + e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}\right) - \sqrt{\left(A + B + e^{\left(\frac{\Delta H - T\,\Delta S}{R\,T}\right)}\right)^2 - 4\,B\,A}}{2B}$$

Equation of the hybridized template fraction as a function of temperature
I remember that A and B are the initial concentrations of the two filaments (B is the lower one)

This is the equation that governs the hybrid fraction as a function of temperature.
Tables and/or graphs can be obtained.
See examples on our platform (biozarivan.it or dna_promix.it) in the Oligo Melting app.

This is an example obtained with Oligo Melting

Fraction of hybridized template as a function of temperature (at equilibrium)




```
5'       ACCTTGACCGATTGAACCCA ->   3'
         ||||||||||| ||||||||
3' nn--  TGGAACTGGCTTACTTGGGT --nn 5'
```

Length oligo = 20 n.GC = 10 (50.00 %) n.mismatch = 1

Template Fraction in duplex form (at equilibrium)

Temp. °C	41.64	43.64	45.64	47.64	49.64	51.64	53.64	55.64	57.64	59.64	61.64	63.64	65.64	67.64	69.64	71.64	73.64
Oligo only match % template in duplex	100.00	100.00	100.00	100.00	100.00	99.98	99.91	99.63	98.52	94.30	80.72	51.85	21.96	6.95	1.97	0.55	0.15
Duplex with mismatch % template in duplex	100.00	99.99	99.97	99.88	99.54	98.21	93.42	78.89	50.00	21.38	6.99	2.06	0.60	0.17	0.05	0.02	0.00

Note: I developed this equation many years ago, but it is difficult to find this equation or other equations cited for determining the fraction of hybridized template.

Useful Notes and Observations: In the next part I will share the experiences gained together with the group of Prof. Giorgio Valle at the Department of Biology of the University of Padua (.pdf)

DNA Hybridization Reaction

Useful Notes and Observations on Annealing Temperature and Time in PCR

dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

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):

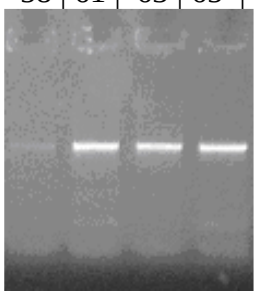
I remind you that the annealing temperature should be set 2-4 °C lower than the primer melting temperature (this is the temperature at which, at equilibrium, 50% of the template should be hybridized to the primers).

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.

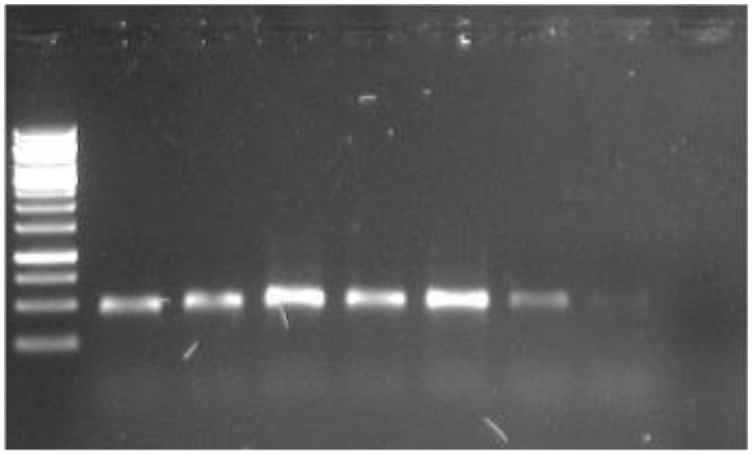
What could be the reasons?
- primers, at low temperatures, can be sequestered by the long template (if many similar regions exist);
- primers form secondary structures (self and hetero dimer or hairpin loop) preferentially at low temperatures;
- the template forms secondary structures in the primer hybridization 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:

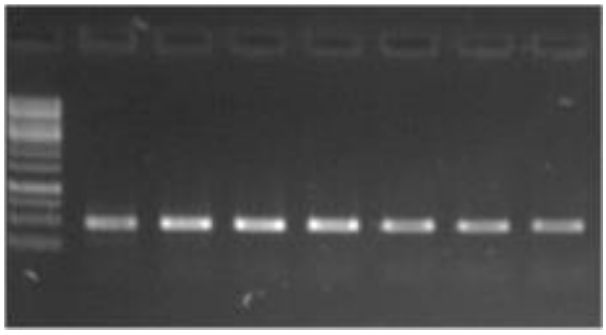
Usually this parameter is not given importance, it is usually set to 20'' - 40''.
From my experiences and from the experiences gained with Dr. Riccardo Schiavon during the HLA project at the Department of Biology of the University of Padua, it turned out that the annealing time should be well evaluated.
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).

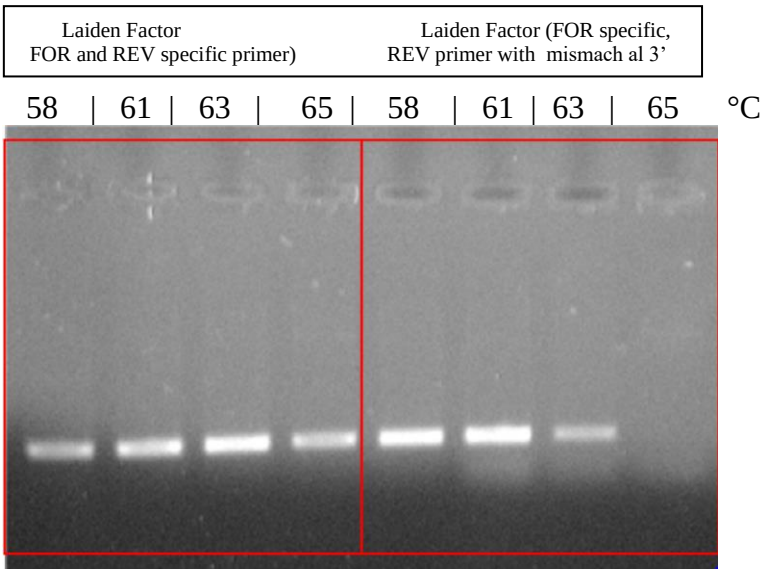
The annealing time to set may also depend on the type of thermal cycler and/or the support used:
The solution inside the well takes a certain amount of time to change temperature and reach equilibrium. This time depends on the speed with which the thermal cycler changes its temperature and the speed 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.

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dr. Ivano Zara (ivano.zara.bio@gmail.com) web: dna_promix.it or biozarivan.it.

Attachment

Some methods used to estimate DNA hybridization reaction and melting temperature

In literature there are many studies to estimate the melting temperature and to estimate the effect of the solutions, here I briefly report some of them

Methods that use content in bases:

- Wallac (1979) Tm=2(A+T)+4(G+C)
- Howley et al. (1979) Tm=81.5+0.41(%GC)-500/N ('N' is the oligo length.)

Methods Nearest Neighbor (determination of enthalpy and entropy changes):

- SantaLucia JJr et al. (1998)
- Breslaur KJ. et al. (1986)

Methods for salt correction (monovalent cation):

- Wetmur (1991): Tm=Tm1+ 16.6 log([Na+]/(1+0.7[Na+])) + 3.85; where 'Tm1' is the melting temperature with [Na+]=1M.
- SantaLucia et al.(1996): Tm = Tm1 + 12.5 log([Na+]; where 'Tm1' is the melting temperature with [Na+]=1M.
- SantaLucia et al.(1998): ΔS = ΔS1 + K (N-1) ln([Na+]) where ΔS1 is ΔS with [Na+]=1M; 'K'=0.368 for ΔS in cal/Mol°K 'K'=1.5397 for ΔS in J/Mol°K;

Methods for determinate Equivalent Sodium ion concentration of Mg2+:

- von Ahsen et al. (2001): [Na+]eq = 3.79 x sqr[Mg2+];
- Nakano et al. (1999): [Na+]eq = 140 x [Mg2+];

Ecc. Ecc.

The use of different studies leads to different Tm estimates (even substantial ones).

On my website (**biozariva.it or dna_promix.it**) in the Oligo Melting app it is possible to compare some of these models and get details by entering sequences of the oligos and composition of the solutions.

In our works, to estimate the thermodynamic parameters, we have adopted the following methods which we have found most reliable and which we have also used in numerous experiments:
Nearest neighbor **SantaLucia JJr et al. (1998)** to determine the thermodynamic parameters (entropy and enthalpy).
For salt (Na+) correction: **SantaLucia et al.(1998)**,
For salt Mg++ correction **von Ahsen et al. (2001**).
Zuker M. (2003) In case of multi-mismatch or terminal mismatches it use Mfold parameter .