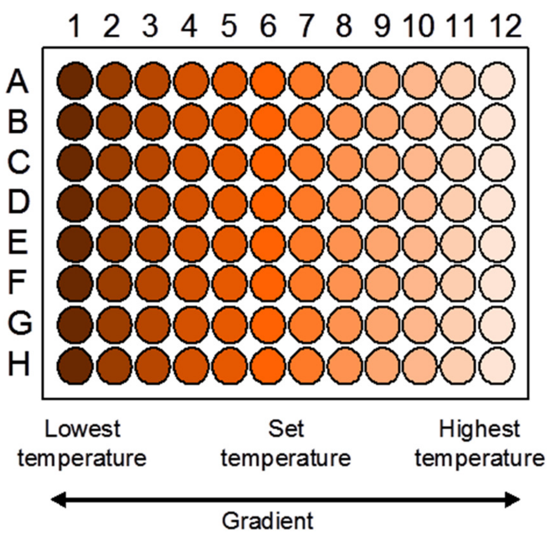
Introduction

The sequence and length of PCR primers generally determine the annealing temperature of the thermal cycling reaction for a specific assay. Although primers are usually supplied with theoretical melting temperatures (T_m), these can be calculated in different ways which may give widely varying values. Using too low an annealing temperature can produce non-specific products whereas if the temperature is too high the PCR yield may be reduced.

An annealing temperature optimisation step can often avoid these problems and is especially important when changing a sensitive assay from one thermal cycler to another. Block tolerances can vary between instruments and small differences in temperature may significantly affect results. Therefore by using the gradient feature of a thermal cycler, the PCR can be optimised for each particular instrument.

Block gradient

When programming a gradient on the 3PrimeG, PrimeG or Prime Elite, the programmed temperature is the temperature in the centre of the block and the gradient is the variation at the two extremes, with the left hand column (column 1) being the coolest and the right hand column (column 12 in a 96-well block) the hottest (Fig. 1).



The Prime is capable of producing a gradient of temperatures across its block by using the four independent heating channels that divide the block into quarters from left to right. The gradient function controls each of these channels at a different temperature, producing a near linear gradient across the block.

The maximum temperature gradient which can be set is dependent on the model and block type with up to 30°C on a 96-well block for the Prime Elite or 14°C on a 3PrimeG.

***Fig. 1**: Gradient spread in a 96-well block.*

Programming a gradient

As a starting point, a gradient should be set up such that it spans the calculated annealing temperature of the primers ±10°C. This range can then be further reduced to fine-tune the assay. This step can be combined with various primer concentration combinations to optimize both annealing temperature and primer concentration simultaneously. The user manuals for the Prime range give instructions on how to program a temperature gradient for a particular step. Samples should be placed in wells across the block so there is at least one sample in each temperature zone.

Once the experiment has ended, the samples can be run and visualised on an agarose gel to assess the results. The optimal annealing temperature and primer combination will give the band with the highest intensity (yield) with no non-specific products.

Fig. 2 illustrates an idealised optimisation experiment using a gradient across a 96-well block. The experiment uses three different primer concentration combinations. In this example it can be deduced that the primer concentrations of combination 2 at the annealing temperature of 58.4°C gives optimal amplification (maximum amount of product with no non-specific amplification).

In reality, primers might be found to work over a considerable temperature range; in which case, the highest temperature should be selected for the annealing temperature to give the most stringent reaction conditions.

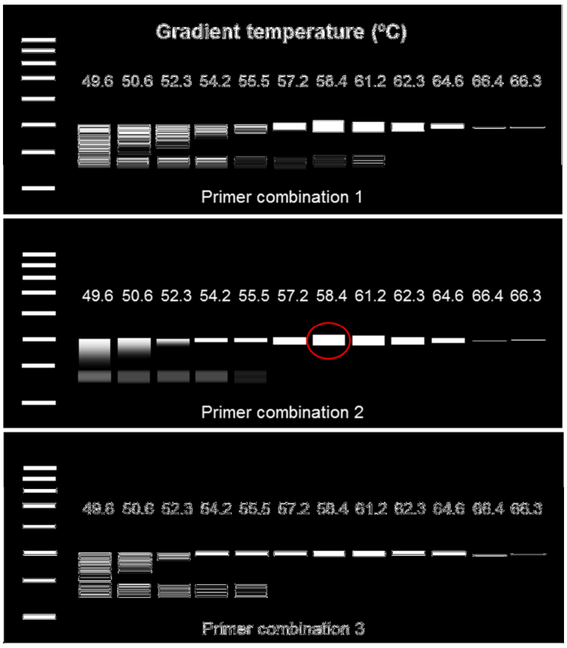


***Fig.2**: Representation of an annealing temperature and primer concentration optimisation experiment. The shadowy, low molecular weight bands are non-specific amplification products.*

The gradient calculator

A gradient calculator is included in the Settings menu of all gradient-enabled Prime units. The calculator is used to display the actual temperatures of the block for any given gradient. While the gradient is mainly linear there are small variations at the extremes of the gradient due to thermal losses from the edges of the block and a gain of heat from the adjacent columns. This is the reason why the first column does not reach the lowest ideal temperature of the gradient and the last column cannot reach the highest temperature.

The values given in the gradient calculator reflect the physical characteristics of the block. The temperatures have been measured empirically to ensure they are correct so, even if they are not quite linear, the value given in the gradient calculator will be the temperature of the corresponding column in the block.

An example of the gradient calculator for the 96-well block is shown in Fig. 3. The gradient is 10°C with a hold temperature of 55°C; the actual block temperatures range from 50.7°C in column 1 to 59.7°C in column 12.

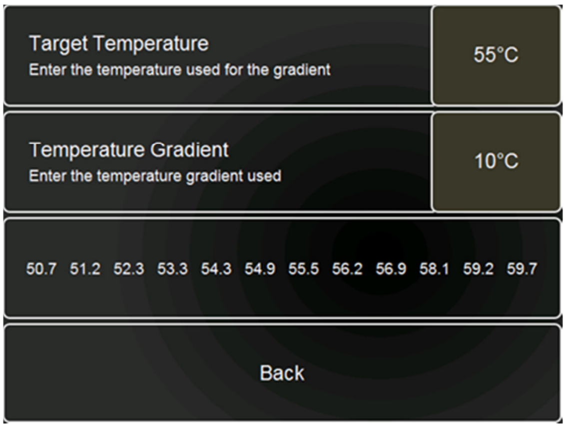


***Fig. 3**: Example of the gradient calculator found in the Settings menu of gradient-enabled instruments.*

Conclusions

The gradient function of the Prime thermal cyclers is a useful feature which can be used to improve the results of a PCR by allowing a simple optimisation step that can be performed in a single run. It could also be used to perform the initial optimisation of a number of different assays with various sets of primers, thus saving the user a significant amount of time.