Protocol P01-001B

Touchdown PCR: Programing decreasing temperatures

Prime

Introduction

One of the most common problems encountered in PCR, especially when amplifying products from genomic DNA, is the presence of non-specific products or primer-dimers. Since these are formed by mis-priming events and are generally shorter than the desired target they can be preferentially amplified at the expense of the target and rapidly overwhelm the reaction. The problem becomes worse if there is only a small amount of target template present in the sample.

In general, the way to overcome amplification of non-specific products is to optimise the PCR. This can include testing various concentrations of the reaction components such as Mg^{2+}, dNTPs, primers and template. However one of the most important parameters is the annealing temperature of the reaction. This can be determined theoretically using various software programmes to calculate the melting temperature (T_m) of the primers. The Prime range of units has an oligonucleotide T_m calculator in the Settings menu for this purpose.

Primer annealing

The T_m is defined as the temperature at which one half of a DNA duplex will dissociate to become single stranded. The annealing temperature for a PCR is usually set at about 4 to 5°C below the theoretical T_m of the primers and ideally, the two primers used in a reaction should not differ in T_m by more than 5°C.

However the behaviour of primers in the actual reaction is difficult to predict. A rapid way to determine the ideal annealing temperature is to use a temperature gradient across the thermal cycler block for the annealing step. The PCR products are then run on an agarose gel to visualise the bands. The temperature of the block column giving the best yield of specific product can be determined by viewing the gradient calculator on the instrument. This temperature is then used as the annealing temperature for subsequent PCRs with these primers. The Techne 3PrimeG, PrimeG and Prime Elite all have gradient blocks which will allow the user to do this.

Without a gradient block, annealing temperature optimisation can be a lengthy process, requiring repeated runs to test each different annealing temperature. However there is a method that can be used to increase the specificity of the reaction without actually determining the optimal annealing temperature. This method is known as touchdown PCR[1].

Touchdown PCR

In touchdown PCR the annealing temperature is gradually decreased during the cycling process. At the beginning of the cycling stage, the annealing temperature is set 5 to 10°C higher than the T_m of the primers. While the temperature is high, this favours only the most specific base pairing between the primer and template and therefore only specific products will be amplified.

In subsequent cycles the temperature is decreased in small amounts so that by the end of the amplification stage, the annealing temperature is 2 to 5°C below the T_m. Since the specific products have already been amplified and are present in excess, these will be preferentially amplified at the lower, more permissive annealing temperatures.

Programming might at first appear complex; however this is made simple with the increment/decrement time/temperature feature available on all Techne thermal cyclers.

Increment/decrement time/temperature

Under normal circumstances, the hold temperature of the various steps remains constant throughout a stage. However, it is possible to automatically increment or decrement the temperature of a specified step of a cycled stage. To do this, it is simply a matter of activating the increment/decrement function and setting the temperature decrease or increase per cycle. Note: this feature cannot be set unless the stage has >1 cycle programmed.

The final annealing temperature can be calculated as follows:

Final temperature (°C) = Initial temperature (°C) + [(Number of cycles -1) x ΔT(s)]

Where ΔT(s) is the temperature increment/decrement in s.

Increment/decrement time works in a similar way except that the hold time is increased or decreased with each cycle. This can be useful in applications such as long PCR.

Programming for Touchdown PCR

The following steps demonstrate how to program the 3Prime, Prime and Prime Elite thermal cyclers for touchdown PCR.

3Prime

1. First ensure the number of cycles for the stage has been set then enter the required hold temperature for the step.
2. Touch the step **Temp** button. This will open the temperature screen (Fig. 1).
3. Touch **Increments and Decrements**.

The increment, decrement and gradient (3PrimeG only) screen will open.

4. Touch the button next to the required parameter to turn it **ON**.
5. Next touch the temperature button to set the increment or decrement per cycle (Fig. 2).

A new screen will open which is similar to the simple temperature entry screen.

6. Enter the required value followed by **Back**.

If an entered temperature value is invalid, a prompt will indicate the acceptable range.

7. Touch **Accept** to return to the previous screen.
8. Touch **OK** to accept the modification and return to the program.

The temperature value will now appear orange in the programming screen indicating that it contains a modified function (Fig. 3).

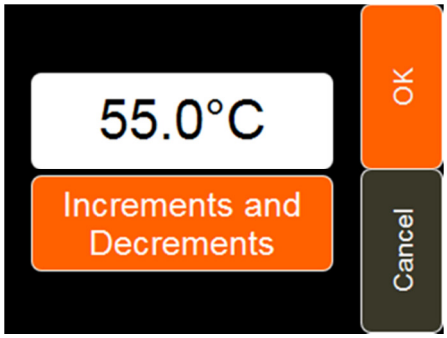

Fig. 1: Increments and Decrements.

Fig. 2: Turn on Decrease Per Cycle.

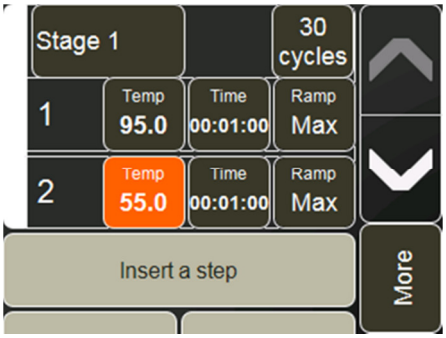

Fig. 3: Modified step shown in orange.

Prime and Prime Elite

1. First ensure the number of cycles for the stage has been set then enter the required hold temperature for the step.
2. Touch the step **Temperature** button. This will open the temperature entry screen (Fig. 4).
3. For a decrease in temperature per cycle, touch the button below **Decrease Per Cycle** to turn this function **ON**.
4. Touch the temperature entry button below this.

A temperature entry screen will open. The maximum decrease in temperature per cycle (based on the number of cycles and the temperature limits of the unit) will be shown (Fig. 5).

5. Enter the value required followed by **OK**.

If an entered temperature value is invalid, a prompt will indicate the acceptable range.

6. Touch **OK** again to return to the programming screen.

The temperature value will now appear orange in the programming screen indicating that it contains a modified function (Fig. 6).

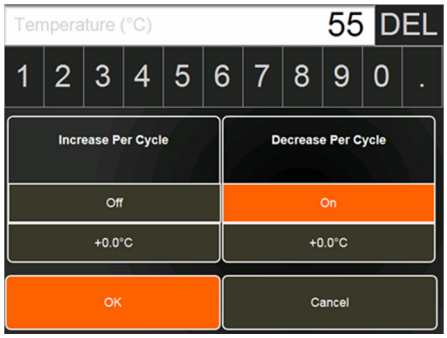

Fig. 4: Increments and Decrements.

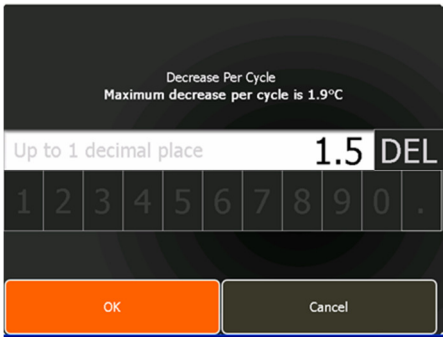

Fig. 5: Enter the required Decrease Per Cycle.

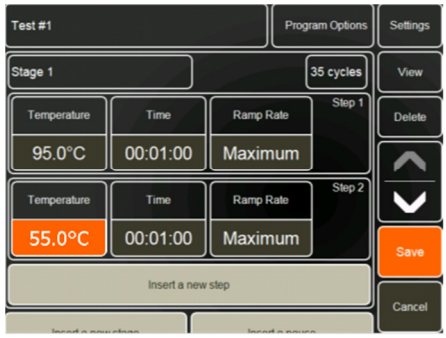

Fig. 6: Modified step shown in orange.

Conclusions

All Techne thermal cyclers have flexible programming options which allow the user to set up just about any thermal cycling profile. The increment/decrement time and temperature features simplify programming for complex applications such as touchdown PCR, allowing the user to easily edit existing programs with these features.

References

1. R.H. Don, P.T. Cox, B.J. Wainwright, K. Baker and J.S. Mattick. “Touchdown” PCR to circumvent spurious priming during gene amplification. *Nucl. Acids. Res*: **19**, 4008.