



Long PCR: using the increment time programming feature of Techne thermal cyclers

■ Introduction

Many applications in PCR are simply to identify the presence or absence of a particular target in a complex pool of DNA. This usually requires amplification of only a short fragment of the DNA for identification purposes, usually between 100 and 500bp in length. However in some cases it may be required to amplify longer segments of DNA, or even complete genes, in order to clone them and determine their function. In such instances it may be necessary to amplify lengths of 5 to 10 kb or even longer. This is known as long PCR.

■ Long PCR

As well as amplifying long stretches of DNA, long PCR also needs to be accurate. For this reason, many protocols use a mix of thermostable DNA polymerases, usually *Taq* DNA polymerase for high processivity and another DNA polymerase such as *Pfu* or *Pwo* with 3'-5' proofreading ability. Proofreading polymerases remove any misincorporated bases which may halt extension of the PCR product, therefore allowing longer primer extension than can be achieved with *Taq* alone.

Due to the use of proofreading polymerases in the reaction, the enzyme must not be mixed with the primers until immediately before thermal cycling as it will degrade them. Alternatively, a hot start procedure can be used whereby the polymerase is added either during a programmed hot start step or during the first annealing/extension step.

Primers for long PCR tend to be slightly longer than those for standard PCR (24 to 30 bases). This allows for a higher annealing temperature in the PCR and a combined annealing/extension step at 68°C is often used. Due to the size of the product, long annealing/extension times are required. This is generally around 25 to 35s per kb in the first 10 to 15 cycles. In the subsequent 15 to 20 cycles the extension time is increased by 15 to 20s per cycle to allow for the increased amount of product. This can be programmed using the increment time feature of the Techne thermal cyclers.

■ Increment/decrement time/temperature

Under normal circumstances, the hold time of a particular step is constant for each cycle within a stage. However, it is possible to automatically increment or decrement the hold time of a specified step of a cycled stage. To do this, an initial hold time and a final hold time need to be defined for the step. During the cycling stage the time will increase or decrease by an amount depending on the number of cycles in the stage.

For example, if the initial hold time of a step is 1 min and the final hold time is 6 min and there are 11 cycles, in the first cycle the step will have a hold time of 1 min (the first cycle is never incremented/decremented, only subsequent cycles); in the second cycle, the step will have a hold time of 1 min 30s and so on until the 11th cycle in which the step will have a hold time of 6 min. Therefore the time increment or decrement per cycle (ΔT) is given by:

$$\Delta T = (\text{Final hold time} - \text{Initial hold time}) / (\text{Number of cycles} - 1)$$

Or to calculate the final hold time:

$$\text{Final hold time} = \text{Initial hold time} + [(\text{Number of cycles} - 1) \times \Delta T]$$

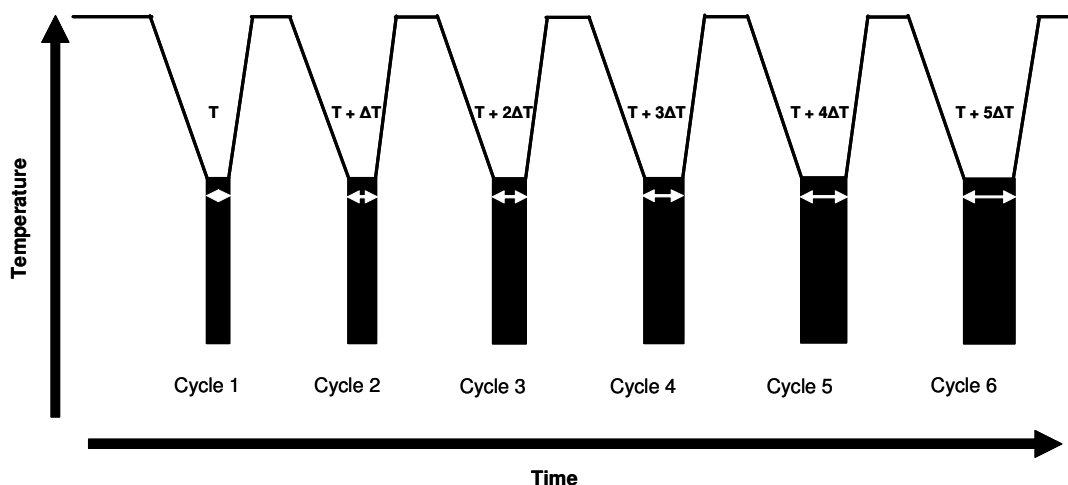


Figure 1: Incremented time function.

Increment/decrement temperature works in a similar way except that the temperature of a step is increased or decreased with each cycle. This can be useful in applications such as touchdown PCR.

■ Programming for Long PCR

The following examples demonstrate how to program the various Techne thermal cyclers for a typical long PCR protocol with a combined annealing/extension step.

TC-5000 and TC-512

1. Open the 2 STEP PCR TEMPLATE and touch EDIT.
2. Adjust the default parameters as required. If a hot start is required for addition of the polymerase mix, touch 'Hot start', set to 'Enabled' and set the required temperature. Note that this acts as a pause step and user intervention is required to continue the cycling program.
3. Set the number of cycles for the first amplification stage. This is often 10 to 15 cycles.
4. Touch the first segment of the amplification stage to edit the required denaturation temperature and hold time. For example, 94°C for 10 to 20s.
5. Touch the second segment of the amplification stage to edit the required annealing/extension temperature and hold time. This is often 68°C for 25 to 35s per kb.
6. Touch 'Next step'. In the 'Edit program function' screen select 'Number cycles'.
7. Set the number of cycles for the second amplification stage. This is often 15 to 20 cycles.
8. Touch 'Next step'. In the 'Edit program function' screen select 'Segment'.
9. Edit the required denaturation temperature and hold time. For example, 94°C for 10 to 20s.
10. Touch 'Next step'. In the 'Edit program function' screen select 'Inc/Dec'.
11. On the top line where it says 'First' enter the annealing/extension temperature e.g. 68°C.
12. Next, enter the starting hold time (e.g. 25 to 35s per kb).
13. On the next line where it says 'Last' enter the annealing/extension temperature e.g. 68°C.
14. Next enter the final hold time e.g. 25 to 35s per kb + [(number of cycles -1) x required increment per cycle*]. *Often 15 to 20s per cycle.
15. Edit the remaining defaults, such as final extension, as required.
16. Touch 'Save As' to save and give the program a name.

TC-4000, TC-412, TC-3000, TC-3000X and TC-3000G

1. Open the 2 STEP PCR TEMPLATE and select 'Copy program'. Give the program a suitable name.



2. Adjust the default parameters as required. If a hot start is required for addition of the polymerase mix, select 'Hot start' and set the required temperature. Note that this acts as a pause step and user intervention is required to continue the cycling program.
3. Set the number of cycles for the first amplification stage. This is often 10 to 15 cycles.
4. Edit the first step of the amplification stage to the required denaturation temperature and hold time. For example, 94°C for 10 to 20s.
5. Scroll down to the second step and edit the required annealing/extension temperature and hold time. This is often 68°C for 25 to 35s per kb.
6. Scroll down to the flashing dotted line. Press Enter to insert a new stage.
7. Enter the number of cycles for the second amplification stage. This is often 15 to 20 cycles.
8. Scroll down the next step and edit the required denaturation temperature and hold time. For example, 94°C for 10 to 20s.
9. Press the decimal point key to insert a new step.
10. Next, press the pause key. The prompt at the bottom of the screen will ask if you want to change step. Press the enter key for yes.
11. The cursor will flash at the beginning of the step. Press the up or down arrow key until you see 'fst' which indicates the first or starting annealing/extension hold time.
12. Enter the annealing/extension temperature e.g. 68°C followed by the starting hold time (e.g. 25 to 35s per kb).
13. Press enter until the line flashes then scroll down to the next line which begins with 'lst' which indicates the last or final annealing/extension hold time.
14. Enter the annealing/extension temperature e.g. 68°C followed by the final hold time e.g. 25 to 35s per kb + [(number of cycles -1) x required increment per cycle*]. *Often 15 to 20s per cycle.
15. Press enter until the line flashes then scroll down to edit the remaining defaults, such as final extension, as required.
16. When finished, press the end key to save and confirm by pressing the enter key.

■ 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 long PCR, allowing the user to easily edit existing programs with these features.