Using the *in situ* PCR adapter

Introduction

In situ PCR is a technique in which a thin tissue section fixed on a glass slide is subjected directly to PCR to determine loci of specific targets within the tissue. Previously, to perform this application, it has been necessary to purchase an additional thermal cycler or new thermal block specifically for this technique, often at significant expense. The Techne *in situ* PCR adapter (part code PRIME/ISHA) can be used to convert a 96-well Prime or PrimeG gradient thermal cycler to an *in situ* instrument as simply as inserting a PCR plate. The adapter accommodates up to four standard glass microscope slides.

PRIME/ISHA

The *in situ* adapter is a single piece of machined aluminium designed to fit closely in a 96-well thermal cycler block. The flat surface is 3mm thick aluminium which allows efficient and uniform temperature transfer from the block. The design includes corner cut-outs to allow easy removal of slides and an aluminium perimeter ridge which permits the thermal cycler lid to be closed without risking damage to glass slides and PCR samples.

In this application note we look at the thermal profile of the adapter surface in comparison to the 96-well thermal cycler block with a view to recommending ways of optimising PCRs to achieve the required temperature changes whilst taking into account the increased thermal mass of the adapter.

Methods

For all tests, the adapter was placed in the 96-well block of a Prime thermal cycler. Three K-type thermistors were attached to the surface of the adapter across the centre as shown in Fig. 1. The lid was closed to maintain temperature uniformity but was not heated during the tests. Data from the thermistors was collected using a TC-08 Pico Log recorder (Pico Technology Limited), collecting readings at 1 second intervals.

Three experiments were performed. In the first, the adapter was heated through a series of temperatures to check the temperature accuracy of the surface compared to the thermal cycler block. In the second and third, typical 2-step and 3-step PCR programs were run and then modified in an attempt to achieve the required temperature changes and hold times when using the adapter.

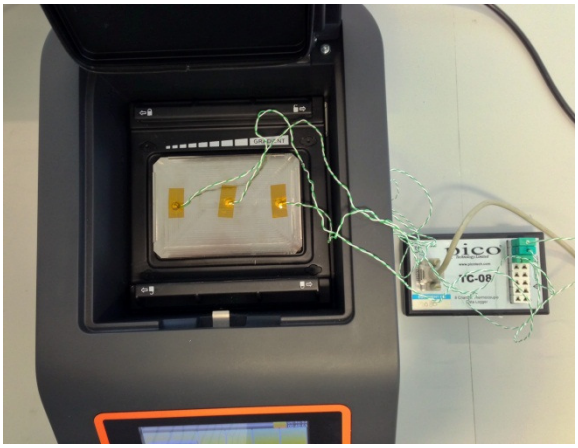

Fig. 1: Set up and positioning of the thermistor probes on the surface of the adapter plate.

Results

Temperature accuracy

The thermal cycler was programed as follows: 90°C for 5 min; 70°C for 5min; 50°C for 5 min; 30°C for 5 min; 10°C for 5 min; 30°C for 5 min; 50°C for 5 min; 70°C for 5 min; 90°C for 5 min: 10°C for 5 min and 90°C for 5 min. This program was designed to see how accurate the surface temperature of the adapter plate is compared to the thermal cycler block when cooling or heating from one temperature to another. The profile of temperature changes recorded by the Pico Log is shown in Fig. 2 and summarised in Table 1.

Block programmed temperature (°C)	90.0	70.0	50.0	30.0	10.0	30.0	50.0	70.0	90.0	10.0	90.0
Adapter measured temperature (°C)	83.5	67.1	49.5	31.3	12.5	29.5	47.5	66.2	85.1	14.0	83.9

Table 1: Block programmed temperatures and adapter surface temperatures measured using K-type thermistor probes. The values shown for the adaptor are those achieved at the midpoint of the holding time and are the averages of the three thermistors. The error of the probes is ±0.5°C.

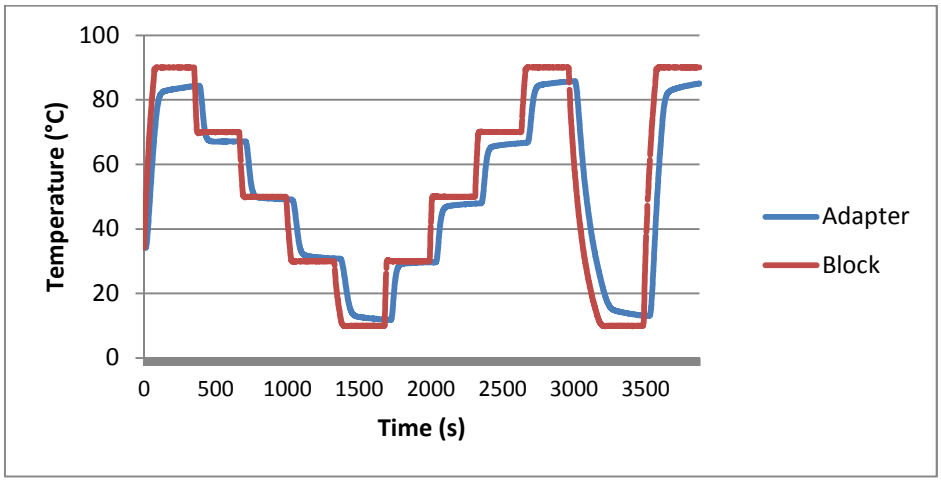

Fig.2: Temperature profile of the thermal cycler block obtained from the temperature log data (red) and average temperature measurements made on the top surface of the adapter (blue).

The results indicate that the surface of the adapter was not able to achieve the highest temperature tested (90°C) within the 5 minute incubation period. This is most likely due to heat loss from the large surface area. When ramping down to lower temperatures, in general the adaptor remained slightly above the block temperature as the heat did not have sufficient time to dissipate completely. When ramping up in temperature the adapter lagged behind the block slightly and did not quite reach the programmed temperature during the incubation period. These results indicate that some changes may need to be made to thermal cycling programs in order to account for this behaviour and to achieve the required incubation temperatures and hold times for a PCR.

2-step PCR program

The Prime thermal cycler was programmed with a 2-step cycling program as detailed in Table 2. The surface temperatures of the adapter were measured and compared with the expected temperature profile. Ten cycles of the cycling stage were performed and the average values for each cycle are given in Table 2.

		Program	Required	Actual
Initial denaturation	Temperature (°C)	95.0	95.0	89.9
	Hold time (s)	300	300	250
Cycling (x 10)	Temperature (°C)	95.0	95.0	87.3
	Hold time (s)	15	15	13
	Temperature (°C)	58.0	58.0	59.3
	Hold time (s)	30	30	17

Table 2: Required and actual temperatures and hold times achieved by the adapter. Where a temperature is given, the value was the maximum (95°C step) or minimum (58°C step) temperature achieved. The hold time was calculated as the length of time the surface temperature remained within 1°C of the actual temperature.

Considering these values, in order to achieve the required temperatures at the surface of the adapter it is clear that certain program modifications need to be made. In this case the program was modified such that the initial denaturation and denaturation step temperatures were increased to 100°C and the annealing temperature decreased by 1°C. Hold times were also increased by 15 seconds. Table 3 gives details of the modified program and the results from these modifications.

		Program	Required	Actual
Initial denaturation	Temperature (°C)	100.0	95.0	95.3
	Hold time (s)	300	300	183
Cycling (x 10)	Temperature (°C)	100.0	95.0	94.7
	Hold time (s)	30	15	18
	Temperature (°C)	57.0	58.0	57.6
	Hold time (s)	60	30	38

Table 3: Modifications to the thermal cycling program designed to achieve temperatures and hold times similar to the required thermal cycling profile. Temperatures and hold times were calculated as described in Table 2.

As Table 3 illustrates, making a few simple changes produced a thermal cycling profile similar to that which would be expected if running the reactions in the block itself. Fig. 3 shows the measured adapter temperature profiles before and after the program modifications.

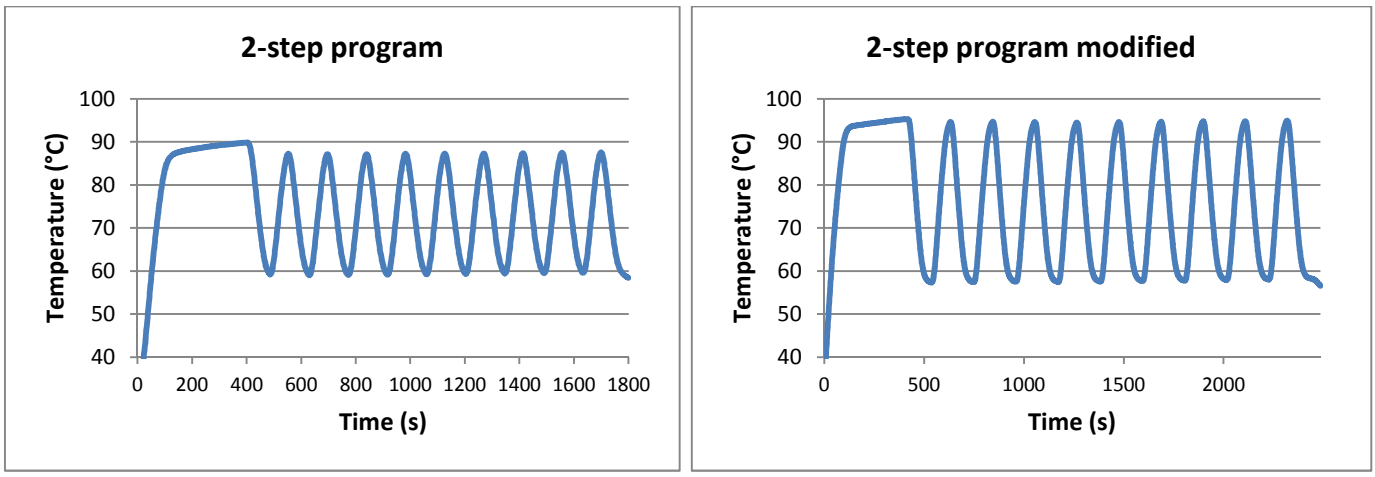

Fig 3: Temperature profiles of the adapter surface when running the unmodified and modified 2-step thermal cycling programs.

3-step PCR program

In a similar test to the 2-step program, the Prime was programmed to run a typical 3-step cycling program as detailed in Table 4, followed by a modified program (Table 5) in an attempt to reproduce more accurately the required thermal cycling profile.

		Program	Required	Actual
Initial denaturation	Temperature (°C)	95.0	95.0	89.2
	Hold time (s)	300	300	147
Cycling (x 10)	Temperature (°C)	95.0	95.0	87.7
	Hold time (s)	15	15	13
	Temperature (°C)	55.0	55.0	55.9
	Hold time (s)	30	30	17
	Temperature (°C)	72.0	72.0	Variable
	Hold time (s)	30	30	5*

Table 4: Required and actual temperatures and hold times achieved by the adapter. Where a temperature is given, the value was the maximum (95°C step) or minimum (55°C step) temperature achieved. The hold time was calculated as the length of time the surface temperature remained within 1°C of the actual temperature. *For the 72°C step, the hold time given is the time the temperature remained between 71°C and 73°C.

		Program	Required	Actual
Initial denaturation	Temperature (°C)	100.0	95.0	95.8
	Hold time (s)	300	300	198
Cycling (x 10)	Temperature (°C)	100.0	95.0	94.6
	Hold time (s)	30	15	18
	Temperature (°C)	54.0	55.0	56.0
	Hold time (s)	45	30	27
	Temperature (°C)	73.0	72.0	Variable
	Hold time (s)	45	30	20*

Table 5: Modified 3-step thermal cycling program. Temperatures and hold times were calculated as described in Table 4. *See Table 4.

Since the 72°C extension step was continually changing in temperature (no steady 72°C was achieved), the hold time was taken to be the time the temperature remained between 71°C and 73°C. Modifying the program as shown in Table 5 improved the hold times and denaturation temperature but the annealing temperature was still a little too high. Fig. 4 shows the corresponding temperature profiles measured at the adapter surface for the unmodified and modified 3-step programs.

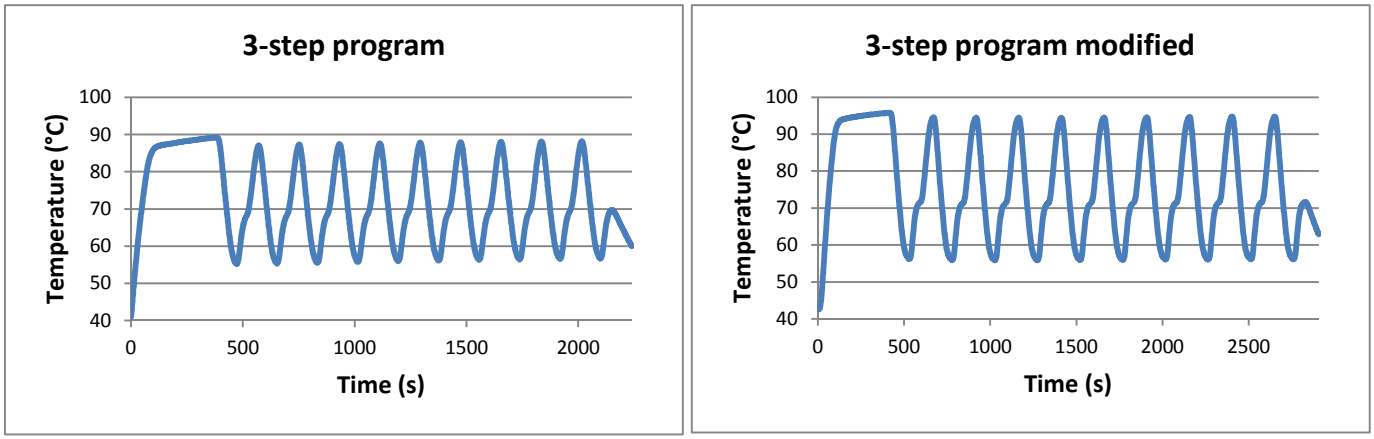

Fig 4: Temperature profiles of the adapter surface when running the unmodified and modified 3-step thermal cycling programs.

Conclusions

It is important to appreciate that, due to the additional thermal mass of the adapter, when it is used in a block existing PCRs will require optimization in order to achieve similar thermal cycling profiles. The adapter itself will take slightly longer to equilibrate in temperature and due to the large surface area, at higher temperatures natural heat loss will mean that it cannot achieve the same temperature as the block. Likewise, cooling will take longer as the heat cannot dissipate as easily. To account for these features, the following guidelines are recommended when optimizing the PCR:

- Increase the block temperature for initial denaturation and denaturation steps to 100°C to achieve 95°C.
- When cooling to an annealing step, program the annealing temperature approximately 1°C to 2°C lower than usual.
- Add approximately 15 seconds to each hold time to allow the adapter to reach and hold the required temperature for the required length of time.