Establishing PCRs successfully – how advanced gradient thermal cycler technology will help to achieve efficient and robust amplification

INTRODUCTION

The importance of PCR protocol optimisation is obvious, not only during a pandemic, when there is high pressure to develop effective qPCR diagnostic kits. To achieve reliable results, emphasis is put on using high quality reagents, consumables and instruments, also primer sequences are selected carefully. Still a PCR might fail resulting in either no detectable or unspecific amplification. To succeed in establishing PCRs that robustly perform within a wider scope of template quality and quantity, optimisation of the PCR temperature protocol is crucial. Probably most important in this context is the determination of the optimal annealing temperature.

CALCULATING ANNEALING TEMPERATURE T_A

Identifying an annealing temperature (T_a) that will probably work isn't too complicated. As a common rule T_a should be about 3 to 6 °C lower than the melting temperature (T_m) of the primers [1,2] which defines the temperature at which 50% of complementary DNA strands will be dissociated. T_m depends on primer length and composition (see info box for design recommendations) and it can be calculated by rather simple equations [e.g. T_m = 2 °C * (A+T) + 4 °C * (G+C)] or more precise algorithms based on thermodynamics [3, 4].

Also, modern PCR instruments like the VWR family of thermal cyclers are equipped with software tools to calculate T_m reasonably accurately.

GENERAL RECOMMENDATIONS FOR PCR PRIMER DESIGN

- Primer length 18 to 30 bases
- GC content 40 to 60%
- 3' end of a primer G or C to ensure binding
- ΔTm of both primers ≤4 °C
- Tm 60 to 75 °C
- No ≥4 runs of one base
- No relevant (≥3 bases) of intra- and inter-primer homology to avoid self-annealing/primer dimers, especially at 3' end

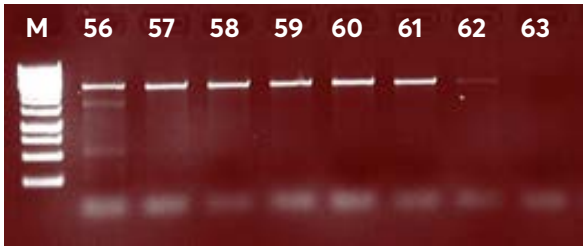

FIGURE 1: Assessing effect of 8 different annealing temperatures on PCR product yield and specificity via agarose gel electrophoresis.

GRADIENT PCR FOR T_A IDENTIFICATION

Having T_m calculated and applying the common rule to subtract ~5 °C from it is a good starting point, but how do you identify the T_a that will deliver maximum specificity and yield in practice, meaning under the specific conditions of an individual PCR? On complex templates like large plant genomes, there will be a higher chance for primers hybridising to non target sequences. Also, the higher the salt concentration within the PCR reaction, or the stronger the processivity of the DNA polymerase, the higher the likelihood of unspecific amplification. Increasing T_a will help to ensure specificity in such a situation. In contrast, when amplifying an insert from a simple plasmid template, T_a might get lowered to increase PCR yield while specificity won't be a problem. However, beside all theory, the straightest way is to determine the optimal T_a empirically by applying different T_a values and checking PCR products via agarose gel electrophoresis (Figure 1).

To test the effect of applying several different T_a values within a certain temperature range in a time saving parallel manner, gradient PCR is the method of choice. A PCR instrument offering gradient functionality won't always have the same temperature across the entire thermal plate but can be programmed to realise different temperatures in different areas of the thermal plate. A basic approach to achieve this is to apply the highest temperature on one half of the thermal plate and the lowest temperature on the 2nd half of the thermal plate. Depending on the temperature conductivity of the thermal plate and influenced by edge effects a temperature 'gradient' of mixed temperatures will establish in between (Figure 2A).

GRADIENT THERMAL CYCLER TECHNOLOGY

Within a thermal cycler, Peltier elements attached to the bottom of the thermal plate are responsible for heating or cooling. Building a temperature gradient will at least need two Peltier elements, each owning an individual temperature probe and control circuit. In practice, because of Peltier element size constraints, often six elements can be found underneath a 96-well thermal plate, with three of them being combined, sharing the same temperature probe/control circuit to reduce manufacturing costs (Figure 2A).

Gradient PCR cyclers using this basic principle of two control circuits might be economical, however, temperature measurement reveals one relevant drawback: Connecting the temperatures of the individual rows of the thermal plate results in a sigmoid curve.

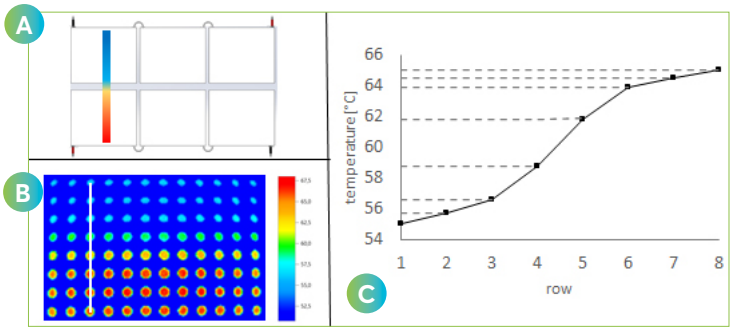

FIGURE 2: The basic approach 2A) Schematic drawing of arrangement of 6 Peltier elements, 3 Peltier elements each set in series in 2 distinct control circuits; colour bar indicates course of temperature gradient. 2B) Thermal image of gradient PCR on 96-well plate, white line indicates wells where measurement was taken. 2C) Diagram of temperatures measured during gradient PCR.

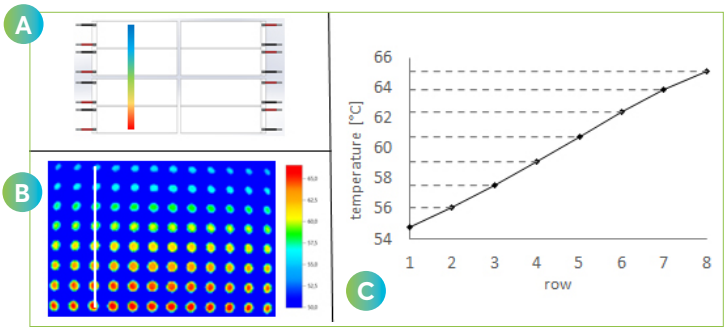

FIGURE 3: The VWR® XT96/XTender96 approach. 3A) Schematic drawing of arrangement of 8 Peltier elements, each with an individual control circuit; colour bar indicates course of temperature gradient. 3B) Thermal image of gradient PCR on 96-well plate, white line indicates wells where measurement was taken. 3C) Diagram of temperatures measured during gradient PCR.

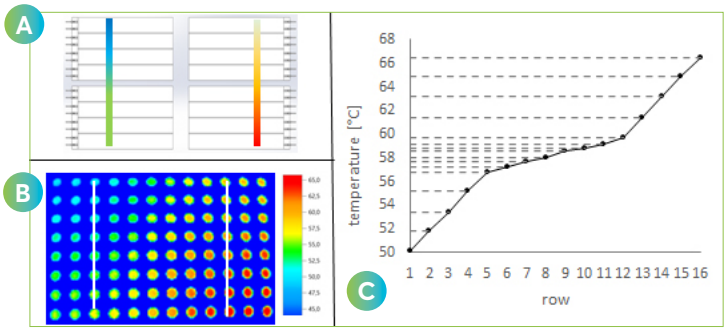

FIGURE 4: The VWR® UNO96 approach. 4A) Schematic drawing of the arrangement of 16 Peltier elements, each with an individual control circuit; colour bar indicates course of temperature gradient. 4B) Thermal image of gradient PCR on 96-well plate, white line indicates wells where measurement was taken. 4C) Diagram of temperatures measured during gradient PCR after intentionally increasing the resolution around the selected center temperature.

It becomes more obvious that ΔT from one well to the next in the centre of the thermal plate is much larger than towards the edges (Figure 2C). Thus, the different T_a values that can be tested at once are unevenly spread and there is a lack of information/resolution in the centre of the plate. However, it is the centre which is of special interest as it represents calculated T_a and temperatures close to it have the highest chance to turn out as best T_a in practice.

ADVANCED TECHNOLOGY

Advanced gradient PCR cyclers will own more than two control circuits to turn the sigmoid curve more linear, resulting in individual temperatures that are more evenly spread over the rows of the thermal plate. Still economical, the XT-family of VWR thermal cyclers is equipped with eight peltiers that are individually controlled by eight control circuits, with four of them being responsible for gradient temperature generation. The result is a very linear gradient enabling for a much more efficient identification of optimum T_a (Figure 3).

While a linear temperature gradient curve identifies the premier league of gradient PCR cyclers, couldn't it still be better? What if the temperature of each row could be controlled separately by its own Peltier element/control circuit? VWR® UNO96 Gradient provides the answer.

16 Peltier elements, each individually controlled, allow for the definition of 16 different temperatures at once (Figure 3A and B). Thus, not only twice the number of different T_a values can be tested per run, but even the temperature distribution over the thermal plate can be changed, e.g. to increase the resolution close to calculated T_a (Figure 3C). In addition to offering todays most advanced gradient PCR functionality for establishing new PCRs, VWR® UNO96's sophisticated temperature control regimen allows for running different PCR protocols with distinct T_a values at the same time.

CONCLUSION

Although supporting an established methodology, gradient PCR cyclers can differ a lot. As a rule of thumb, a higher number of control circuits that are used to adjust the temperature within the thermal plate will enable researchers to establish more robust PCR protocols in a shorter time.

Manufactured in Germany and equipped with up to 16 Peltier element control circuits, the VWR family of PCR thermal cyclers defines the highest standard of gradient PCR technology.

XT96

DOPPIO 96

UNO96

Ordering information

Description	Cat. No.
VWR UNO96 Gradient with 96-well universal block	732-2549
VWR Doppio Gradient with 2x48-well blocks	732-2552
VWR XT[96] Gradient with 96-well block	732-3428
VWR XTender[96] Gradient with 96-well block	732-3658

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