PCR Types Performed by the xxpress® Thermal Cycler

Since the polymerase chain reaction (PCR) was first described by Kary Mullis in 1983, the process has been greatly developed and modified, originating a new range of PCR techniques and applications. This white paper investigates the wide variety of techniques that utilise PCR and demonstrates how the speed and accuracy of the xxpress® qPCR thermal cycler makes it ideal for performing these methods.

1. End-Point PCR

In end-point or standard PCR, millions of copies of a region of interest are created using specific temperature cycles. Unlike in qPCR, the amplified product can only be detected once the reaction has completed, using downstream processing techniques such as electrophoresis analysis to characterise the amplicon by its size. A major drawback for standard PCR is that it is not quantitative, because the final yield of product is not primarily dependent upon the concentration of the target sequence in the sample. For this reason, this technique is used mostly to amplify specific DNA for use in other molecular techniques such as sequencing or cloning (Logan, Edwards and Saunders, 2009).

Although the xxpress® is a qPCR thermal cycler, it can also perform end-point PCR by turning off the xxpress® optics system, as no fluorescent molecule is added to the reaction.

2. Real-Time PCR

Real-time PCR detects and measures the accumulation of amplified product as the reaction progresses. This is achieved by including a fluorescent molecule in the reaction that reports the increase in the amount of DNA with a proportional fluorescent signal. It can provide both qualitative, showing the presence or absence of the DNA sequence of interest, and quantitative analysis, determining the initial DNA concentration with accuracy and high sensitivity. It also eliminates the necessity for post-PCR manipulation, which reduces the risk of contamination and saves a considerable amount of time.

There are two general approaches to obtain a fluorescent signal from the synthesis of product: the use of non-specific DNA intercalating dyes, such as SYBR Green I and EvaGreen, or the use of sequence specific oligonucleotide probes (Kubista *et al.*, 2006; Logan, Edwards and Saunders, 2009; Navarro *et al.*, 2015).

Intercalating dyes bind to the minor groove of the double-stranded DNA, which causes an increase in their fluorescence, allowing it to be detected in the extension phase of each cycle of the PCR (Figure 1). Its main advantage is the reagents are much cheaper than those used for probe-chemistries (Navarro *et al.*, 2015).

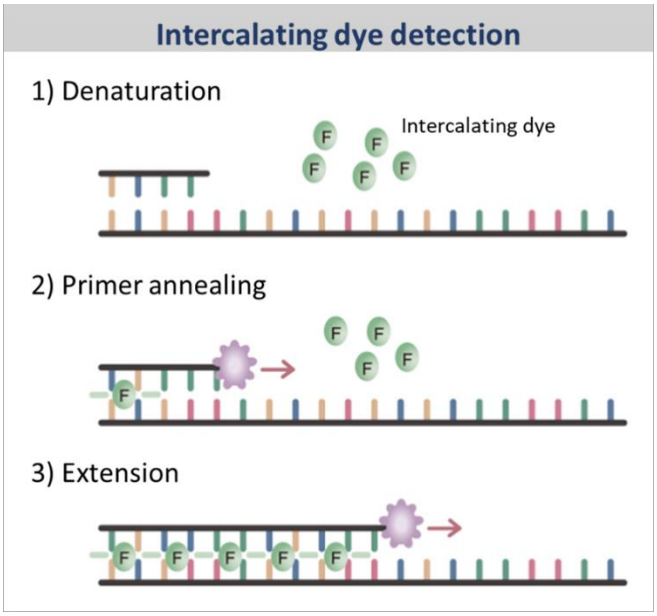

Figure 1. Mechanism of intercalating dye in a qPCR reaction. Modified from http://genomictree.com.

Figure 2 shows a PCR graph of a standard dilution series performed with KAPA SYBR Fast (a master mix including the intercalating dye SYBR Green) on the xxpress® thermal cycler.

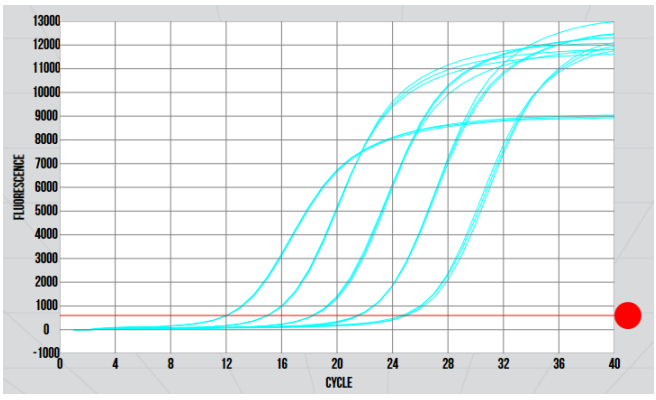

Figure 2. PCR reaction performed on the xxpress® using the intercalating dye SYBR Green and 10-fold DNA dilutions from 100 ng to 0.01 ng. Efficiency: 105%; R^2: 0.999.

Fluorophore-labelled oligonucleotides can be classified in three different types: primer-probes, which are oligonucleotides that combine a primer and a probe in a single molecule; probes, which have a donor and/or acceptor fluorophore attached and can also classify on

hydrolysis and hybridization probes; and finally, the nucleic acid analogues, that are similar in structure to naturally occurring RNA and DNA (Navarro *et al.*, 2015). Figure 3 represents the mechanism of action of the most used qPCR probes, Taqman probes, belonging to the hydrolysis group.

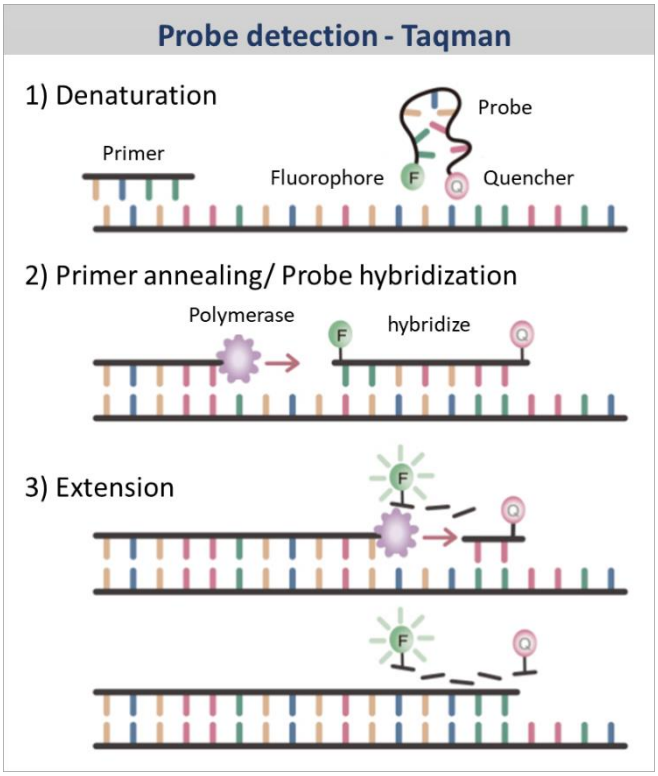

Figure 3. Mechanism of Taqman probe in qPCR. Modified from http://genomictree.com.

In Figure 4 there is an example of a PCR reaction performed with a fluorophore-labelled probe using the xxpress®. The fluorophore used for these reactions was FAM, measured in the optical channel 1 of the xxpress® instrument.

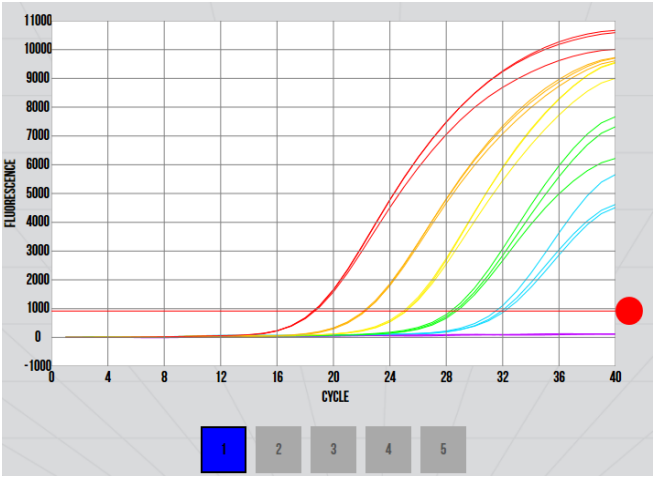

Figure 4. PCR reaction performed on the xxpress® using a hybridisation probe marked with the fluorophore FAM, and DNA 10-fold dilutions from 50 ng to 0.005 ng. Efficiency: 102%; R^2: 0.998.

3. Three-Step vs. Two-Step PCR

Typically, a PCR cycle is composed of three steps: a 95°C step to denature the DNA double helix and separate the strands; an annealing step at around 55°C to allow the primers to bind to the single-stranded DNA templates; and an extension step at 72°C, the optimum temperature for the Taq polymerase to synthesise a new DNA strand complementary to the template. This cycle is usually repeated between 30 and 40 times, taking often more than 2 hours to complete a whole PCR procedure (Kubista *et al.*, 2006; Jing *et al.*, 2011).

However, PCR can be performed with a two-step cycling programme combining the primer annealing and the extension in only one step. This type of thermal protocol is applied when an amplicon in qPCR is small, using 60°C as the temperature. The use of this protocol can lead to significant time-savings compared with the standard three-step protocol (Kubista *et al.*, 2006; Jing *et al.*, 2011).

Figure 5 shows the thermal profile of a three step and a two-step PCR performed on the xxpress® instrument. The ultra-fast ramp rates achieved by the xxpress® (10°C/s) enable to perform either a two-step or a three-step 40 cycles-PCR in just over 10 minutes. This implies significant time-savings in comparison with other thermal cyclers, which usually achieve ramp rates of ~3.3°C/s.

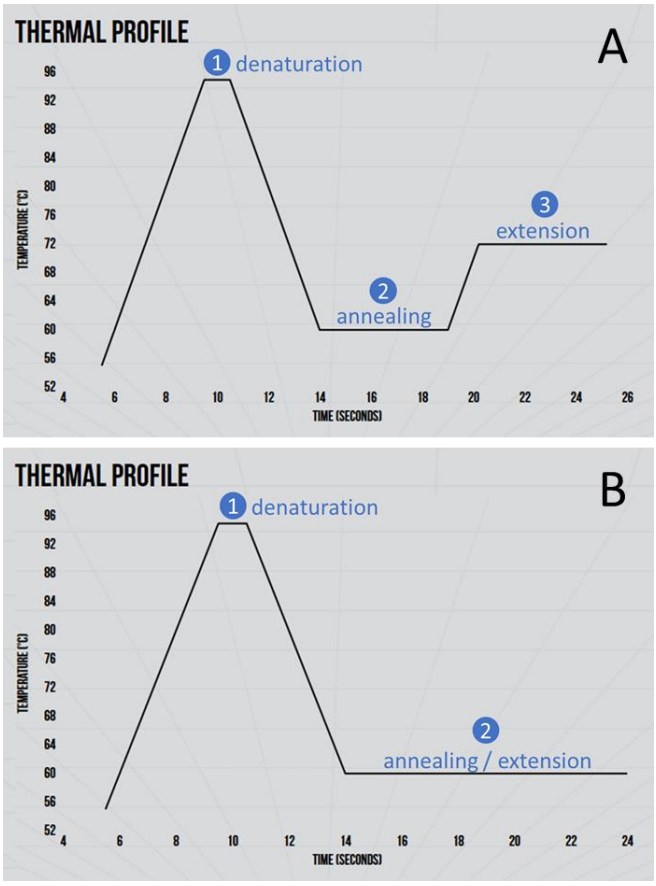

Figure 5. Thermal profile of a three-step PCR cycle (A) and a two-step PCR cycle (B) on the xxpress® thermal cycler.

4. RT-PCR

Reverse transcription (RT) is the reaction by which DNA is synthesised from an RNA template. RT followed by PCR is a powerful method to amplify and detect mRNA. Also, real-time quantitative RT-PCR (RT-qPCR) makes it possible to quantify the expression of a gene. It is the most sensitive and reliable method, especially for low abundant transcripts in tissues with low RNA concentrations (Dorak, 2006).

The levels of expression can be measured by two different types of quantification strategies: an 'absolute' quantification, that relates the PCR signal to input copy number using a calibration curve, and a 'relative' quantification, that measures the relative change in mRNA expression levels (Dorak, 2006).

It is very simple to set up an RT-qPCR on the xxpress® thermal cycler. When configuring the thermal profile, an additional step for RT can simply be added before the initial denaturation. The temperature and time for this step is chosen depending on the reverse transcriptase used. Figure 6 shows a screenshot of the xxpress® user interface where a RT-qPCR has been programmed.

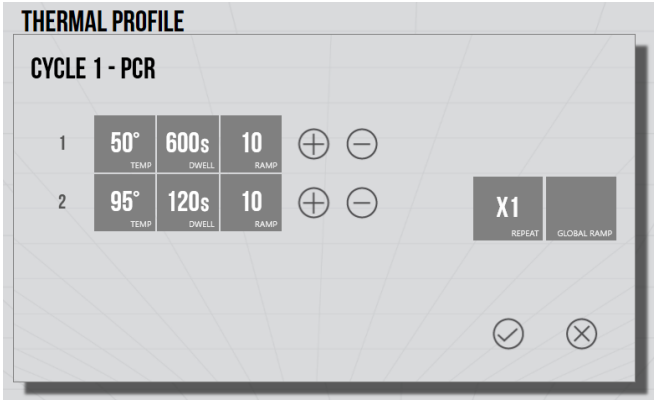

Figure 6. RT-qPCR configuration on the xxpress®. The screenshot shows the settings for the RT step (1) and the initial denaturation step (2).

5. Multiplex PCR

This technique performs the amplification and specific detection of two or more sequences in the same PCR reaction, using a specific dye for each particular target. Multiplexing may be used to produce qualitative or quantitative results. Its advantages are the increased throughput, as more samples can be assayed per plate; the reduced sample usage and reduced reagent usage, and the elimination of well-to-well variability due to the use of a normalizer gene. Typically, multiplex reactions are used to amplify a normalizer gene and a gene of interest in the same reaction (Kubista *et al*., 2006).

The xxpress® thermal cycler enables multiplexing in any combination of up to 5 channels. The dyes that can be used in each channel -or generic equivalents of those- are shown in Table 1. Each channel has its own LED for illumination, and a CCD camera is used for fluorescence detection.

CHANNEL	DYES
1	FAM, SYBR Green
2	JOE, VIC, HEX
3	ROX, Texas Red
4	Cy5
5	Cy5.5

Table 1. Dyes used for each channel on the xxpress

6. Melting Curve Analysis

The PCR product melting curve analysis confirms that specific amplification has taken place and that no other products have been formed, such as primer-dimers. It is performed at the end of the PCR by heating the product from around 50°C to 90°C while taking fluorescence measurements. As the DNA melts, the intercalating dye is released, resulting in a decrease of the fluorescence intensity. Then, a first derivative plot of the data is produced. This indicates the Tm (melting point) of the PCR product which should be the same as the predicted one for that particular amplicon. It also indicates the presence of primer-dimers, which usually produce a peak at a lower temperature than the specific PCR product (Dorak, 2006; Logan, Edwards and Saunders, 2009).

Figure 7 shows a melt curve analysis performed on the xxpress®. The red line demonstrates an example where only specific amplification has taken place, while the green line shows both the peaks for the amplicon and a lower peak corresponding to primer-dimers.

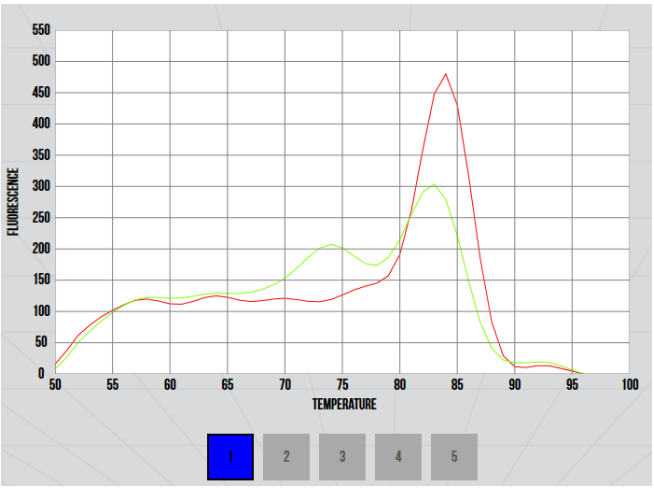

Figure 7. First derivative melt curve produced by the xxpress®.

7. HRM

High resolution melting analysis (HRM) enables mutation detection and genotyping using double-stranded DNA dyes instead of probes (Dorak, 2006; Logan, Edwards

and Saunders, 2009). Differences in PCR products even as small as a single-base-pair mutation are detectable by HRM analysis because they lead to changes in the shape of DNA curves. HRM analysis is a more sensitive approach to traditional melt curve analysis, and it requires a PCR instrument with upgraded optical and thermal capabilities and highly accurate thermal control and consistency (Wittwer, 2009; Słomka *et al*., 2017).

The xxpress® thermal cycler performs quick, simple HRMs and displays the data in an intuitive melt graph, as shown in Figure 8 below.

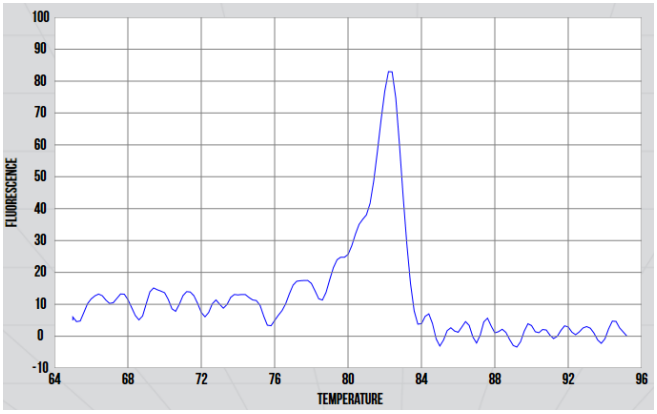

Figure 8. First derivative HRM curve performed by the xxpress®.

8. References

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