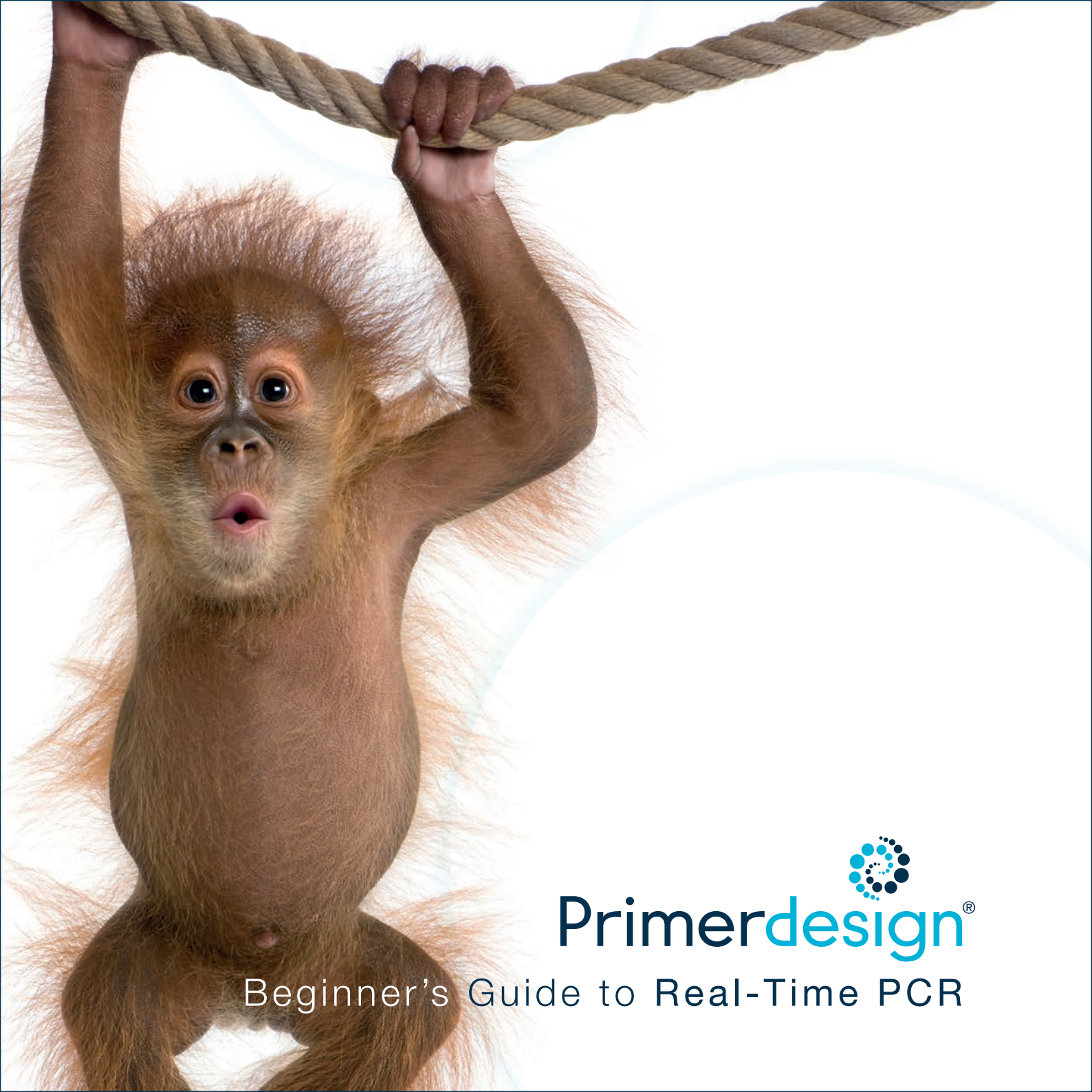


Primerdesign®

Beginner's Guide to Real-Time PCR

02 Real-time PCR basic principles

PCR or the Polymerase Chain Reaction has become the cornerstone of modern molecular biology the world over. Real-time PCR is an advanced form of the Polymerase Chain Reaction that maximizes the potential of the technique.

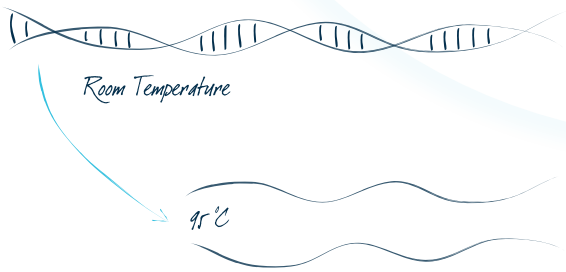
To understand real time PCR it is easier to begin with the principles of a basic PCR:
PCR is a technique for amplifying DNA. There are 2 reasons why you may want to amplify DNA. Firstly you may want to simply create multiple copies of a rare piece of DNA. For example a forensic scientist may want to amplify a tiny piece of DNA from a crime scene. More commonly however you may wish to compare 2 different samples of DNA to see which is the more abundant. DNA analysis requires amplification in order for there to be enough DNA to give a detectable signal for quantification. If you amplify both samples at the same rate, you can calculate which sample had the highest copy number of the target of interest to begin with.

It is a thermostable polymerase enzyme that drives a PCR. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point.

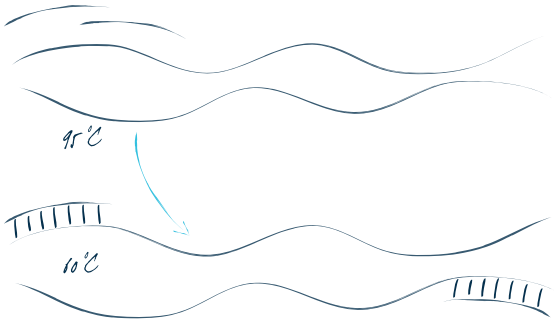
This is very useful because you can choose which gene you wish the polymerase to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to your gene of interest. These small pieces of DNA are known as primers because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest.

During a PCR, changes in temperature are used to control the activity of the polymerase and the binding of primers.

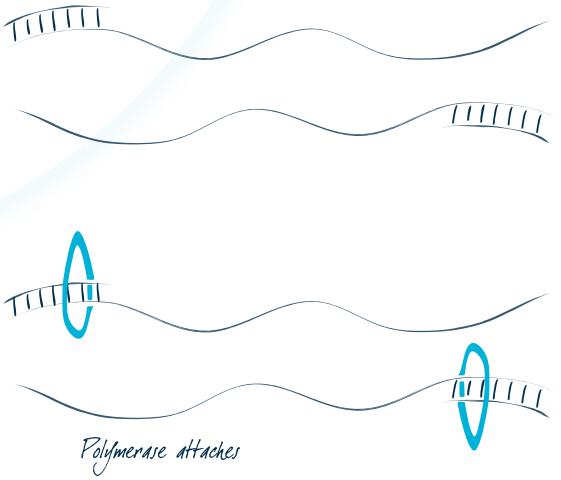
To begin the reaction the temperature is raised to 95°C. At this temperature all double stranded DNA is "melted" in to single strands:

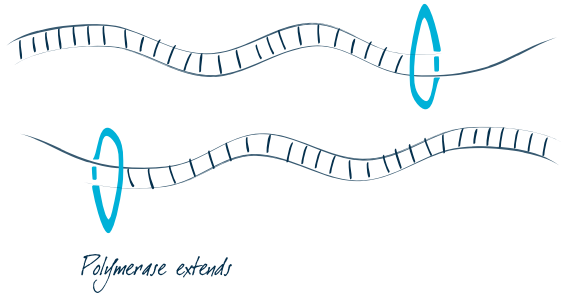


The temperature is then lowered to ~60°C. This allows the primers to bind to your gene of interest:



Thus the polymerase has somewhere to bind and can begin to copy the DNA strand:





The optimal temperature for the polymerase to operate is 72°C so at this point the temperature is sometimes raised to 72°C to allow the enzyme to work faster.

There are now twice as many copies of your gene of interest as when you started:

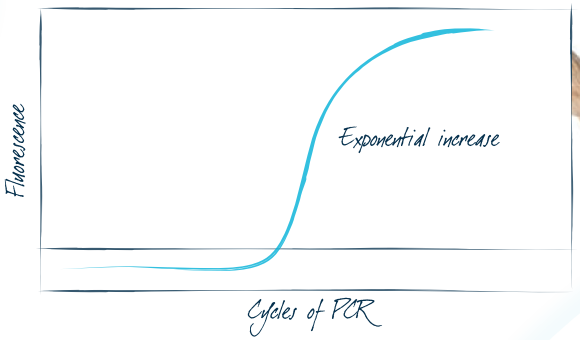
This temperature change is repeated through around 40 'cycles'. Thus one copy becomes 2, 2 become 4, 4 become 8, and so on until billions of copies are created.

After amplifying your gene it is possible to run the amplified DNA out on an agarose gel and stain it with a dye which makes it visible. The brighter the visible band, the more copies of your target you have created.

Real-time PCR

This same principle of amplification is employed in real-time PCR. But instead of looking at bands on a gel at the end of the reaction, the process is monitored in "real-time". Literally, the reaction is placed in to a real-time PCR machine that watches the reaction occur with a camera or detector.

There are a many different techniques that are used to allow the progress of a PCR reaction to be monitored but they all have one thing in common. They all link the amplification of DNA to the generation of fluorescence which can simply be detected with a camera during each PCR cycle. Hence, as the number of gene copies increases during the reaction, so the fluorescence increases.



Real time PCR has many benefits over the old fashioned approach:

- Firstly it gives you a look in to the reaction. You can literally see which reactions have worked well and which have failed.
- The efficiency of the reaction can be precisely calculated.
- There is also no need to run the PCR product out on a gel after the reaction as the melt curve analysis effectively does this for you.
- The greatest advantage of all however, is that real-time PCR data can be used to perform truly quantitative analysis of gene expression. In comparison, old fashioned PCR was only ever semi-quantitative at best.

Probe vs SYBR green

Choosing the correct detection chemistry for your experiment

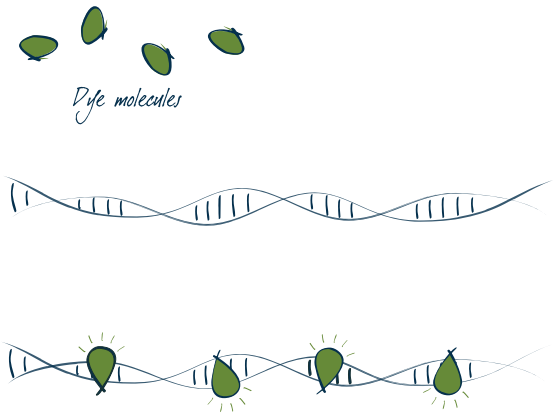
05

Introduction

When designing a real time PCR experiment a significant decision is choosing the correct detection chemistry for your application. There are two major options to choose from. Most people use either an intercalating dye (e.g. SYBR green) or a hydrolysis probe based detection solution (e.g. Taqman® or PerfectProbe). Both technologies are designed to generate fluorescence during the PCR, which allows your real time PCR machine to monitor the reaction in "real time".

SYBRgreen (or other intercalating dye)

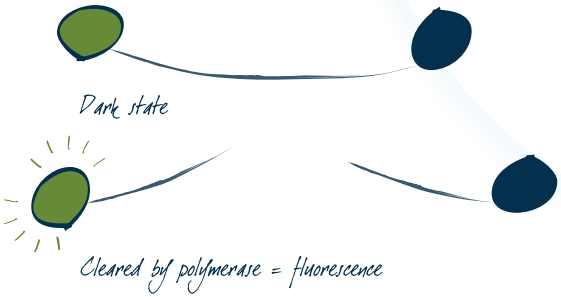
SYBRgreen is by far the most commonly used intercalating dye. There are others available but it's very likely that SYBRgreen is the one you have heard of. All of these dyes operate via a simple mechanism.



The dye is fluorescent in it's own right but in the presence of double stranded DNA, the dye intercalates with (binds in to) the DNA double helix. This alters the structure of the dye and causes it to fluoresce more. So very simply as the PCR creates more DNA, more dye can bind and more fluorescence is generated.

Hydrolysis Probes

Probes are fluorescently labelled DNA oligonucleotides. They are designed to bind downstream of one of the primers during the PCR reaction and to give a fluorescent signal during the reaction. The 5' end of the probe is labelled with a fluorescent reporter molecule. FAM is a green reporter and is the most commonly used but there are others such as VIC, JOE, CY5 etc which emit light at different wave lengths and can be read through separate detecting channels. On the 3' end of the probe is a quencher molecule. This is another molecule that effectively quenches the output from the reporter. Therefore, when the reporter and quencher are physically close to one another the overall level of fluorescent output is low.



Hydrolysis probes give more convenient data to work with...

During the PCR the probe binds downstream of the primer. The probe is then cleaved by the polymerase enzyme during the reaction. By cleaving the probe the reporter and quencher are separated which means that the quencher no longer has its effect over the reporter and the level of fluorescence increases. This means that with every cycle of PCR more probe is cleaved and more fluorescence is generated.

Things to consider: Cost

Cost is an important part of any experimental design. It is more expensive to use a hydrolysis probe than an intercalating dye. This means that if you have an experiment looking at lots of different genes or targets then you will probably want to choose an intercalating dye as your detection chemistry. However, if you only have a few targets of interest and you know that they will be your focus, you should choose to use a hydrolysis probe.

Things to consider: Specificity

Hydrolysis probes: Overall, hydrolysis probes give more convenient data to work with than intercalating dyes. This is because of their inherent specificity. When you get a signal from a hydrolysis probe you can be sure that the signal has come from genuine amplification of your target sequence. It is only possible to get a signal when the primers and the probe bind in the correct place, to the correct target. So no post run analysis is required to confirm the correct target has been amplified. This means the data is inherently reliable and simple to work with.

Intercalating dyes: the weakness of intercalating dyes is that they are non specific. If your PCR amplifies the wrong target, or even more than one target, you will still get an amplification plot that looks identical to a genuine signal. Intercalating dyes will bind to and report on any double stranded DNA that is formed during the reaction regardless of what it is. This means that additional analysis is required to be certain of your results. This analysis is in the form of a 'melt curve' (sometimes called 'dissociation curve'). They require more time and more experience to interpret correctly. It is crucial to interpret the melt curve along side the amplification plot in order to work with your data.

Things to consider: Target abundance

Because of its inherent lack of specificity, intercalating dyes are less effective when your target of interest is rare. When a target is rare the primers are more likely to form primer dimers or to bind to the wrong target. Even the very best primers will eventually form a primer dimer and give a false signal after enough cycles of PCR if no authentic target of interest is present. However, when there is plenty of your target of interest present then intercalating dyes work very nicely.

So as a rule of thumb, if your target of interest is rare (Cq values > 30), then using an intercalating dye may well be troublesome and a hydrolysis probe is recommended. But if your target of interest is abundant (Cq values < 30) then using an intercalating due will be perfectly sufficient.

Kits, reagents and expertise

Real-Time PCR is an exceptionally powerful research tool. With the correct kits, reagents and experimental design it is quick and easy to generate high quality meaningful data in no time with real-time PCR. Whether you are an expert or a complete beginner Primerdesign Ltd can make your life very easy. We are experts and can help you by providing everything you need to generate the very best possible real-time PCR data.

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Who we are

Primerdesign are a UK based company specialising in real-time PCR. Founded in 2004 the company has more than a decade of expert experience and supplies the likes of the US army, the US CDC, the UK NHS, as well as thousands of other customers in more than 100 countries.

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