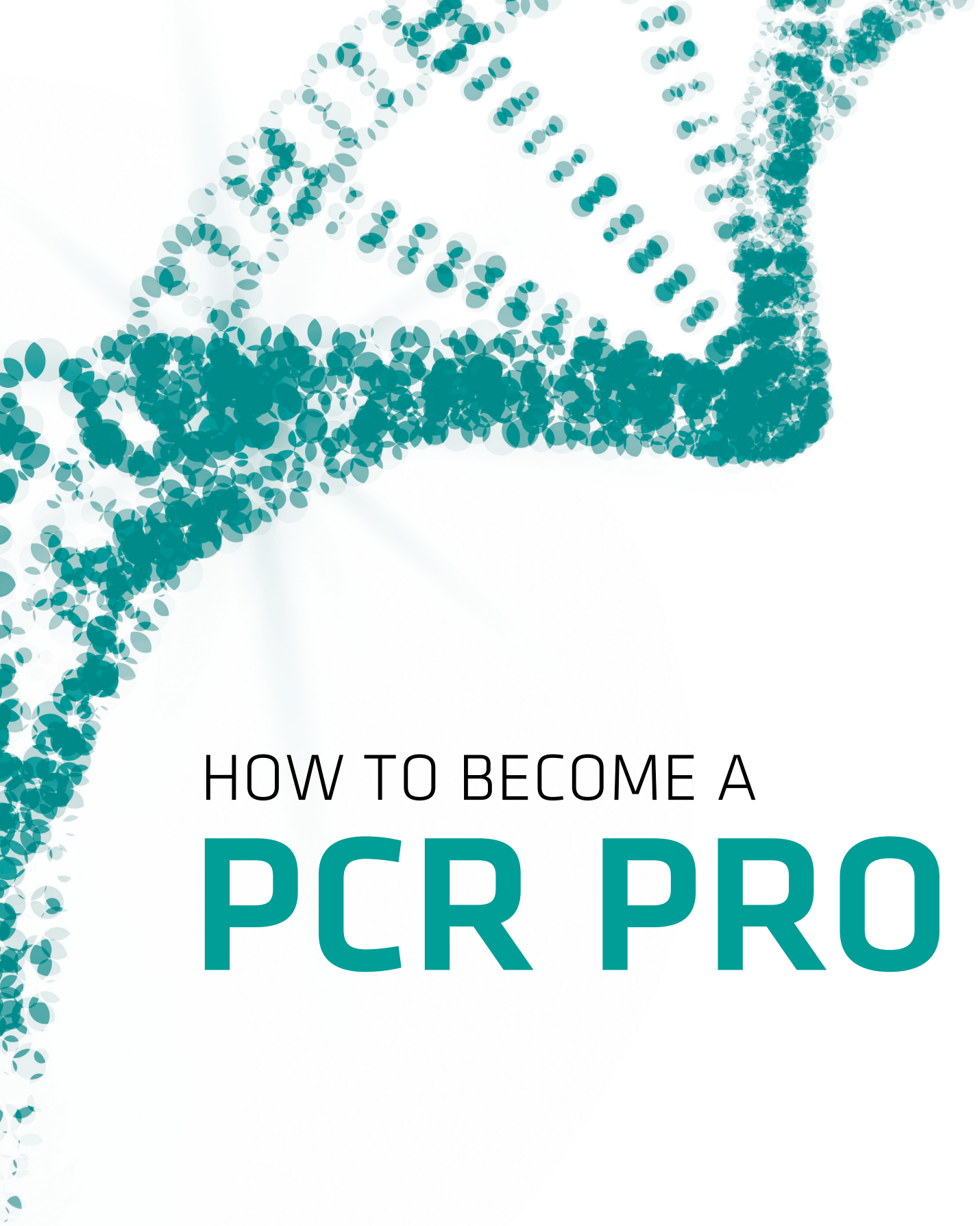

HOW TO BECOME A PCR PRO

INTEGRA

FOREWORD

The polymerase chain reaction (PCR) is a key life sciences technique. It has been used in molecular biology – including molecular diagnostics – for many years, and a number of different types, for example, RT-PCR, qPCR, vPCR and ddPCR have been developed over time. Today, PCR is a vital tool for the detection of pathogens, such as the SARS-CoV-2 virus, and is essential for genotyping and NGS library preparation. However, PCR is well known for being difficult to run successfully and several parameters must be considered when planning the PCR protocol.

We have therefore compiled this eBook – consisting of in-depth educational articles, relevant app notes and customer testimonials – to help you understand how PCR works, and what needs to be considered to perform effective PCR reactions. We also demonstrate how our solutions can help you to enhance the throughput of your lab, and become a PCR pro in no time.

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CHAPTER 1:
What you need to know about PCR

In this chapter, we will cover topics such as PCR's fascinating history, its mechanism and different variations, and techniques for troubleshooting common issues you may encounter. We'll also go through tips for establishing a PCR lab, as well as a comprehensive overview of all things related to qPCR and primer design.

1.1 The complete guide to PCR

Polymerase chain reaction (PCR) methods have been carried out in labs around the world since the 1980s, opening the door for an array of new applications, such as genetic engineering, genotyping and sequencing. In this article, we take a deep dive into this fascinating technique by explaining its mechanism, exploring its history, looking into the different types of PCR, discussing troubleshooting tips and much more.

What is PCR?

The polymerase chain reaction (PCR) is a fast and inexpensive technique for amplifying a DNA sequence of interest. It consists of three steps:

- **Denaturation:** The sample is heated to separate the DNA into two single strands.
- **Annealing:** The temperature is lowered to allow primers to anneal to specific single-stranded DNA segments, flanking the sequence to be amplified.
- **Extension:** The temperature is raised to the optimum working temperature of the polymerase enzyme, which then makes a complementary copy of the DNA sequence of interest.

One such repetition or 'thermal cycle' theoretically doubles the amount of the DNA sequence of interest present in the reaction. Typically, 25 to 40 cycles are performed – resulting in millions or even billions of DNA copies – depending largely on the amount of DNA in the starting sample and the number of amplicon copies needed for post-PCR applications.

The three steps of a PCR reaction are carried out automatically by a thermal cycler, but can only be successful if the master mix has been correctly prepared. The following sections explain the components that make up the master mix and how they interact with the template DNA during thermal cycling.

PCR master mix components

The PCR master mix consists of six components:

- **PCR-grade water:** Certified to be free of contaminants, nucleases and inhibitors.
- **dNTPs:** Containing equal concentrations of the four nucleotides (dATP, dCTP, dGTP and dTTP), which are the 'building blocks' to create complementary copies of the DNA sequence of interest.
- **Forward and reverse primers:** Short, single-stranded DNA sequences that anneal specifically to the plus and minus strands of the template DNA, flanking the sequence to be amplified. For some assays – such as protocols amplifying much-studied genes or DNA sequences of common bacteria – ready-to-use primers can be purchased. However, many experiments require custom PCR primers tailored to the region of interest of the template DNA and the reaction conditions.

- **DNA polymerase:** *Taq*-polymerase is the most commonly used enzyme for PCR reactions. It uses dNTPs to create complementary copies of the DNA sequence of interest. For some applications, such as mutagenesis, *Taq*-polymerase is not accurate enough and the use of high fidelity polymerases is recommended. Just like *Taq*-polymerase, they sometimes add an incorrect nucleotide when replicating the template DNA but, as they have a 3' to 5' exonuclease activity, they 'proofread' the newly synthesized strands and correct any mistakes.[1] This proofreading step is highly beneficial for accuracy but it also slows down PCR reactions, and high fidelity polymerases (also called slow polymerases) therefore need about twice the time of *Taq*-polymerase to create a complementary DNA strand. The most popular high fidelity DNA polymerase is Pfu-polymerase.[2]

- **Buffer:** Provides a suitable environment for the DNA polymerase, with a pH between 8.0 and 9.5.[3]

- **Magnesium chloride:** Increases the activity of the DNA polymerase and helps primers to anneal to the template DNA for a higher amplification rate.[4] This cofactor is sometimes included in the buffer in a sufficient concentration.[5]

The template DNA , which may be genomic DNA (gDNA), complementary DNA (cDNA) or plasmid DNA (pDNA), is then added after master mix preparation.

The 3 steps of PCR

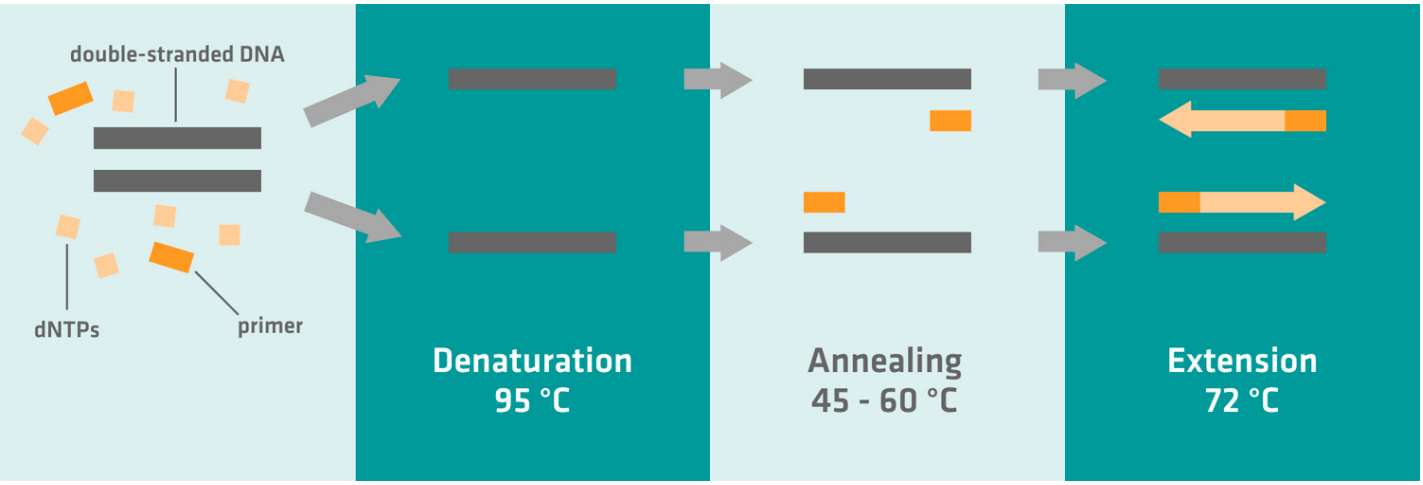
After preparing the PCR master mix and adding the template DNA samples to it, you can load your reaction tubes, PCR strips or microplates into the thermal cycler. They will then go through the following steps:

- **Denaturation:** The thermal cycler first heats the reaction mix to 95-98 °C to denature the template DNA, separating it into two single strands. Depending on your sample, this usually takes 2-5 minutes during the first thermal cycle, and 10-60 seconds for subsequent cycles.

- **Annealing:** When the temperature is lowered, the primers anneal to the sequences flanking the template DNA region of interest. Depending on the sequence and melting temperature of your primers, this step usually takes 30-60 seconds, and the optimal annealing temperature typically lies between 45 and 60 °C.

- **Extension:** The temperature is increased to 72 °C, which is the ideal working temperature for the *Taq*-polymerase. Depending on the synthesis rate of your polymerase, and the length of the target sequence, it usually takes 20-60 seconds to create complementary copies of the DNA sequence of interest.[6] After approximately 25-40 cycles – depending on the amount of DNA present at the start, and the number of amplicon copies needed for post-PCR applications[7] – the last extension step should be extended to 5 minutes or longer, allowing the *Taq*-polymerase to finish the synthesis of uncompleted amplicons.[5] If you can't immediately take your samples out of the thermal cycler after the final extension step because you're busy with other experiments, program it to cool your samples to 4 °C. For overnight runs where you

leave your samples in the thermal cycler for hours after the final extension step, you should opt for a holding temperature of 10 °C instead of 4 °C, as it causes less wear and tear on your machine.



As shown in the image above, the amount of PCR product theoretically doubles at every thermal cycle, leading to an exponential increase of PCR product. However, in reality, the phase of exponential amplification eventually levels off and reaches a plateau because the reagents have been consumed and the DNA polymerase activity decreases.

The different types of PCR

After performing a standard PCR reaction, you can determine the concentration, yield and purity of the amplified DNA sequences using gel electrophoresis, spectrophotometry or fluorometry. However, you can't determine the amount of template DNA present in a sample before amplification using standard PCR. If this is a requirement for your experiment, you have to perform a qPCR reaction.

qPCR

qPCR – also called real-time PCR, quantitative PCR or quantitative real-time PCR – is a technique used to detect and measure the amplification of target DNA as it is produced. In contrast to conventional PCR reactions, qPCR requires a fluorescent intercalating dye or fluorescently-labeled probes, and a thermal cycler that can measure fluorescence and calculate the cycle threshold (Ct) value. Typically, the fluorescence intensity increases proportionately to the concentration of the PCR product being formed, measuring quantities of the target in real time.

qPCR can be divided into dye-based methods (e.g. SYBR® Green) and probe-based methods (e.g. TaqMan®).

RT-PCR and RT-qPCR

Another limitation of standard PCR is that it can only be used to amplify DNA sequences. If you want to amplify RNA target sequences, you have to use RT-PCR.

RT-PCR

Reverse transcription PCR (RT-PCR) is used to amplify RNA target sequences, such as messenger RNA or RNA virus genomes. This type of PCR involves an initial incubation of the RNA samples with a reverse transcriptase enzyme and a DNA primer – comprising sequence-specific oligo (dT)s or random hexamers – prior to the PCR amplification.

You can also perform a qPCR reaction instead of executing a standard PCR reaction after the reverse transcription step, which produces cDNA from RNA. This PCR variant is called RT-qPCR.

vPCR

The third limitation of standard PCR is that it cannot distinguish between the DNA of viable and non-viable cells. You should use vPCR if this is important to your application, for example, because you want to know if the infectious microorganisms in a clinical sample are dead or alive.

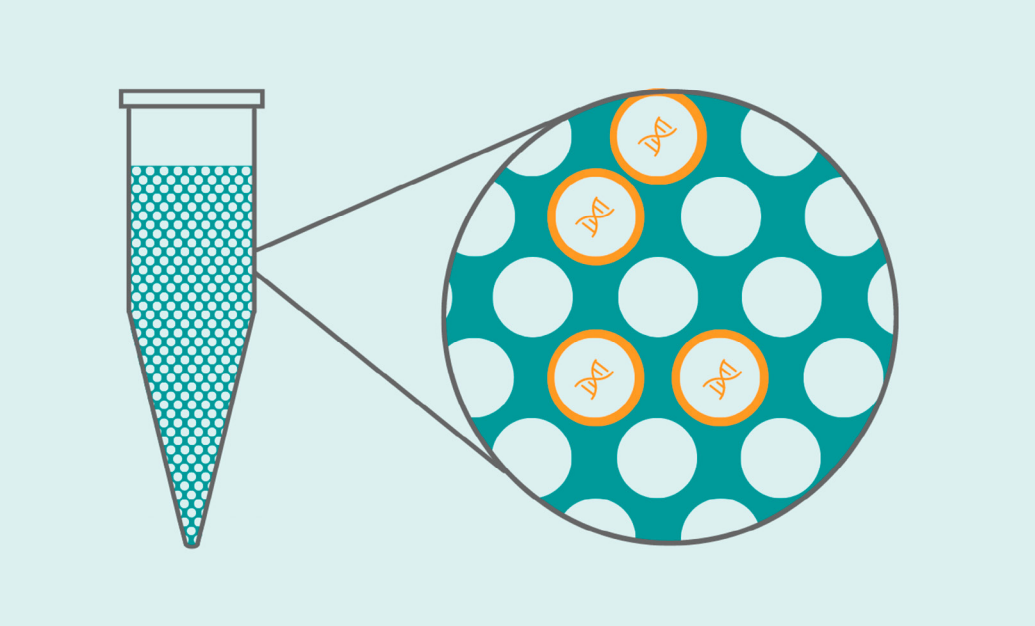
vPCR

For viability PCR (vPCR), each sample needs to be split into two aliquots. One aliquot is incubated with a photoreactive intercalating dye that is unable to diffuse through intact cell membranes of live cells. This means that it only intercalates into the DNA of dead cells. When this aliquot is subsequently treated with a blue light, the dye binds irreversibly to the DNA. Both aliquots are then subject to DNA purification and qPCR amplification. If they exhibit similar qPCR signals, the target microorganisms in the sample are mostly viable. If the dye-treated aliquot exhibits a weaker signal, the target microorganisms are mostly dead. vPCR is an important technique in diagnostics, agriculture and food safety.

ddPCR

Digital droplet PCR (ddPCR) is another relatively new type of PCR. It uses fluorescently labeled probes to detect DNA sequences of interest, and a water-oil emulsion system to split each sample into about 20,000 nanoliter-sized droplets. After amplification, every droplet of the sample is analyzed on its own. Droplets that contain at least one DNA sequence of interest emit a fluorescent signal – and are consequently positive – while droplets without the DNA sequence of interest don't fluoresce, and are therefore negative. Using the Poisson distribution, you can then determine the concentration of the DNA sequence of interest in the original sample by analyzing the ratio of positive to negative droplets for absolute quantification.[8]

ddPCR



An advantage of ddPCR compared to qPCR is that it's more precise. While qPCR can detect two-fold differences in DNA target sequence variation, e.g. discriminate 1 copy from 2 copies of a gene, ddPCR can discriminate 7 copies from 8 copies, which means that it can detect differences as small as 1.2-fold.[9] On top of that, ddPCR is better suited for multiplexing assays if you want to determine the ratio of low abundance to high abundance DNA sequences of interest, such as rare mutations on wild type backgrounds. When using qPCR, the fluorescent signal from the high abundance sequences can dominate and obscure the signal from the low abundance sequences. This risk is ruled out with ddPCR, as each droplet behaves as an individual PCR reaction and contains either zero, one or, at most, a few sequences of interest.[10,11]

Due to these advantages, ddPCR is often preferred over qPCR for the detection of mutations and SNPs (single nucleotide polymorphisms), allelic discrimination, gene expression studies, and the analysis of copy number variations.[12]

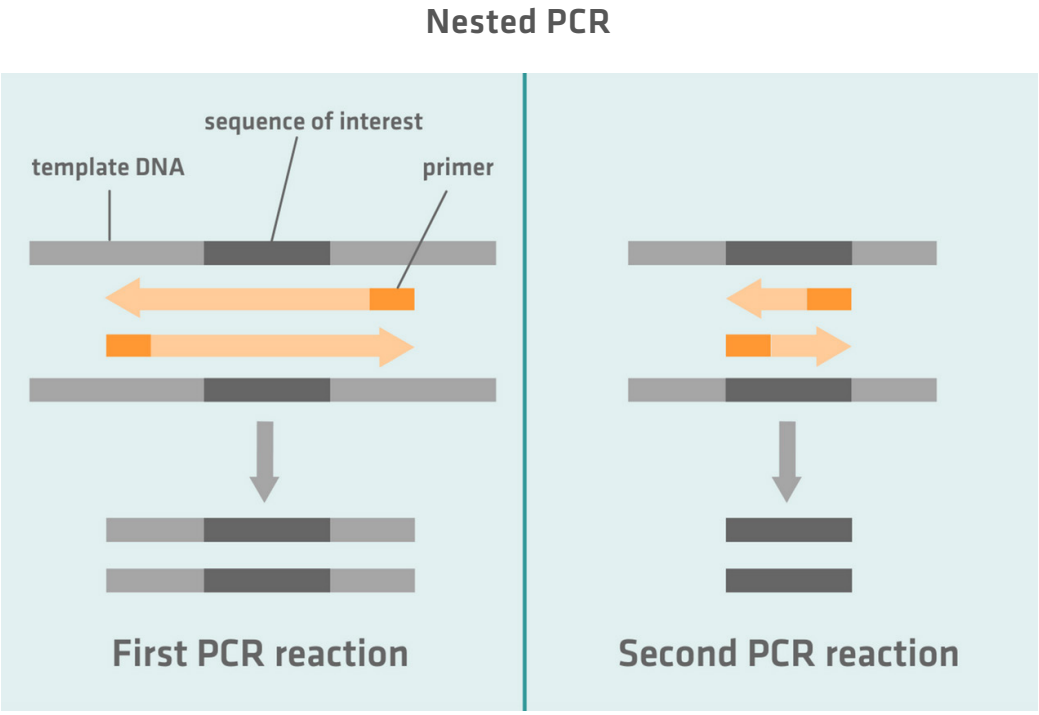
Hot start PCR

If your PCR reaction results in non-specific amplification, you can try to increase the reaction specificity using a hot start polymerase. This enzyme remains inactive during master mix preparation and sample addition at room temperature, eliminating the risk that unintended products and primer dimers are formed during PCR set-up.[13]

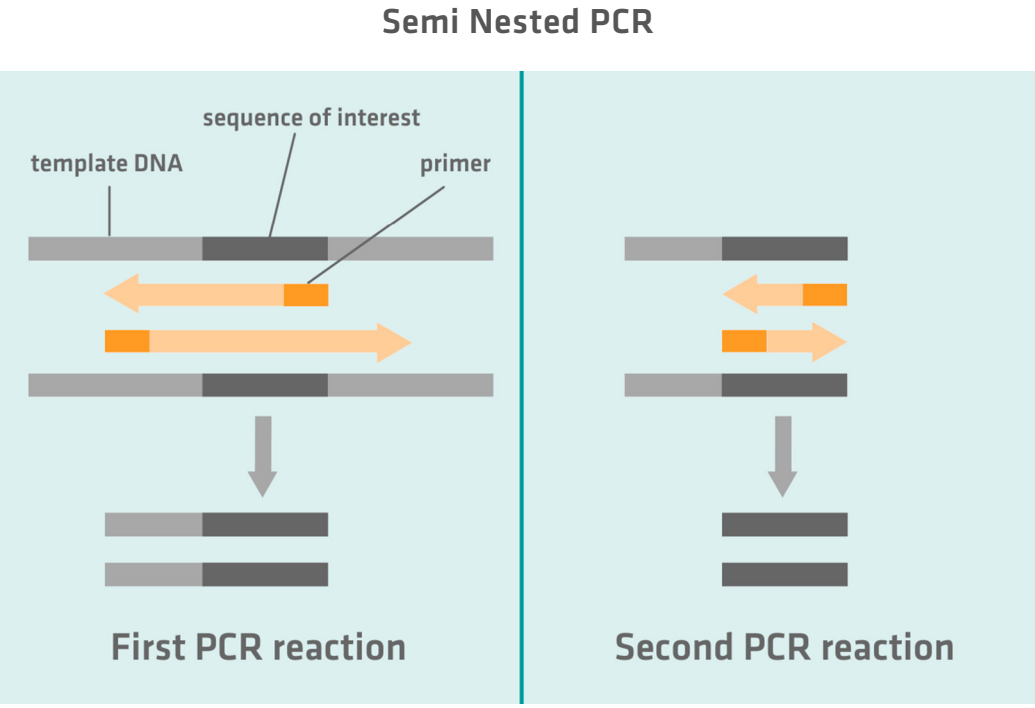
Nested and semi-nested PCR

Nested or semi-nested PCR are alternatives to hot start PCR that increase reaction specificity.

Nested PCR uses two sets of primers and two successive PCR reactions. The first set of primers is designed to amplify a DNA sequence slightly longer than the sequence of interest. During the second PCR reaction, the second set of primers that is specific to the sequence of interest anneals to the amplicons of the first PCR reaction and helps to amplify the sequence of interest.[14,15]



Semi-nested PCR works similarly to nested PCR. During the first PCR reaction, one primer anneals to the sequence of interest and the second primer to a region flanking the sequence of interest. This primer is then replaced with a second primer annealing to the region of interest during the second PCR reaction.



The idea behind nested and semi-nested PCR is that, if non-specific products were amplified during the first PCR reaction, these will not be amplified during the second PCR reaction, as the primers cannot anneal to them.

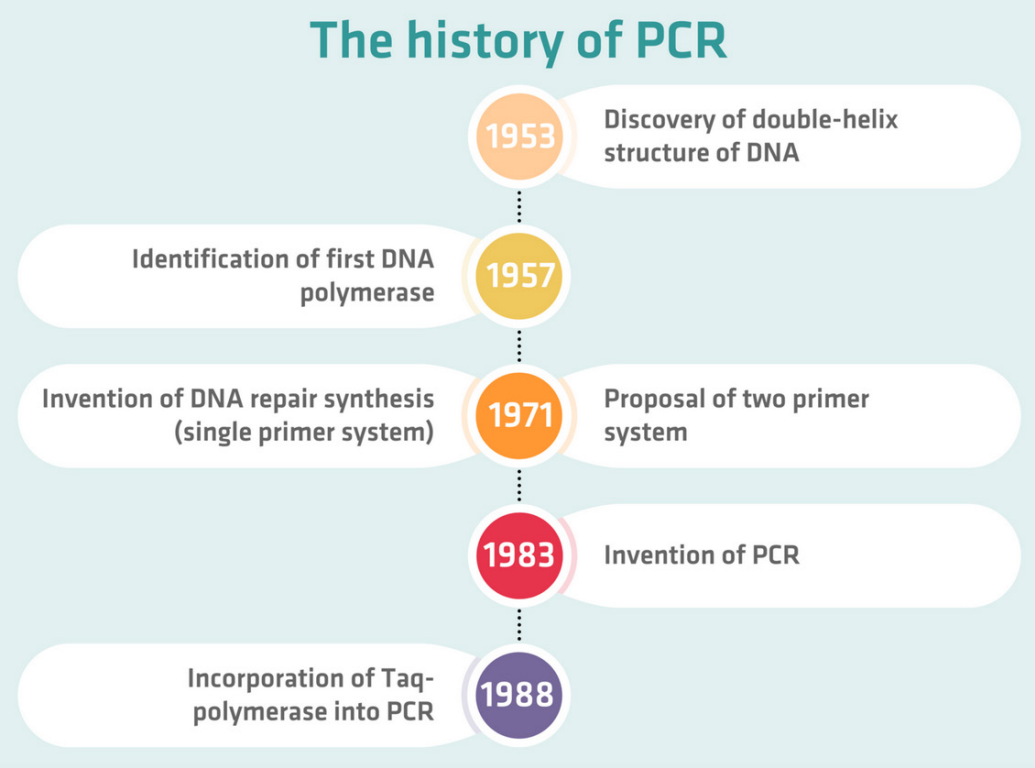
Touchdown PCR

A third type of PCR developed to increase reaction specificity is touchdown PCR. The assay set-up for touchdown PCR is identical to the set-up for standard PCR. The only difference lies in the annealing step. During the first thermal cycle, the annealing temperature should be several degrees above the optimal primer annealing temperature, then be lowered by 1-2 °C every second cycle.[16] These high temperatures during the first cycles avoid PCR primers forming primer-dimers or binding to regions outside the DNA sequence of interest. The downside is that the PCR primers don't all sufficiently bind to the template DNA, which leads to low yields.[17] However, this can be tolerated, as the low yield of specific amplicons is then exponentially amplified with every thermal cycle that is performed at the optimal annealing temperature.

The history of PCR

As we've shown, there are many different types of PCR, and some of them have only recently been developed. However, the foundation for PCR was laid in the 1950s:

- In 1953, James Watson and Francis Crick discovered the double-helix structure of DNA, and suggested that there might be a possible copying mechanism for DNA.
- Four years later, Arthur Kornberg identified the first DNA polymerase that was able to copy the template DNA, although only in one direction.
- In 1971, Gobind Khorana and his team started to work on DNA repair synthesis. Their technique used DNA polymerase repeatedly, but employed only a single primer template complex, which did not allow exponential amplification.
- At the same time, Kjell Kleppe from Khorana's lab proposed a two primer system that would double the amount of DNA in a sample, but no one actually conducted the experiment to find out whether it worked. The reason for this was probably that there was not yet a DNA polymerase that could withstand the high temperatures of the denaturation step. This means that they would have had to add a fresh dose of enzyme after every thermal cycle.
- In 1983, Kary Mullis, working at Cetus Corporation, added a second primer to the opposite strand, and realized that repeated use of DNA polymerase triggers a chain reaction that will amplify a specific DNA sequence, thus inventing PCR. The patent got approved in 1987, and he won the Nobel Prize in Chemistry six years later.
- In 1976, the thermostable enzyme *Taq*-polymerase – which is typically used in PCR today – was first isolated from the bacterium *Thermus aquaticus*, which had been discovered in a hot spring of Yellowstone National Park in 1969. When it was finally incorporated into PCR workflows in 1988, it removed the need to add a new dose of enzyme after every thermal cycle, paving the way for the invention of automated thermal cyclers.[18,19,20]



PCR troubleshooting

One of the most important troubleshooting mechanisms is to always include positive and negative control samples.

If the sequence of interest wasn't amplified in your positive control sample, your master mix, template DNA or thermal cycler could be the source of the problem:

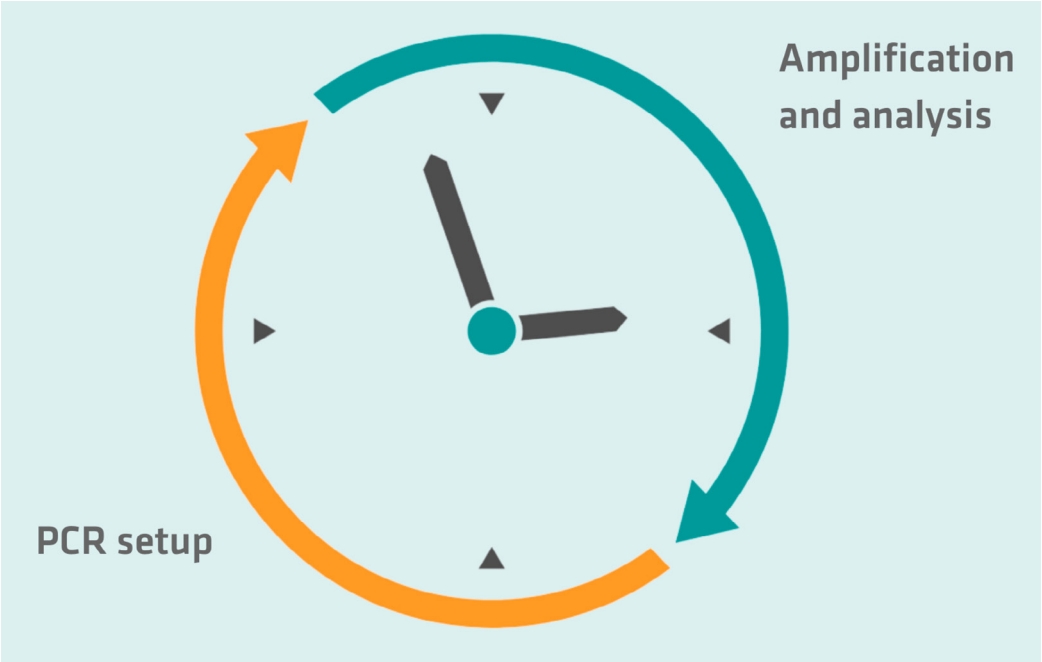
- **Master mix:** Have you added the right volume and concentration of each reagent, and have you cooled your reagents during master mix preparation?
- **Template DNA:** Have you run an agarose gel to ensure that your template DNA isn't degraded? Is your template DNA pure enough and, if not, have you purified it?
- **Thermal cycler:** Is the number of thermal cycles sufficient for your assay? Have you programmed the device correctly, and is it calibrated to ensure that it performs the reaction steps at the right temperatures?

If the sequence of interest was amplified in your negative control sample, one or more components of your master mix is contaminated. PCR reactions are very sensitive, and create large number of copies of DNA sequences from minute amounts of starting material, so contamination is a common issue. To prevent it, the right lab set-up is crucial.

Lab set-up

Ideally, your PCR lab should have two rooms, each divided into two areas. The first room should be exclusively used for pre-PCR activities, and divided into a master mix preparation area and a sample preparation area. The second room should have a dedicated area for amplification, and another one for product analysis.

If you're lacking in space or budget for a two-room PCR lab, you can set up the pre-PCR and amplification and analysis areas in the same room, but ensure they are as far from one another as possible. In addition to the spatial separation, you could also consider setting up your PCR reactions in the morning, and performing the amplification and analysis steps in the afternoon. Temporally separating the different steps of your PCR reactions may limit your flexibility and make you lose some time, but lowers the risk of aerosols with high DNA concentrations from the analysis area contaminating your master mix and samples in the pre-PCR area.



On top of these precautionary measures, you should always work in biosafety cabinets or laminar flow hoods when setting up your PCR reactions, use different sets of pipettes for master mix preparation, sample preparation and analysis, and make sure that you use filter tips and consumables that are free of DNase, RNase and PCR inhibitors.

Specificity

Another major PCR challenge is specificity. As explained before, it can be improved using hot start, nested, semi-nested or touchdown PCR. A further option to prevent the amplification of regions outside the DNA sequence of interest, as well as the formation of secondary structures, is to redesign your primers.

Use this checklist to see whether your primers meet all the requirements:

- Are your primers between 18 and 24 bp long?
- Is your target sequence length between 100 and 3000 bp for standard PCR assays, or 75 and 150 bp for qPCR assays?
- Do your primers have melting temperatures between 50 and 60 °C, and within 5 °C of each other?
- Have you performed a gradient PCR to determine the optimal annealing temperature?
- Does the GC content of your primers lie between 40 and 60 %?
- Have you avoided runs or repeats of four or more bases or dinucleotides?
- Have you made sure that your primers are not homologous to a template DNA sequence outside the region of interest?
- Have you checked that your primers can't form stable secondary structures?

PCR equipment

The most important PCR instrument is certainly the thermal cycler but, as the right pipetting devices can help create faster and more efficient workflows with fewer errors, we'll also look at a few different liquid handling options in this section.

Thermal cyclers

Before the development of thermal cyclers, scientists had to manually move their samples between water baths of different temperatures. The first thermal cycler prototype called 'Mr. Cycle' also used water baths to heat and cool the samples, and was developed by engineers at Cetus Corporation, where Kary Mullis worked when he invented PCR.[21] Today's instruments work with electric heating and refrigeration units, and many different models with various additional features are available.

For standard PCR, a thermal cycler that can heat and cool your samples to the required temperatures might be sufficient to complete the different reaction steps. However, your thermal cycler will need additional properties – such as gradient capability or an integrated fluorometer – if you want to perform gradient PCR assays to optimize primer annealing temperatures, or qPCR assays to determine the amount of template DNA present in a sample before amplification.

Pipettes

While the thermal cycler is the star of PCR labs, the right pipettes help you to process more samples in less time, while ensuring maximal accuracy and precision. Electronic pipettes offering a Repeat Dispense mode, for example, are a great option to boost the efficiency of aliquoting master mix into an entire well plate. Adjustable tip spacing pipettes, paired with low dead volume reagent reservoirs, can be a useful alternative to single channel pipettes when transferring reagents and samples between different labware formats. And, if you want to significantly cut your PCR set-up and purification time, pipetting robots or 96 and 384 channel pipettes might be the right tool for you.

Conclusion

We hope that this article has been useful in helping you understand the mechanisms behind the different types of PCR, and has shown you different ways to avoid contamination and non-specific amplification.

1.2 Simple PCR tips that can make or break your success

Since the outbreak of the COVID-19 pandemic, PCR is on everybody's lips. However, only people working in the lab know how difficult it can be to get the desired results using this well-established technique. Out of this frustration came the popular joke that PCR should stand for 'pipette, cry, repeat'. To ensure that this stays a joke from now on, and that your PCR reactions never drive you to despair again, we have compiled the most important tips and tricks for a successful PCR set-up.

What is PCR?

The polymerase chain reaction (PCR) is used to amplify specific DNA sequences for downstream use. Its inventor Kary Mullis, whose patent on PCR was approved in 1987, was awarded the Nobel Prize in Chemistry six years later,[1] and since this time, PCR has remained one of the most essential molecular biology techniques. Genetic engineering, genotyping, sequencing and the identification of familial relationships, to name a few examples, wouldn't be possible without it.

PCR tips and tricks

To perform PCR reactions, you need to prepare a master mix, add template DNA, and amplify the sequence of interest using a thermal cycler. This might seem straightforward, but it is far from it. Calculating the required amounts of master mix reagents correctly to get the right volume, at the right concentration, is the first challenge.

Once this is accomplished, the reagents need to be mixed together. The difficulty here is that the liquids usually have to be cooled and they are often highly viscous, sticky and needed in minimal quantities. In addition, work must be performed in a concentrated manner, as distractions or interruptions can quickly lead to a situation where you no longer know which reagents have already been added to the master mix. Errors such as skipping a tube or well can

also easily occur when filling PCR strips or plates with master mix and adding template DNA, especially when using single channel pipettes.

The last and probably biggest challenge is to keep your PCR reactions free from contamination. PCR is a very sensitive assay that can create a large number of nucleic acid copies from a tiny amount of starting material, so amplicon and sample contamination can be a huge problem.

Master mix calculations

Let's first have a look at the mathematical calculations needed to set up a PCR master mix. We'll assume that you want to set up several PCR reactions with a volume of 50 µl each.

To calculate the required volume for each reagent, it is best to create a table (see Table 1) with the necessary components, and fill in the stock concentrations and desired final concentrations for the buffer, the $MgCl_2$, the dNTPs and the primers. Then, calculate the dilution factors by dividing the stock concentration by the final concentration. To determine the volume needed for a single PCR reaction, divide the desired reaction volume by the dilution factor.[2]

For the polymerase, a slightly different equation is needed. The manufacturer of the enzyme will tell you the amount of polymerase in one µl, e.g. 5 Units/µl. Fill in this value in the column for the stock concentration and put the desired amount – e.g. 1.25 Units – in the column for the final concentration. The volume needed can then be calculated as follows: 1.25 Units x (1 µl / 5 Units) = 0.25 µl.[3]

The template DNA volume required depends on your sample type. You should add about 1 pg to 10 ng of plasmid or viral DNA, and 1 ng to 1 µg of genomic DNA. In the example below, we calculated how much you would need to use for 0.5 µl of a 1 µg/µl template DNA.[4]

Finally, add the required volumes for all the reagents. The difference between the desired total reaction volume (50 µl) and the result obtained gives you the volume of PCR-grade water.[5]

REAGENT	STOCK CONC.	FINAL CONC. (C_F)	DILUTION FACTOR (= STOCK CON. / C_F)	VOLUME NEEDED (= 50 ML / DIL. FACTOR)
Buffer	10X	1X	10	5 µl
$MgCl_2$	25 mM	1.5 mM	16.66	3 µl
dNTPs	10 mM	0.2 mM	50	1 µl
Forward primer	10 µM	250 nM	40	1.25 µl
Reverse primer	10 µM	250 nM	40	1.25 µl
Polymerase	5 Units/µl	1.25 Units	-	0.25 µl
Template DNA	1 µg/µl	-	-	0.5 µl
PCR-grade water	-	-	-	37.75 µl

Table 1: Example of a PCR master mix table

After determining the required reagent volumes for one PCR reaction, you can simply multiply them by your sample number (plus the negative and positive controls) to get the total volumes for the entire PCR set-up. We recommend adding one additional aliquot to that result, as some of the master mix may be lost during pipetting due to evaporation, adherence to the tip, or pipetting inaccuracies.

That's it, you are now ready to set up your PCR reactions by following the best pipetting practices listed below.

Best PCR pipetting practices

Start by preparing your master mix from all the components listed above, except the template DNA. The huge advantage of preparing the entire quantity of master mix needed for an experiment, and subsequently transferring single aliquots into PCR strips or plates, is that you can pipette higher volumes with better accuracy. On top of that, it reduces pipetting steps, making the entire process less tiring and error prone. Since pipetting mistakes cannot be completely ruled out, you should add the master mix components in order of their price, starting with the most affordable reagent. This way, you waste less money if you have to start over.[6]

Once your master mix is finished, well mixed and dispensed into tubes or plates, you can add the template DNA. As the DNA samples are usually highly viscous and needed in small quantities, you should either dispense them into the master mix or onto the wall of the tube or well. After dispensing, keep the plunger depressed while dragging the tip gently along the wall of your labware to remove any residual liquid. In addition, we recommend using low retention tips.

If you're not using a hot start polymerase, cool your reagents throughout the entire process of master mix preparation and sample addition, to prevent non-specific amplification.

When you are ready to load your samples into the thermal cycler, check that they are tightly capped or sealed, and spin them down to ensure that no droplets remain on the labware wall during amplification.

Pipetting solutions for PCR reactions

Before discussing various pipetting solutions, we would like to address one of the most important aspects of liquid handling. No matter which pipettes you choose, ensure that they are well maintained by regularly calibrating them and checking their performance in between uses.

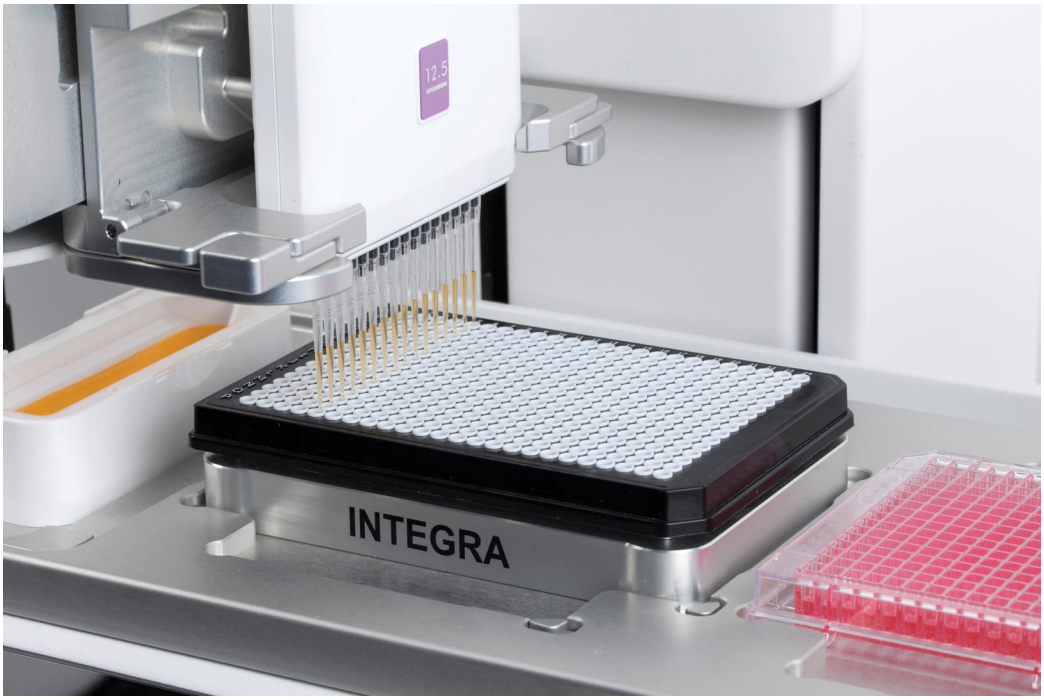
The most affordable pipettes for master mix preparation would be manual single channel models. However, as you need to accurately measure and mix several very expensive reagents, we recommend investing in electronic single channel pipettes. The motor-controlled piston movement guarantees that they always dispense the exact desired volume, minimizing variability to increase the precision and accuracy of pipetting.

For the container, you can either prepare the master mix in a tube or, if you intend to transfer it with an electronic multichannel pipette, in a low dead volume reagent reservoir. The

combination of an electronic multichannel pipette and a reservoir is ideal for this step, because you can fill several tubes or wells simultaneously. On top of that, electronic multichannel pipettes usually feature a Repeat Dispense mode, allowing you to aspirate a large volume of master mix, then dispense it into multiple smaller aliquots. It is also possible to use an electronic single channel pipette if you have a low throughput.

To add template DNA to the master mix aliquots, an adjustable tip spacing pipette can be very handy if the labware format of your samples doesn't match the container used for PCR amplification. For example, it allows you to transfer several template DNA samples from microcentrifuge tubes to an entire row or column of a 96 well plate in one step.

High throughput labs might even want to take advantage of automated solutions for master mix plating and sample transfer, such as a pipetting platform that is capable of automating electronic pipettes.



ASSIST PLUS pipetting robot transferring master mix into a 384 well PCR plate

How to prevent PCR contamination

Several preventative measures should be taken to avoid contaminating your master mix or template DNA with amplicons that were generated during previous PCRs.

One of the most effective means is to physically separate the master mix preparation, template DNA addition, amplification and analysis areas from one another, and to work in laminar flow or biosafety cabinets. Each work zone, and its corresponding equipment, should be cleaned before and after an experiment, and tools used in one area should never enter another one.

When it comes to consumables, make sure you purchase sterile products that are certified to be free from DNase, RNase and PCR inhibitors. Pipette tips should form a perfect seal with the pipette to eliminate contamination that may occur when tips drip or fall off. Using filter tips will also avoid the risk of aerosols entering your pipettes and contaminating subsequent PCR reactions.

As you're a potential source of contamination too, always wear gloves to prevent introducing enzymes, microbes and skin cells to the reaction, and change them when going from one area to another. On top of that, keep your tubes closed whenever possible during the entire PCR set-up.

Despite these preventative measures, you can't completely eliminate the possibility of contaminated PCR reactions. To avoid having to throw away your entire stock of a certain reagent if this occurs, prepare single use aliquots of your master mix components. You can also prepare aliquots of positive and negative controls, as well as serial dilutions of standards for quantitative PCR (qPCR) assays, ahead of time. Electronic pipettes with repeat dispense and serial dilute modes can be helpful for this task, not only to reduce the risk of contamination, but also to increase the efficiency of PCR set-up.

Conclusion

PCR is a fundamental technique in research, diagnostics and forensics. It often involves pipetting minuscule reagent volumes with tricky properties, so it can be difficult to obtain the desired results. On top of that, contamination can have a huge impact on results, as it's a very sensitive assay. We hope that the tips and tricks provided in this article will help you make your future PCR reactions a success. Many of these recommendations can also be applied to other amplification assays, such as reverse transcription and qPCR, loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA).

1.3 Setting up a PCR lab from scratch

PCR reactions are very sensitive and create a large number of copies of nucleic acids from minute amounts of starting material. This makes them a fundamental and highly effective molecular biology technique. However, because it is prone to amplicon and sample contamination, planning and designing of your PCR lab space will need careful consideration.

Designing your PCR lab

Ideally, a PCR lab should have two rooms with two areas, each designed for specific tasks. The first room should be exclusively used for pre-PCR activities and divided into a master mix preparation area and a sample preparation area. Air pressure should be slightly positive to prevent aerosols from flowing in.

The second room should have a dedicated area for nucleic acid amplification, and another one for product analysis. Air pressure should be slightly negative to ensure that amplicon aerosols don't leave the room.

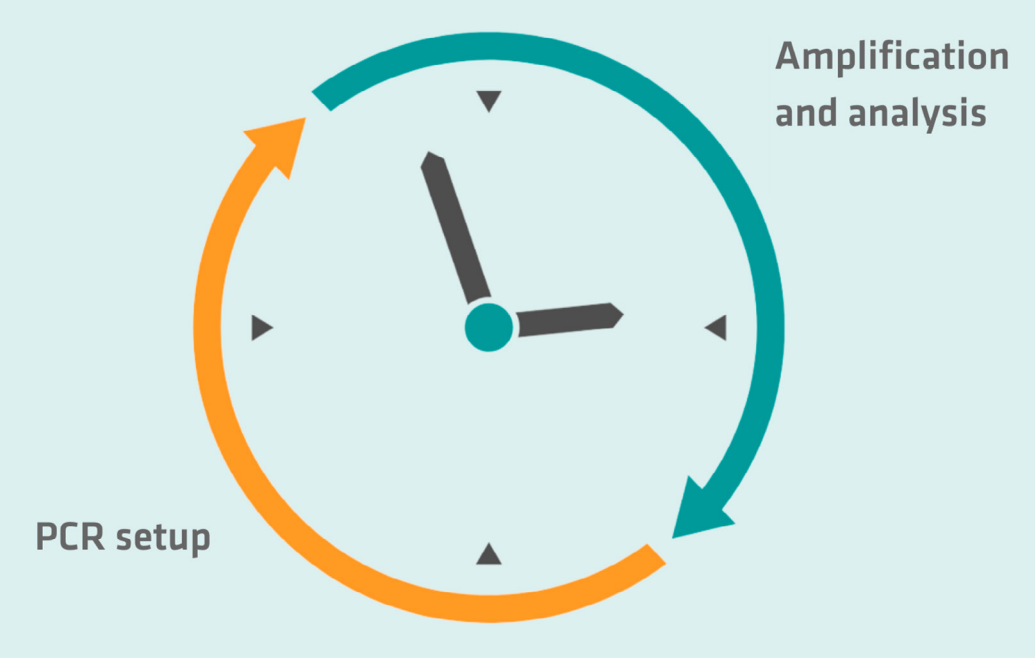
If you're lacking in space or budget for a two-room PCR lab, you can set up the pre-PCR and amplification and analysis areas in the same room, but ensure they are as far from one another as possible.

Having pre-PCR activities spatially separated from the amplification and analysis area – either in different rooms or in separate benches – is very important, because you usually have a low amount of the nucleic acid sample during preparation and a very high concentration after

amplification. This means that if you analyze your PCR in the same space as you prepare your master mix and samples, you may get false-positive results due to amplicon contamination.

You should also ensure that your lab set-up follows a unidirectional workflow. No materials or reagents used in the amplification and analysis areas should ever be taken into the pre-PCR space without a thorough decontamination. This means that you'll need dedicated equipment for each area, e.g., two different sets of pipettes. This unidirectional workflow should also apply to lab staff. If you've been working in the amplification and analysis areas, and you need to go back to the pre-PCR area, change your personal protective equipment, as it may have been contaminated by amplicon aerosols.

Another precautionary measure to take into account when setting up your PCR lab, in addition to the spatial separation, is temporal separation. You could, for example, consider setting up your PCR reactions in the morning, and perform the amplification and analysis steps in the afternoon. This may limit your flexibility, but will prevent contamination issues and having to repeat your experiment.



PCR equipment tips

PCR labs typically require a variety of equipment, such as centrifuges, vortex mixers, pipettes, fridges and freezers, thermal cyclers and analysis instruments (e.g., electrophoresis systems). Depending on the size of your lab and your applications, the amount of equipment you'll need may vary. Instead of providing you a 'shopping list', we will outline what you should look for when purchasing equipment and consumables in order to keep contamination of your PCR reactions to a minimum.

Laminar flow or biosafety cabinet

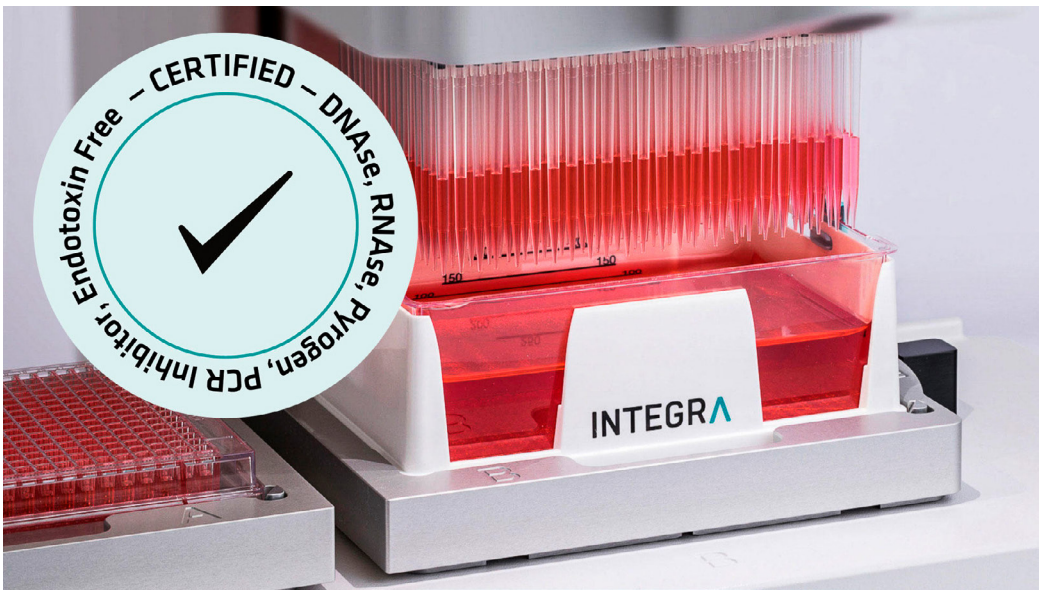
Since you can never be 100 % certain that there are no amplicon aerosols in your pre-PCR space, you should set up your PCR reactions in a laminar flow hood or biosafety cabinet, decontaminated with a bleach solution prior to starting and after you finish your work.

Pipette tips and other consumables

Despite being more expensive than normal pipette tips, using filter tips for your PCR set-up will avoid aerosols entering and contaminating your pipette, and avoid aerosols that might already be present in your pipette contaminating your master mix or samples. To minimize your filter tip consumption, first fill all your tubes with the master mix using only one tip or set of tips – if you're using multichannel pipettes – and follow with your samples, using one tip per sample. Adding the sample last is also recommended because it's easier to dispense it into a liquid than into an empty tube, and because it reduces the risk of aerosolizing your sample as you pipette.

For consumables, you should make sure that you have enough small vials available in your lab when your PCR reagents arrive. Aliquoting them into smaller containers will increase their shelf life and prevent them from going through too many freeze/thaw rounds. If your reagents get contaminated, it will also save you from throwing away your entire supply, as you'll have clean aliquots available for a second PCR.

Finally, you'll need to make sure that all consumables and equipment are free of DNase, RNase and PCR inhibitors. Always choose sterile products from manufacturers that can certify that their tips and consumables are free of any of these potential contaminants.



Cleaning and contamination control

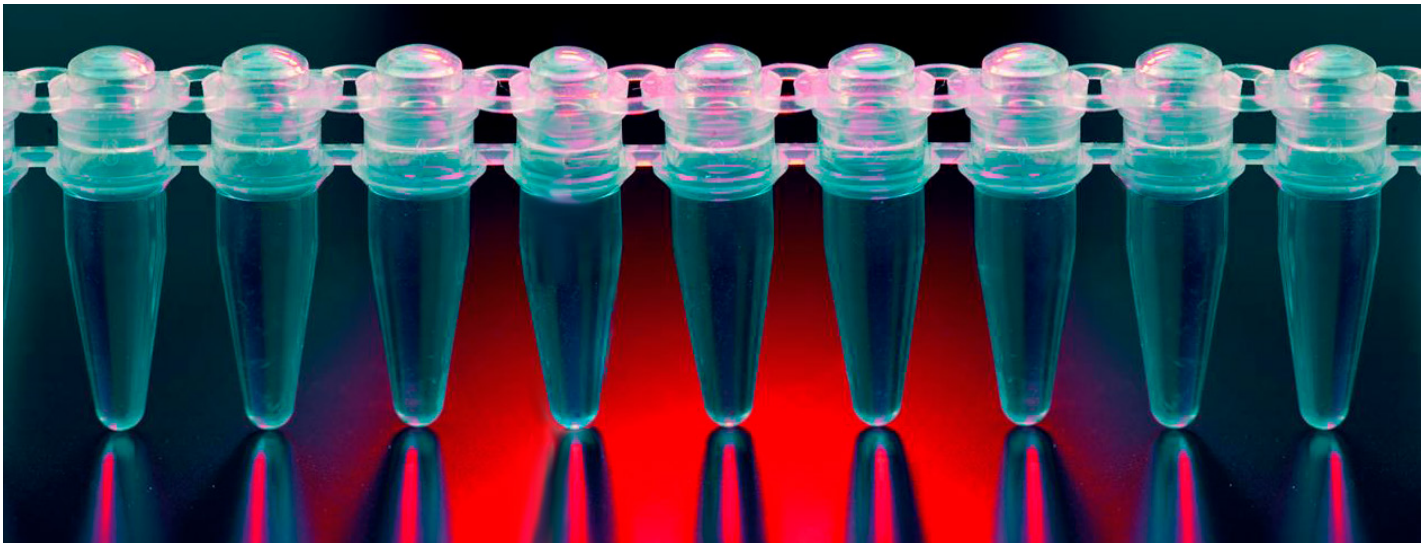
You won't need to worry about cleaning or contamination control when setting up your lab, but you will when your lab is up and running. We will briefly address this topic below.

Whether you decide to set up your PCR reactions in a laminar flow hood, a biosafety cabinet or an open bench, you will need to decontaminate your work space before and after set-up by wiping it with a freshly made bleach solution and distilled water. The same process should be performed in the amplification and analysis areas. You should also make sure you clean your pipettes, equipment, doorknobs, and the handles of your fridges and freezers regularly.

Because PCR assays are so sensitive, all the preventative measures described here may still not guarantee that your experiments will never get contaminated. It is therefore necessary to include the appropriate controls to detect contamination early. Always include negative and positive controls, as this will help identifying master mix contaminations, and confirm the performance of the extraction protocol, reagents and amplification steps. Additionally, you should monitor the positivity rate in your lab, and ensure that unexpected increases in detection have identifiable causes, e.g., a seasonal outbreak.

Conclusion

In this article, we covered how to set up your PCR lab to ensure spatial and temporal separation, and prevent contamination. We also outlined the key factors to consider when purchasing equipment and consumables for your lab, to maintain safety and reduce wastage. Lastly, we highlighted the importance of regular workspace cleaning and the use of appropriate controls to detect any contamination early on. We hope that you are still just as excited about setting up your PCR lab, and that this article has made the task less daunting for you.



1.4 qPCR: How SYBR® Green and TaqMan® real-time PCR assays work

qPCR, or real-time PCR, is a widely used method to quantify DNA sequences in samples. This article gives you a comprehensive introduction to the topic, explaining how dye-based and probe-based qPCR assays (like SYBR Green and TaqMan) work, how to validate your amplification experiments, and how to analyze your qPCR data.

qPCR vs PCR vs RT-PCR

Before explaining how qPCR works, we would like to briefly outline its difference from standard PCR and RT-PCR.

Whereas standard PCR monitors DNA amplification upon reaction completion, qPCR uses fluorescent signals to monitor DNA amplification as the reaction progresses. This is why qPCR is also referred to as real-time PCR, quantitative PCR or quantitative real-time PCR.

RT-PCR, not to be confused with real-time PCR, stands for reverse transcription PCR and can be used to amplify RNA target sequences. It involves an initial incubation of the sample RNA with a reverse transcriptase enzyme and a DNA primer before amplification.

How qPCR works

qPCR relies on fluorescence from intercalating dyes or hydrolysis probes to measure DNA amplification after each thermal cycle. The most common dye-based method is SYBR Green, and the most common probe-based method is TaqMan, which is why this article will focus on these two qPCR techniques.

SYBR Green qPCR

Like standard PCR, the SYBR Green protocol consists of denaturation, annealing and extension phases. The difference being that you add some double-stranded DNA binding dye, SYBR Green I, to your master mix during qPCR setup. This fluorescent dye intercalates into double-stranded DNA sequences during the extension phase, where it shows a strong increase in fluorescent signal. Measuring this signal at the end of every thermal cycle will allow you to determine the quantity of double-stranded DNA present.

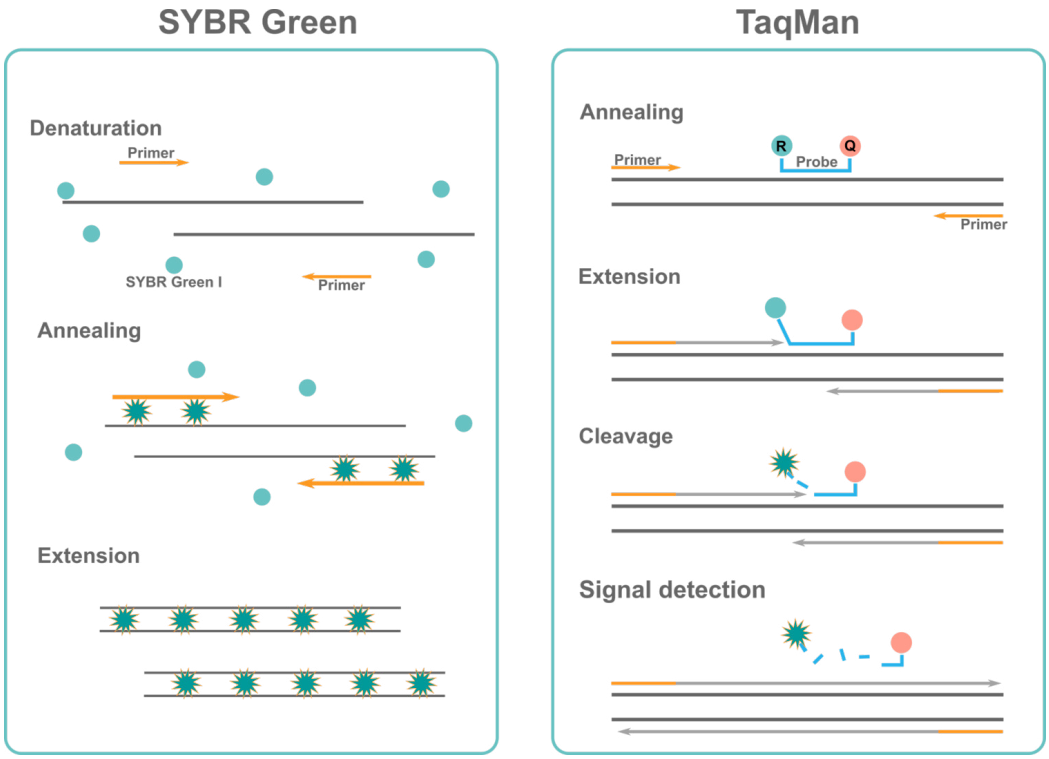
The downside of the SYBR Green assay is that the dye binds to any double-stranded DNA sequence. This means that you could also detect fluorescence emitted from non-specific qPCR products, such as primer dimers. To eliminate this risk, check the reaction specificity by performing a melting curve analysis, explained later in the article, or use the TaqMan method.

TaqMan qPCR

Instead of using intercalating dyes, this assay uses TaqMan probes with a 5' fluorescent reporter dye and a 3' quencher dye. These probes are target-specific, and only bind to the DNA sequence of interest downstream of one of the primers during the annealing step. When the enzyme *Taq*-polymerase encounters the TaqMan probe during the extension phase, it displaces and cleaves the 5' reporter dye. Once the reporter dye has been separated from the quencher dye, its measurable fluorescent signal at the end of every qPCR cycle increases significantly. The second DNA strand is synthesized in parallel but, as no probe is attached to it, this process can't be monitored by fluorescence measurements.

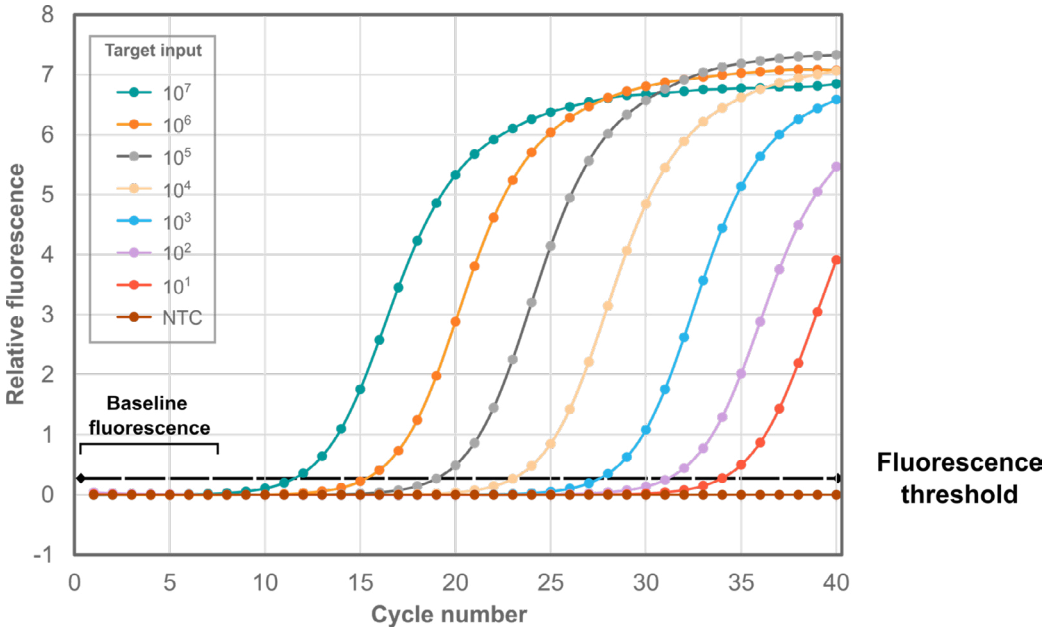
Compared to the SYBR Green assay, the use of TaqMan probes is more expensive, but also offers two significant advantages:

- the TaqMan assay only measures amplification progression of the target sequence, as the probes are target specific.
- you can monitor the quantity of various qPCR products in a single reaction by adding different primers and TaqMan probes with different reporter dyes to the master mix. This multiplex approach allows you to detect several fluorescent signals at the end of every thermal cycle.



Amplification plot

For both qPCR methods, data is visualized in an amplification plot, with the number of thermal cycles on the x-axis, and the fluorescent signals detected on the y-axis:



As can be seen, fluorescence remains at background levels during the first thermal cycles. Eventually, the fluorescent signal reaches the fluorescence threshold, where it is detectable over the background fluorescence. The cycle number at which this happens is called the threshold cycle (Ct). If the Ct value for a sample is high, it means that little starting material was present, and vice versa. Please note that you should always analyze at least three replicates of each sample, as tiny pipetting errors during qPCR set-up can result in huge differences in Ct values.

The Ct value is sometimes also referred to as crossing point (Cp), take-off point (TOP) or quantification cycle (Cq) value, with MIQE guidelines suggesting using Cp value to standardize terminology.[1] In this article we will continue to call it Ct, as this is the most commonly used term.

MIQE guidelines

The MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines describe the minimum information necessary for evaluating qPCR experiments. When publishing a manuscript, the scientist needs to provide all relevant experimental conditions and assay characteristics described by the MIQE guidelines, allowing reviewers to assess the validity of the protocols used, and enabling other scientists to reproduce the experiments.

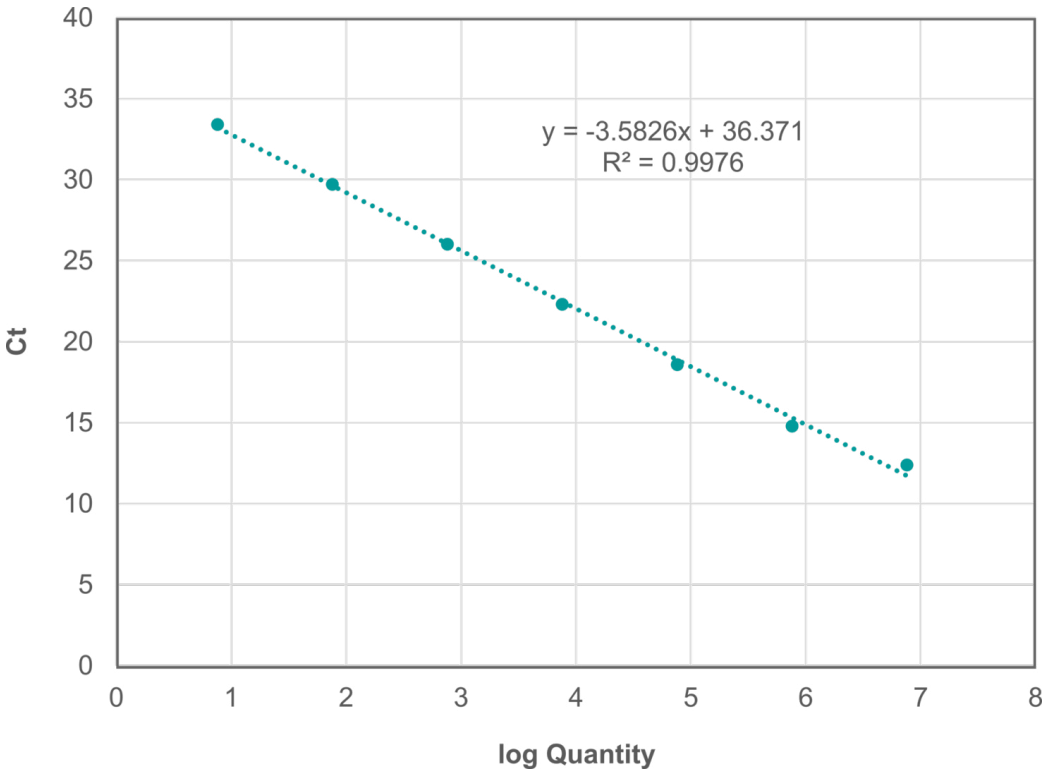
Validation of qPCR assays

qPCR amplification plots can be analyzed using absolute or relative quantification. However, before explaining qPCR data analysis, we need to quickly discuss how to determine reaction efficiency and specificity. You don't need to perform these steps after every qPCR experiment, but should always validate these two values when setting up a new qPCR protocol or changing your current workflows.

Reaction efficiency

A perfect qPCR assay would have a reaction efficiency of 100 %, which means that the number of template DNA copies would double at every thermal cycle. As this is almost impossible to achieve in practice, reaction efficiencies between 90 and 110 % are considered to be ideal.

To calculate the reaction efficiency of your assay, you need to set up a 10-fold serial dilution of an undiluted sample with a known amount of template DNA. After running a qPCR, create a standard curve with the log of the starting quantity on the x-axis and the Ct values on the y-axis.



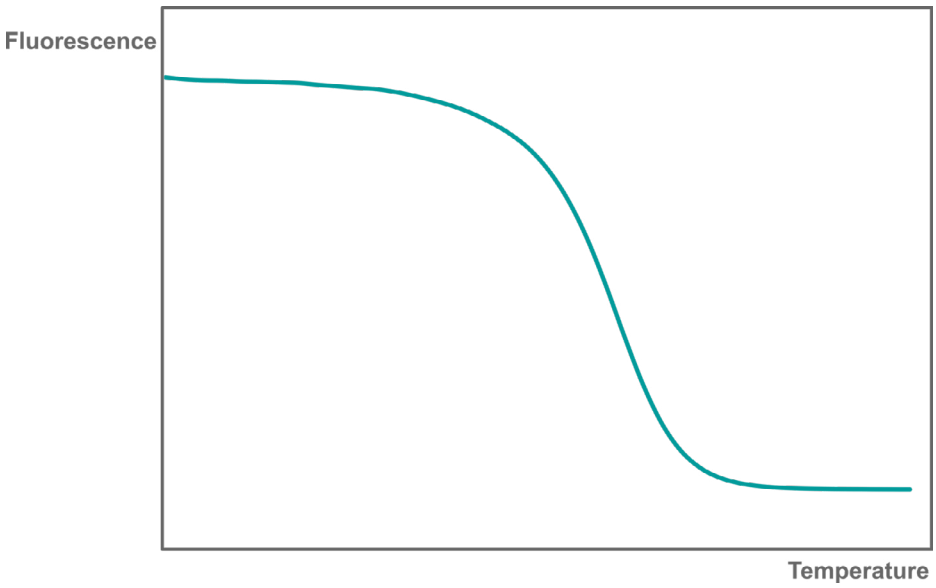
Using the equation for the linear regression line (y = mx + b), you can now determine the reaction efficiency as follows[2]:

Efficiency = $(10^{(-1/m)}-1) \times 100$

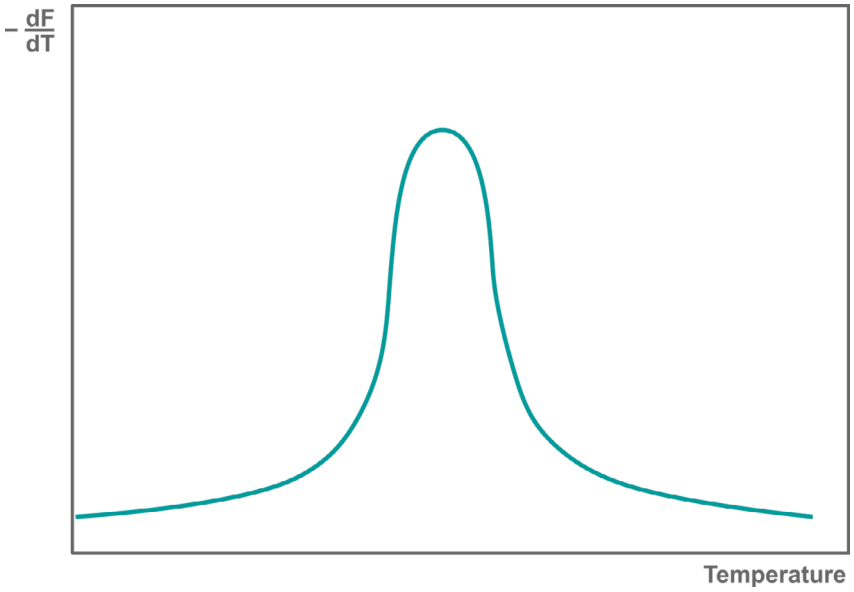
In our example, m would be -3.5826, resulting in a reaction efficiency of 90.1634 %.

Reaction specificity

Reaction specificity can be determined using a melting curve analysis, allowing you to identify non-specific qPCR products and primer-dimers. To perform a melting curve analysis, run a qPCR assay with a fluorescent intercalating dye like SYBR Green I. After amplification, the thermal cycler increases the temperature step by step while monitoring fluorescence. As the temperature increases, the dsDNA qPCR products present will denature, resulting in a decreasing fluorescent signal:



Then, plot the change in slope of this curve as a function of temperature to obtain a melting curve:



If you're observing only one melting peak like the image above, your qPCR assay is specific. If there are several melting peaks, primer-dimers and/or non-specific products were amplified during qPCR, and you should redesign your experiment to increase its specificity.

Analysis of qPCR data

qPCR data can be analyzed by absolute or relative quantification, and the method suitable for your experiment depends on your goal. Absolute quantification allows you to determine the quantity of starting material that was present in a given sample before amplification. For example, this method can be used to determine the viral load of a patient sample. Relative quantification is applied to compare levels or changes in gene expression between different samples. For example, it is helpful to investigate whether the expression of a certain gene is higher in a tumor sample than in a healthy control sample.

Absolute quantification

After qPCR amplification, you will have produced an amplification plot, and know the Ct value of each sample. To find the quantity of starting material present in your samples, you need to compare these values to a standard curve. As seen above in the section on reaction efficiency, a standard curve is obtained by amplifying a serial dilution of a sample with a known amount of template DNA, then plotting the Ct values against the log of the starting quantities.

The equation for the linear regression line of the standard curve (y = mx + b) will then allow you to calculate the quantity of starting material for each sample. As y corresponds to the Ct value, and x to the log quantity, the equation for the linear regression line is equivalent to:

$$Ct = m(\log \text{quantity}) + b$$

Solving this equation for the quantity will give you the formula:

$$\text{Quantity} = 10^{((Ct-b)/m)}$$

This will allow you to quickly determine the quantity of starting material in each sample.

$$Y = mx + b \rightarrow Ct = m(\log \text{quantity}) + b \rightarrow \text{Quantity} = 10^{((Ct-b)/m)}$$

Relative quantification

To compare levels or changes in target gene expression between different samples and a control sample, you first need to define a reference gene whose expression is unregulated. Then, run a qPCR to obtain the Ct values for the reference gene, target gene in your samples, and the control sample.

If the reaction or primer efficiencies for the reference and target genes are near 100 %, and within 5 % of each other, you can then use the ΔΔCt method – also called the Livak method – to determine the expression rate of the target gene in your samples. However, if the efficiencies are further apart, you should use the Pfaffl method. To learn how to calculate reaction efficiencies, please refer to the 'Reaction efficiency' section earlier in the article.

The calculations for the two methods are as follows:

ΔΔCt method

Normalize the Ct of the target gene to the Ct of the reference gene for each sample and the control sample:

ΔCt(sample) = Ct(target gene) – Ct(reference gene)
ΔCt(control) = Ct(target gene) – Ct(reference gene)

Normalize the ΔCt of each sample to the ΔCt of the control sample:

ΔΔCt(sample) = ΔCt(sample) – ΔCt(control)

Since the calculations are in logarithm base 2, you must use the following equation to get the normalized expression ratio for each sample:

Normalized expression ratio = $2^{-\Delta\Delta Ct(sample)}$

Pfaffl method

Calculate the ΔCt of the target gene for each sample:

ΔCt(target gene) = Ct(target gene in control) – Ct(target gene in sample)

Calculate the ΔCt of the reference gene for each sample:

ΔCt(reference gene) = Ct(reference gene in control) – Ct(reference gene in sample)

Calculate the normalized expression ratio for each sample:

Normalized expression ratio = $((E_{target\ gene})^{\Delta Ct(target\ gene)}) / ((E_{reference\ gene})^{\Delta Ct(reference\ gene)})$

$E_{target\ gene}$: Reaction efficiency of the target gene

$E_{reference\ gene}$: Reaction efficiency of the reference gene

The normalized expression ratio obtained using the ΔΔCt or the Pfaffl method is the fold change of the target gene in your sample relative to the control. A normalized expression ratio of 1.2 would mean that you have a gene expression of 120 % relative to the control.

Conclusion

We hope that this article answered all your questions regarding qPCR methods, assay validation and data analysis.

1.5 How to design primers for PCR

PCR is one of the most widespread molecular biology applications, yet it is anything but simple to perform. Common issues – such as a low product yield or non-specific amplification – are often caused by poorly designed PCR primers. We have therefore summarized the most important information on designing PCR primers to help you overcome these challenges.

What is a PCR primer?

Primers – also called oligonucleotides or oligos – are short, single-stranded nucleic acids used in the initiation of DNA synthesis. During PCR reactions, they anneal to the plus and minus strands of the template DNA, flanking the sequence that needs to be amplified.

How to design PCR primers?

PCR primers have to be tailored to both the region of interest of your template DNA and your reaction conditions. This means that, unlike the other components of the PCR master mix, you can't just buy them, but need to design them yourself using a primer design tool. These tools allow you to set parameters such as primer length, melting temperature, GC content and more. Read on to learn what the optimal values for each of these parameters are, and how they affect your PCR assay.

Primer length

The optimal length of a PCR primer lies between 18 and 24 bp. Longer primers are less efficient during the annealing step, resulting in a lower amount of PCR product. Conversely, shorter primers are less specific during the annealing phase, leading to more non-specific binding and amplification. However, there are exceptions to this rule. For example, some scientists have successfully used miniprimers that are 10 bp long to expand the scope of detectable sequences in microbial ecology assays.

Target sequence length

The target sequence to be amplified should ideally be between 100 and 3000 bp for standard PCR assays, and 75 and 150 bp for qPCR assays. Longer sequences usually need special enzymes and reaction conditions to ensure that they are completely and specifically amplified.

Primer melting temperature

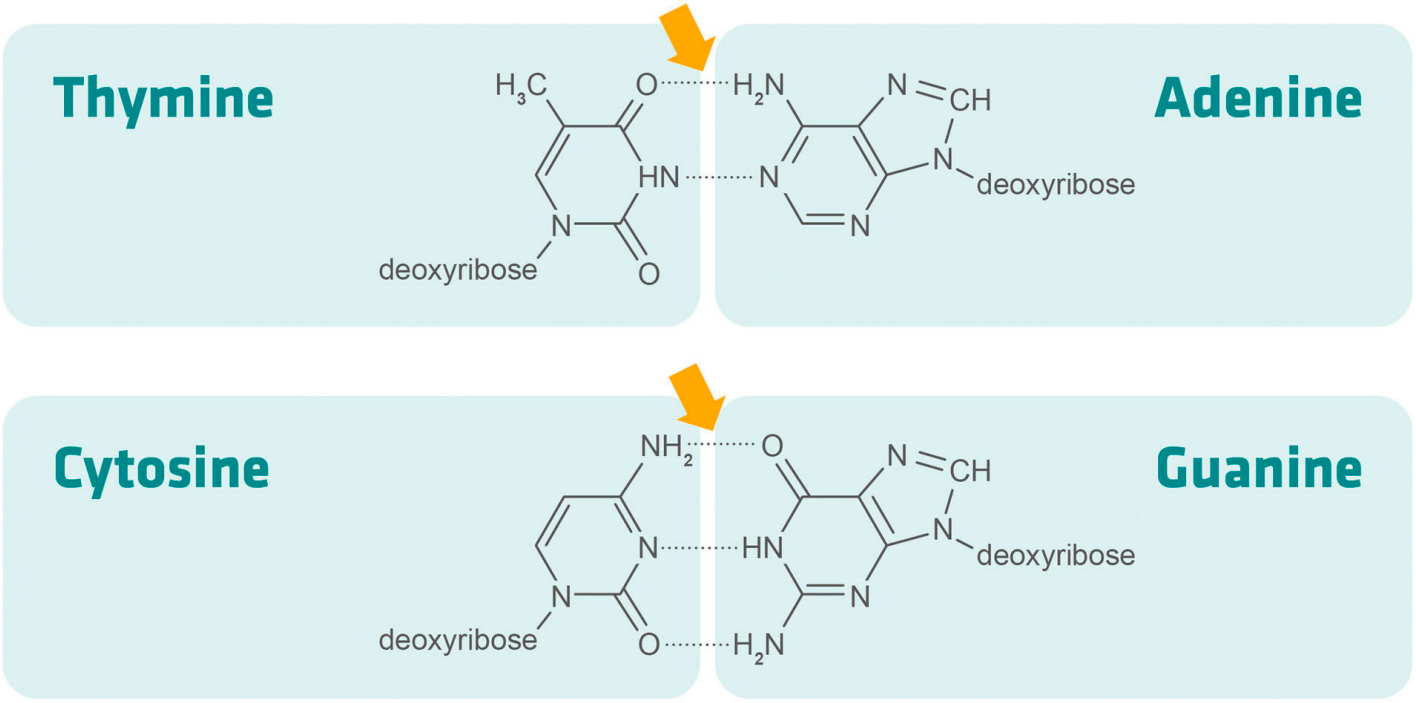
The primer melting temperature (Tm) can be defined as the temperature at which half of the primers dissociate from the template DNA. It is usually between 50 and 60 °C, and the melting temperatures of the forward and reverse primers should be within 5 °C of each other. If the two melting temperatures are further apart, it won't be possible to find an annealing temperature that allows both primers to bind to the template DNA.

Most primer design tools use the nearest neighbor method to calculate primer melting temperatures, as it's the most accurate. However, if you want to make an approximate calculation yourself, you can use this formula:

$$Tm = 4\ °C \times (G+C) + 2\ °C \times (A+T)$$

Tm: melting temperature
G, C, A, T: number of nucleobases (guanine, cytosine, adenine, thymine) in the primer

As indicated in the formula above, G-C bonds are harder to break than A-T bonds – because G-C base pairs are linked by three hydrogen bonds, and A-T base pairs by two – and the length of the primer also impacts its melting temperature. This means that you can either increase the GC content of a primer (provided the template allows for this), or slightly extend its length if its melting temperature is too low.



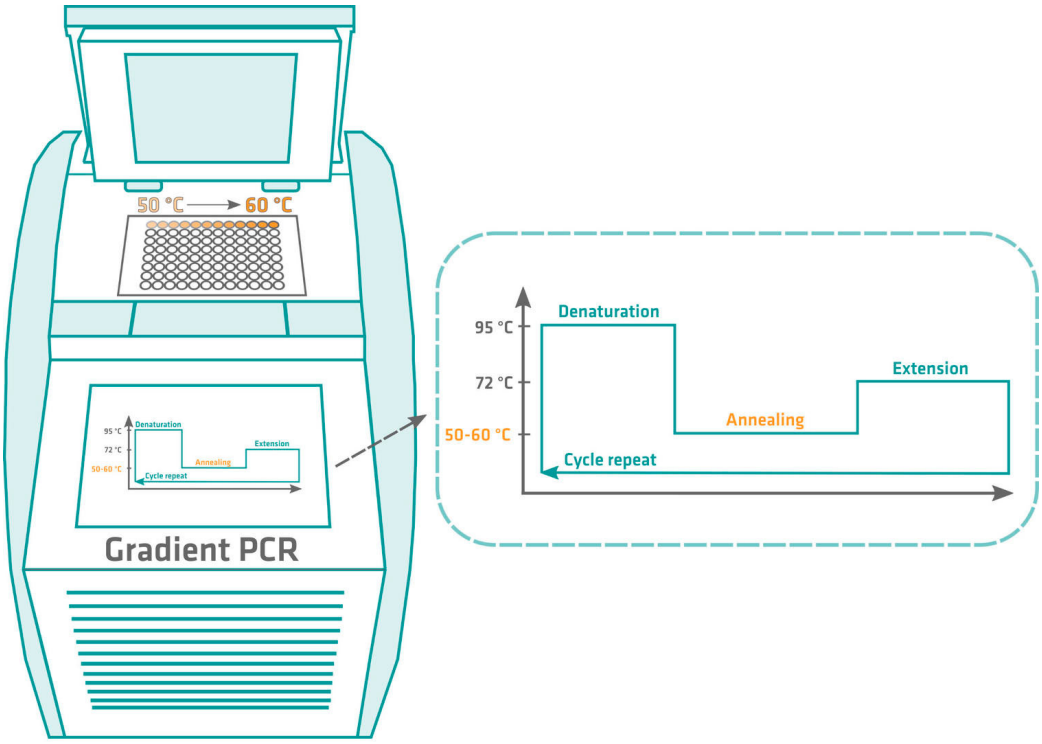
Primer annealing temperature

The primer annealing temperature (Ta) is the temperature needed for the annealing step of the PCR reaction to allow the primers to bind to the template DNA. The theoretical annealing temperature can be calculated as follows:

$$Ta = 0.3 \times Tm_{(primer)} + 0.7 \times Tm_{(product)} - 14.9$$

Ta: primer annealing temperature
$Tm_{(primer)}$: lower melting temperature of the primer pair
$Tm_{(product)}$: melting temperature of the PCR product

Once you've calculated the theoretical annealing temperature, the optimal annealing temperature needs to be determined empirically. To achieve this, perform a gradient PCR, starting a few degrees below the calculated annealing temperature, and ending a few degrees above. After amplification, run a gel, and the sample producing the clearest band contains the largest quantity of PCR product, making its annealing temperature the optimal one for your primers. Usually, you'll get a value that is 5 to 10 °C lower than the primer melting temperature.



It's important to determine the optimal annealing temperature, as primers could form hairpins or bind to regions outside the DNA sequence of interest if it's too low, producing non-specific and inaccurate PCR products. If the annealing temperature is too high, the primers won't sufficiently bind to the template DNA, and you'll obtain little to zero amplicons.

GC content

As seen before, G-C base pairs are stronger than A-T base pairs, which means that a higher GC content ensures a more stable binding between the primers and the template DNA. The optimal GC content of a primer lies between 40 and 60 %, and primers should have two to three Gs and Cs at the 3' end to bind more specifically to the template DNA.

Runs and repeats

Avoid runs of four or more single bases – such as ACCCCC – or dinucleotide repeats (for example, ATATATATAT), as they can cause mispriming.

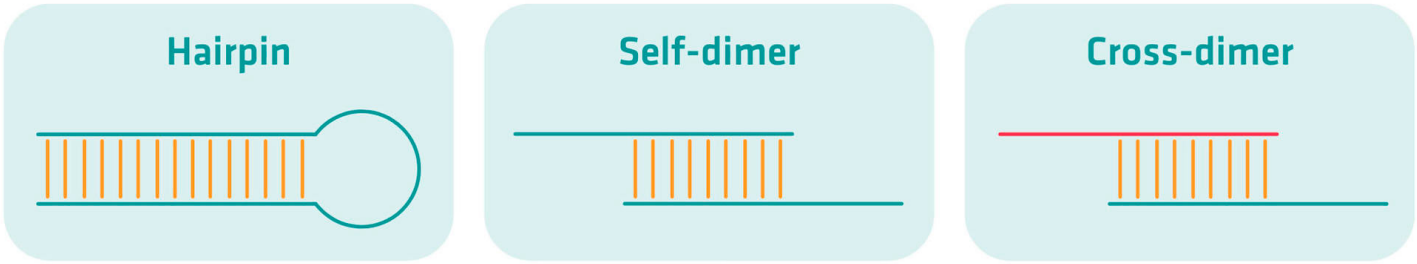
Cross homology

If a primer is homologous to a template DNA sequence outside the region of interest, these sequences will be amplified too. Therefore, you should always test the specificity of your primer design against genetic databases; for example, by ‘blasting’ them through NCBI BLAST software.

Secondary structures

There are three different types of secondary structures – also called primer dimers – that can form during a PCR assay:

- Hairpins: caused by intra-primer homology – when a region of three or more bases is complementary to another region within the same primer – or when a primer melting temperature is lower than the annealing temperature of the reaction.
- Self-dimers: formed when two same sense primers have complementary sequences – inter-primer homology – and anneal to each other.
- Cross-dimers: formed when forward and reverse primers anneal to each other when there is inter-primer homology.



Your PCR product yield will be less if secondary structures form and remain stable above the annealing temperature of your reaction, as the primers bind to themselves or another primer instead of the template DNA. This is why your primer design tool should be able to check for, and warn you of stable secondary structures.

Mismatches and degenerated positions

Mismatches are primer bases that aren't complementary to the target sequence. They can be tolerated to a certain extent, and are sometimes even necessary; for example, when performing a multi-template PCR to amplify a set of similar target sequences from different bacteria with a single set of primers. Degenerate primers could help if mismatches negatively impact the performance of your PCR.

Degenerate primers have several different nucleotides in some of their positions. For example, instead of A you could have an equal concentration of A and T in a certain position. The codes for the different nucleotide combinations available for degenerate primers are as follows:

IUPAC NUCLEOTIDE CODE	BASE
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C or G or T
D	A or G or T
H	A or C or T
V	A or C or G
N	Any base

Conclusion

This article summarized the key points to consider when designing PCR primers to help avoid common issues like low product yield or non-specific amplification. We covered optimal primer and target sequence lengths, and ideal primer melting and annealing temperatures. We also provided helpful tips for other crucial factors such as GC content, runs and repeats, cross homology and the danger of stable secondary structures. Lastly, the article highlighted the value and pitfalls of mismatches and degenerated positions. That's it, after reading about all of this, you are sure to be a 'PCR Primer Pro'!

CHAPTER 2: INTEGRA Biosciences' PCR solutions

PCR is a robust method, but it's comprised of numerous stages, involving multiple precise pipetting steps that often prove time consuming and prone to errors. Temperature-sensitive reagents and samples may affect accuracy, and the varying viscosities of samples, as well as 'sticky' DNA, can be difficult to handle. On top of this, the repetitive nature of this work can also frequently result in user fatigue and handling mistakes.

Fortunately, the right tools can eliminate your pipetting predicaments, vastly improving the reproducibility and productivity of your PCR workflows. Here, we will demonstrate how our range of liquid handling solutions are perfect for PCR applications, allowing you to create a faster and more efficient workflow with fewer errors.

Manual and electronic pipettes

A good starting point for lower throughput PCR applications – up to half a plate per day – is our EVOLVE single or multichannel pipettes, which feature convenient volume adjustment dials to increase the accuracy and speed of manual handling. Our range of VIAFLO electronic pipettes is also suitable for low throughput PCR set-up, and can easily handle up to eight plates per day.

Learn more about **EVOLVE**

Learn more about **VIAFLO**

Adjustable tip spacing pipettes

PCR set-up usually requires transferring liquids between different labware formats which is tedious and highly error prone. Our VOYAGER adjustable tip spacing pipettes solve these problems, increasing speed and eliminating transfer errors, while ergonomic single-handed operation leaves the other hand free to handle labware.

Learn more about **VOYAGER**

96 and 384 channel pipettes

We have a wide range of options perfect for productive high throughput PCR set-up – more than eight plates per day – which are suitable for different lab sizes and budgets. Our VIAFLO 96 and VIAFLO 384 channel handheld electronic pipettes, as well as the MINI 96 channel portable electronic pipette, can reduce handling steps while increasing productivity and reproducibility.

Learn more about **VIAFLO 96** and **VIAFLO 384**

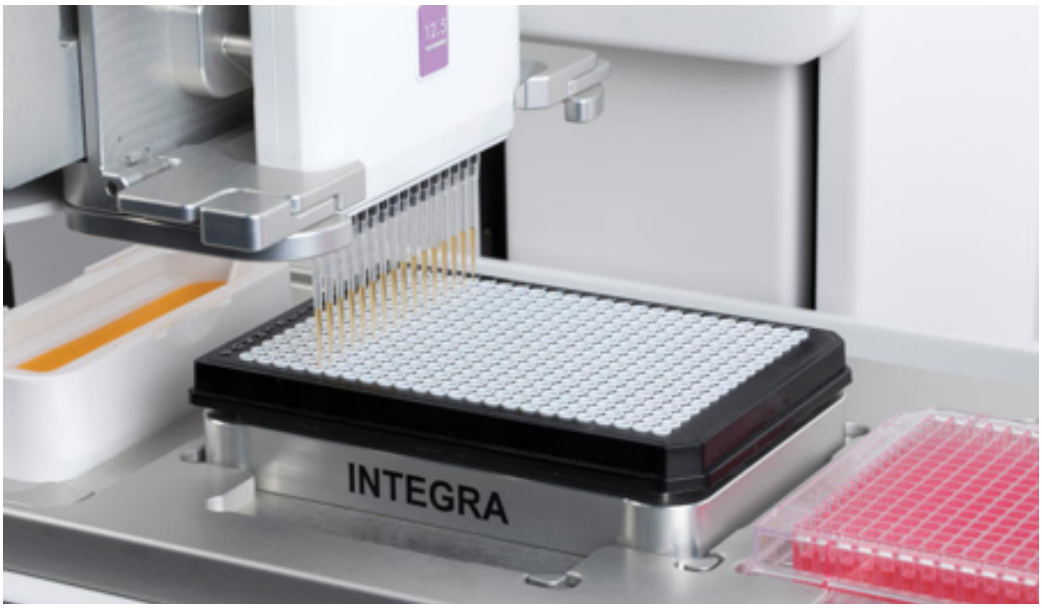
Learn more about **MINI 96**

Pipetting robots

INTEGRA also offers pipetting robots for high throughput laboratories, or for labs that want to reduce the risk of contamination due to manual processing. For example, the ASSIST PLUS pipetting robot can automate the D-ONE single channel pipetting module for master mix preparation, and VIAFLO and VOYAGER multichannel pipettes to take care of the multiple pipetting steps in PCR workflows.

Learn more about **ASSIST PLUS**

Learn more about **D-ONE**



Pipette tips

INTEGRA has developed GRIPTIPS pipette tips to complement its range of pipetting solutions. GRIPTIPS are free from RNase, DNase and PCR inhibitors, and perfectly fit all INTEGRA pipetting solutions, reducing the risk of contamination from tips that leak or fall off.

Learn more about **GRIPTIPS**

Sample reformatting

The transfer of samples between different labware formats is a slow, tedious and highly error-prone procedure when performed manually with a single channel pipette. The combination of the ASSIST PLUS pipetting robot and VOYAGER adjustable tip spacing pipette provide a novel solution for automated, accurate and efficient liquid transfer of multiple samples in parallel. For even higher throughput applications, the VIAFLO 96, VIAFLO 384 and MINI 96 offer a fast solution for whole plate transfers.

Learn more about sample transfers from plate to plate

Learn more about sample transfers from tubes to plates

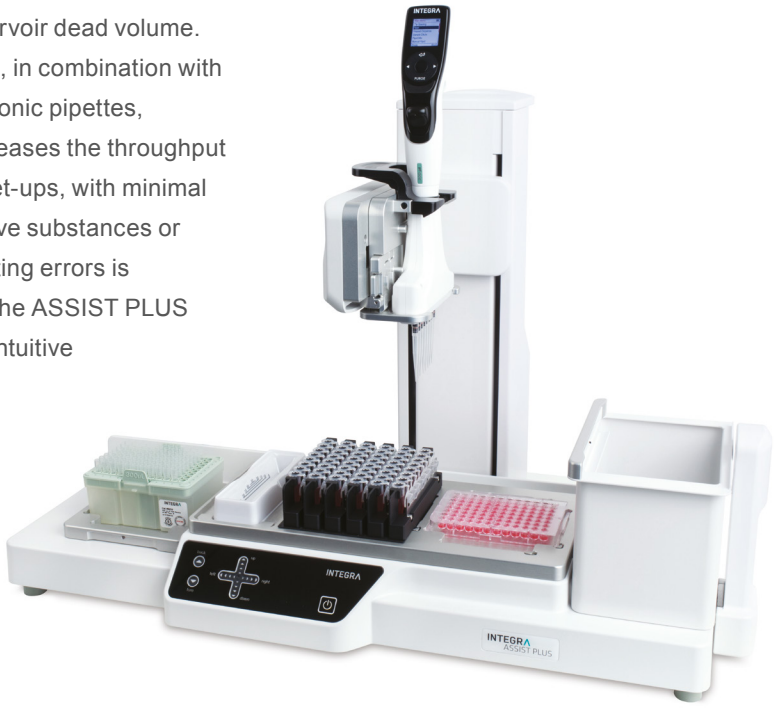
CHAPTER 3: Application notes

Our pipetting instruments are used across a broad spectrum of life sciences applications. To help share this knowledge and experience of using INTEGRA products with the wider scientific community, we have developed an application database which contains a wide range of useful application notes. Here are some of the most relevant app notes related to PCR protocols and workflows.

3.1 Efficient and automated 384 well qPCR set-up with the ASSIST PLUS pipetting robot

Using the ASSIST PLUS pipetting robot to automate set-up for a 384 well plate qPCR

Setting up a qPCR is a tedious process consisting of multiple pipetting steps. One particularly challenging task is reformatting from microcentrifuge tubes into a 384 well plate, which is time consuming and requires a lot of concentration. Another common problem is the loss of valuable and expensive substances, such as master mix and precious samples, due to the reservoir dead volume. The ASSIST PLUS pipetting robot, in combination with the VIAFLO and VOYAGER electronic pipettes, streamlines the workflow and increases the throughput and the reproducibility of qPCR set-ups, with minimal manual input. The loss of expensive substances or valuable samples due to reformatting errors is eliminated. The unique design of the ASSIST PLUS pipetting robot, together with the intuitive VIALAB software, offers exceptional flexibility and straightforward implementation.



Key benefits

- Automating the qPCR set-up with the VIAFLO 16 channel electronic pipette and the ASSIST PLUS pipetting robot allows considerably faster sample preparation, freeing up time for scientists to focus on other experiments.
- Automation of VOYAGER adjustable tip spacing pipettes with the ASSIST PLUS offers a reliable pipetting method that requires minimal manual intervention and eliminates the risk of reformatting errors.
- The use of low retention GRIPTIPS with heightened hydrophobic properties and SureFlo™ low dead volume reservoirs with an anti-sealing array helps to save precious samples and master mix. Combined with the high pipetting accuracy and precision of the ASSIST PLUS pipetting robot, this enables exceptionally low dead volumes to be achieved.
- The ASSIST PLUS pipetting robot, in combination with the intuitive VIALAB software, is quick to set up and easy to use.

Overview: qPCR set-up

The ASSIST PLUS pipetting robot is used to set up a 384 well format qPCR by pipetting 64 samples in triplicate with two different master mixes for the detection of two genes of interest (GOI 1 and GOI 2).

The protocol is divided into two programs that guide the user through all the steps of the qPCR set-up:

- **Program 1:** Mastermix_qPCR
- **Program 2:** Samples_qPCR

The ASSIST PLUS pipetting robot operates a VIAFLO 16 channel 125 µl electronic pipette with 125 µl sterile, filter, low retention GRIPTIPS for **program 1** and a VOYAGER 8 channel 12.5 µl electronic pipette with 12.5 µl sterile, filter, low retention GRIPTIPS for **program 2**.

Experimental set-up: Program 1 - master mix transfer (Mastermix_qPCR)

Prepare the pipetting robot deck as follows (**Figure 1**):

Deck position A:	Dual reservoir adapter – 2 x 10 ml reagent reservoir with SureFlo anti-sealing array (**Figure 2**) containing master mix 1 and 2.
Deck position B:	384 well PCR plate, placed on an INTEGRA cooling block in the landscape position.

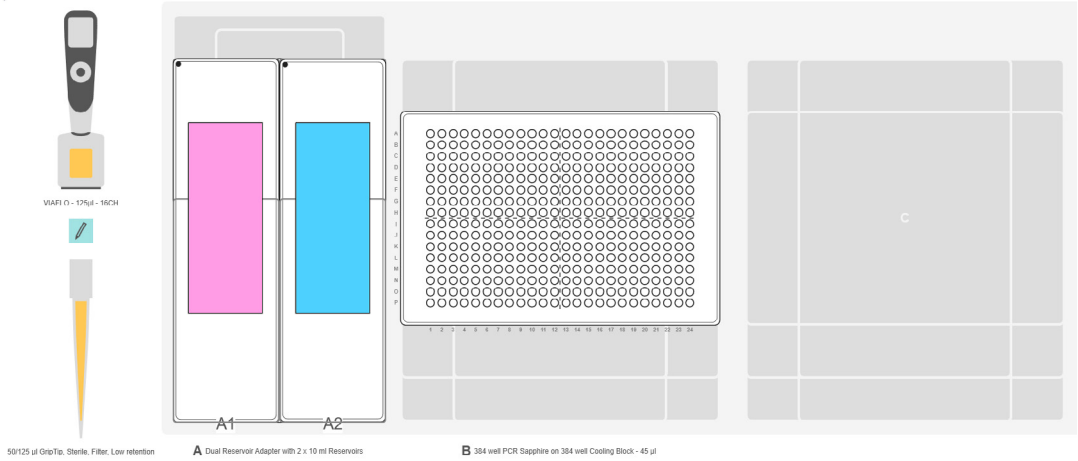


Figure 1: Set-up for the master mix transfer. Position A: dual reservoir adapter with 2 x 10 ml reagent reservoirs with SureFlo anti-sealing array. Position B: 384 well PCR plate, placed on an INTEGRA cooling block.

Figure 2: The INTEGRA dual reservoir adapter accommodates both 10 ml reagent reservoirs on one deck position.

Step-by-step procedure

1. Transfer master mixes into the 384 well plate

Add master mixes 1 and 2 into the left and right sides of the 384 well PCR plate, respectively.

Use an EVOLVE 5000 µl manual pipette with 5000 µl sterile, filter, low retention GRIPTIPS to fill the left 10 ml reagent reservoir with SureFlo anti-sealing array with 1.6 ml of master mix 1 and the right reservoir with 1.6 ml of master mix 2 (**position A**). Select and run the VIALAB program 'Mastermix_qPCR' on the VIAFLO 16 channel 125 µl electronic pipette with 125 µl sterile, filter, low retention GRIPTIPS. The ASSIST PLUS pipetting robot automatically transfers 7.5 µl of master mix 1 (pink) into the left half of the 384 well PCR plate and 7.5 µl of master mix 2 (blue) into the right half (**Figure 3**) using the Repeat Dispense mode with a tip touch on the surface of the liquid to increase pipetting precision. **Figure 4** shows the pipetting robot transferring the master mix into a 384 well plate.

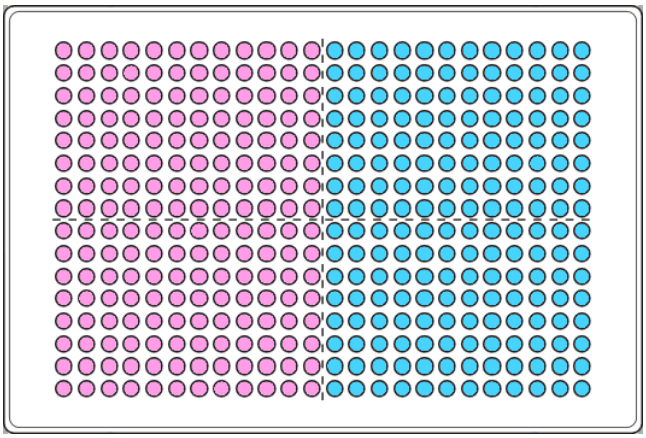


Figure 3: Pipetting scheme for master mixes 1 (pink) and 2 (blue).

Tips:

- Pre- and post-dispense steps are recommended to increase the accuracy and precision of pipetting. The pre- and post-dispense volumes should be between 3 and 5 % of the nominal volume of the pipette.
- The low retention GRIPTIPS are made from a unique polypropylene blend with heightened hydrophobic properties for superior accuracy and precision while pipetting viscous and low surface tension liquids.
- The reservoirs' SureFlo anti-sealing array and a unique surface treatment that spreads liquid evenly enable the pipette tips to sit on the bottom and still aspirate liquids accurately, reducing dead volumes.

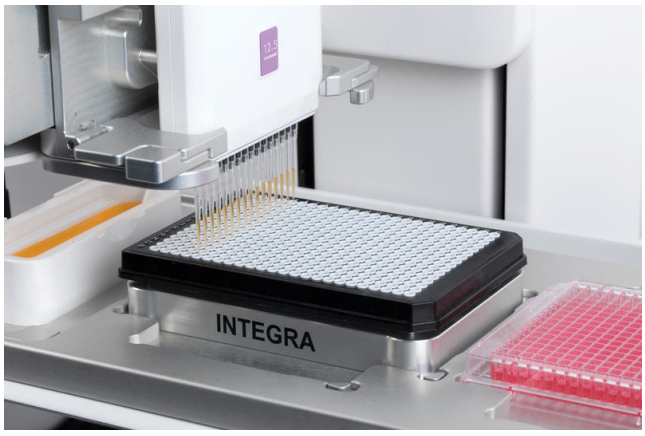


Figure 4: Example of the ASSIST PLUS pipetting robot transferring a master mix into a 384 well PCR plate.

Experimental set-up: Program 2 - sample transfer (Samples_qPCR)

Prepare the pipetting robot deck as follows (**Figure 5**):

Deck position B:	384 well PCR plate, placed on an INTEGRA cooling block.
Deck position C:	INTEGRA 1.5 ml microcentrifuge tube rack, with tubes containing samples 1-32.

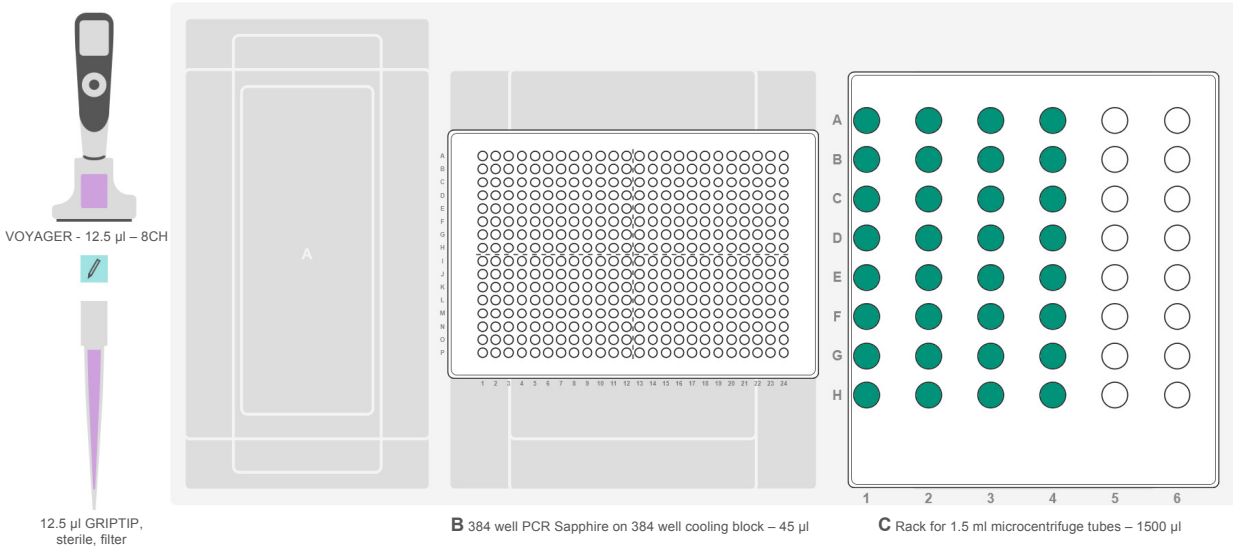


Figure 5: Set-up for the sample transfer protocol. Position B: 384 well PCR plate, placed on an INTEGRA cooling block. Position C: INTEGRA 1.5 ml microcentrifuge tube rack, with tubes containing samples 1-32 (**Figure 6**).

Figure 6: Example of the ASSIST PLUS pipetting samples from the INTEGRA microcentrifuge tube rack into a 96 well plate.

Step-by-step procedure

1. Sample transfer into the 384 well plate

Add the 64 samples in triplicate to the master mixes.

Place samples 1-32 in an INTEGRA 1.5 ml microcentrifuge tube rack on **position C**. Run the VIALAB program 'Samples_qPCR' on a VOYAGER 8 channel 12.5 µl electronic pipette to start the sample transfer. The ASSIST PLUS transfers 2.5 µl of the first 32 samples in triplicate into master mixes 1 and 2 (**Figure 7**, yellow/brown), using the Repeat Dispense mode with a tip touch on the side of the well to make sure that no droplets adhere to the GRIPTIPS. After this step, a prompt informs the user to place the second series of samples (33-64) on **position C**. The ASSIST PLUS pipetting robot continues by transferring 2.5 µl of the samples in triplicate into the other half of master mixes 1 and 2 (**Figure 7**, green).

Tip: Use sterile, filter, low retention GRIPTIPS for optimal liquid recovery of precious solutions, such as the master mix and samples.

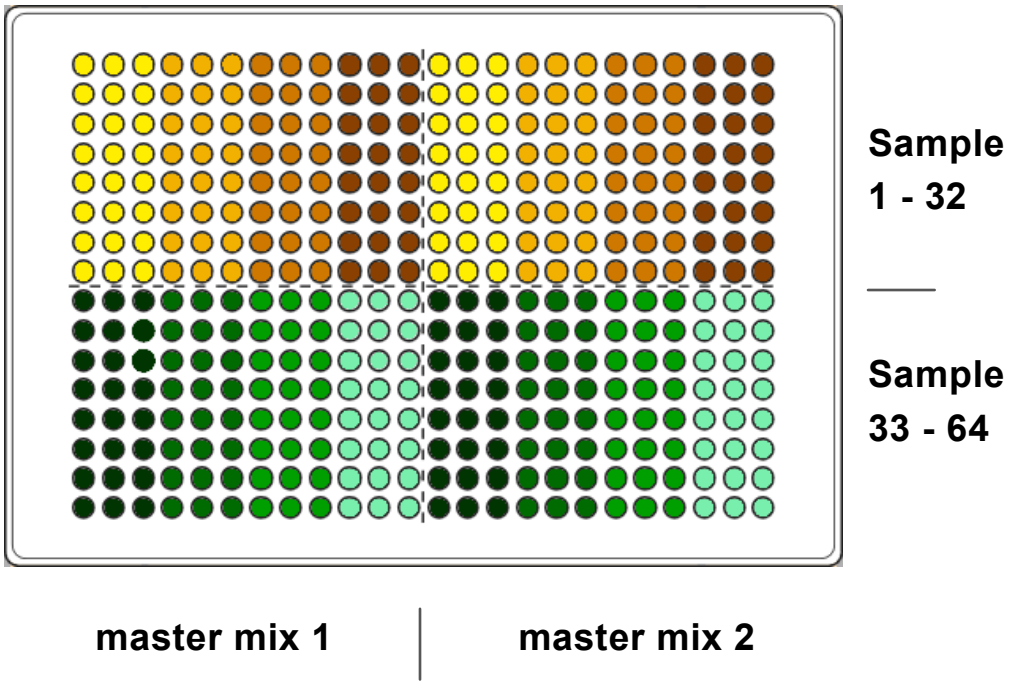


Figure 7: Pipetting scheme of the qPCR assay.

Remarks

VIALAB software:

The VIALAB program can easily be adapted to fit the user's demands, especially if specific labware, incubation times or protocols are needed.

Partial plates:

The programs can be adapted at any time to a different number of samples, giving laboratories total flexibility to meet current and future demands.

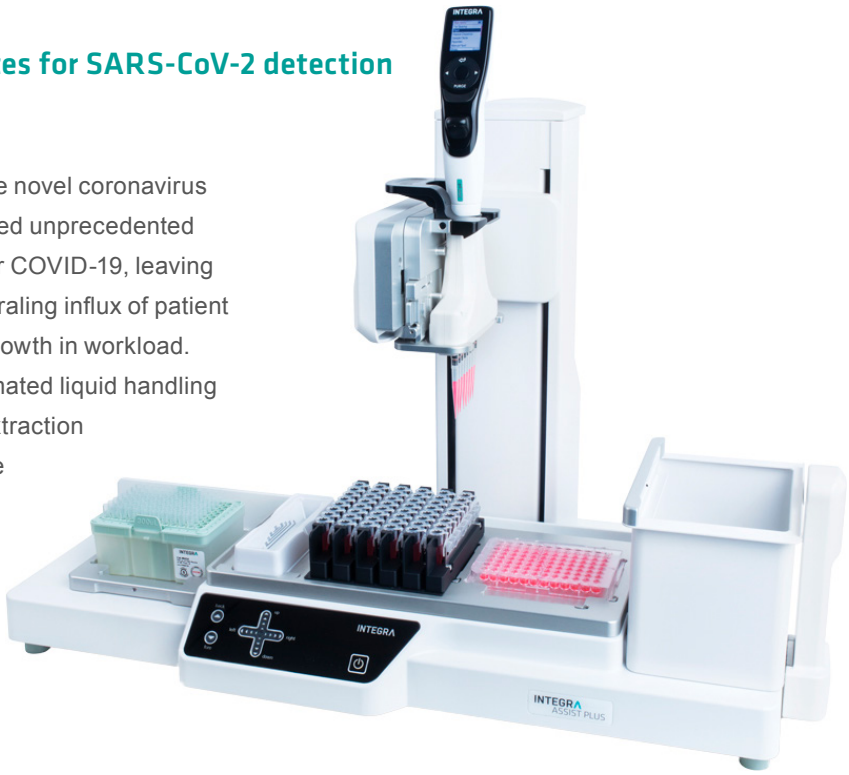
Conclusion

- The time required for a 384 well qPCR set-up can be reduced from 1.5 hours using a single channel pipette to 12 minutes using the ASSIST PLUS pipetting robot in combination with VIAFLO 16 channel and VOYAGER 8 channel pipettes.
- The ASSIST PLUS, together with the VOYAGER adjustable tip spacing pipette, guarantees perfectly reproducible test results and eliminates all risks of reformatting errors when transferring samples from microcentrifuge tubes into a 384 well plate.
- INTEGRA's low retention GRIPTIPS increase pipetting precision for viscous or low surface tension liquids. The reagent reservoirs with SureFlo anti-sealing array reduce the dead volume of costly reagents and precious samples.
- The intuitive VIALAB qPCR program is quick to set up and easy to use or adapt to other pipetting protocols.

For more information and a list of materials used, please refer to our website.

3.2 Automated RT-PCR set-up for COVID-19 testing

How to prepare RT-PCR plates for SARS-CoV-2 detection with the ASSIST PLUS



The emergence and outbreak of the novel coronavirus SARS-CoV-2 (COVID-19) has placed unprecedented demands on laboratories testing for COVID-19, leaving scientific staff to contend with a spiraling influx of patient samples and a rapid, continuous growth in workload. Laboratories need additional automated liquid handling instruments for viral nucleic acid extraction and RT-PCR set-up – which are the most labor-intensive processes in the diagnostic testing workflow – to increase the sample throughput capacity, reduce manual intervention by laboratory analysts and fast track the development of COVID-19 assays.

The ASSIST PLUS pipetting robot together with a VOYAGER adjustable tip spacing pipette, low retention GRIPTIPS and SureFlo 10 ml reagent reservoirs were successfully used for RT-PCR set-up in COVID-19 testing laboratories.

Key benefits

- The full automation capability of the ASSIST PLUS reduces manual intervention and frees highly valuable time for laboratory personnel in this critical COVID-19 pandemic.
- The compact and easy-to-use ASSIST PLUS pipetting robot allows fast set-up regarding installation and programming, allowing labs to immediately increase their sample processing capacity and fast track assay development for COVID-19 sample testing.
- VOYAGER adjustable tip spacing pipettes in combination with the ASSIST PLUS provide unmatched pipetting ergonomics by automatically reformatting patient samples from tube racks into 384 well plates.
- Optimal pipette settings, including tip immersion depth, pipetting speeds and angles, deliver reproducible, precise and accurate results, with no contamination observed in controls or patient samples.
- The use of INTEGRA's low dead volume, SureFlo 10 ml reagent reservoirs, together with low retention GRIPTIPS, demonstrated excellent results, enabling efficient handling of the precious and expensive one-step RT-PCR master mix used for patient testing.

Overview: Automated RT-PCR set-up

The ASSIST PLUS pipetting robot is used to automate testing of suspected COVID-19 positive cases in a 384 well plate. The pipetting robot operates a VOYAGER 12 channel 50 µl electronic pipette with 125 µl sterile, filter, low retention GRIPTIPS. To double the available testing capacity and, concurrently, decrease the cost per test of expensive one-step RT-PCR reagents of dwindling availability, the total PCR reaction volume was miniaturized, reducing it to 10 µl – inclusive of 7.5 µl one-step RT-PCR master mix and 2.5 µl of nucleic acid template. The templates were extracted from combined nasopharyngeal/oropharyngeal flocked swabs or sputum samples. The following procedure is based on the protocol used by the Microbiology and Molecular Pathology Department at Sullivan Nicolaides Pathology (SNP) – part of the Sonic Healthcare Group – in Brisbane, Australia.

The protocol is divided into two parts:

- **Program 1:** Add the master mix (1-COVID-19)
- **Program 2:** Add the nucleic acid template (2-COVID-19)

Experimental set-up: Program 1

Deck position A:	10 ml reagent reservoir with SureFlo anti-sealing array containing 3 ml of one-step RT-PCR master mix.
Deck position C:	384 well plate placed on a PCR 384 well cooling block, allowing the master mix and samples to be kept cold, and enabling exact positioning of the PCR plate on the deck.

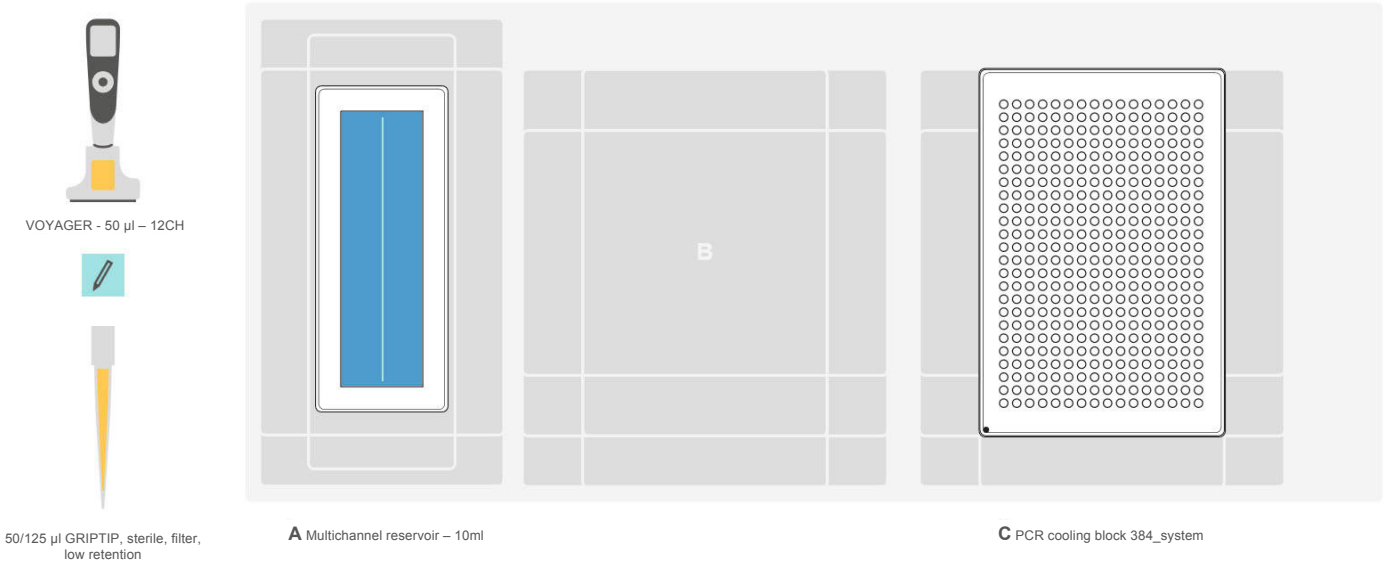


Figure 1: The set-up for program 1-COVID-19.

Step-by-step procedure

1. Add the master mix

Fill the 384 well plate with the one-step RT-PCR master mix.

Place the one-step RT-PCR master mix in a 10 ml sterile, polystyrene reagent reservoir with INTEGRA's SureFlo anti-sealing array. Set up the deck with the required labware, as indicated in **Figure 1**. Select the VIALAB program 1-COVID-19. The VOYAGER pipette automatically transfers the master mix from the reservoir into the 384 well plate (LightCycler® 480 Multiwell Plate, Roche) using the Repeat Dispense mode with tip touch. Each well of the plate is filled with 7.5 µl of master mix.

Tips:

- Using a 10 ml reagent reservoir with SureFlo anti-sealing array allows a very low dead volume (<20 µl) to minimize the loss of expensive reagent of dwindling availability (see **Figure 2**).

- The combination of a low pipetting speed – set at 2 – and low retention GRIPTIPS shows excellent results when pipetting the viscous and foamy master mix.

- Pre- and post-dispense settings, together with the tip touch option, guarantee reproducible, precise and accurate pipetting results (see **Figure 2**).

- The PCR cooling block is used as a support to fix the position of the 384 well plate on the deck, ensuring exact tip positioning when pipetting. The cooling block also helps to keep samples and reagents cool if required by the protocol.

Figure 2: Precise and accurate dispensing of one-step RT-PCR master mix from the low dead volume reagent reservoir to the 384 well plate.

Experimental set-up: Program 2

Deck position A and B:	FluidX Cluster 0.7 ml tubes containing the nucleic acid templates. The tubes are stored in a 96-format rack. A total of four sample racks are used for the protocol (two on position A and two on position B).
Deck position C:	384 well plate placed on a PCR 384 well cooling block.

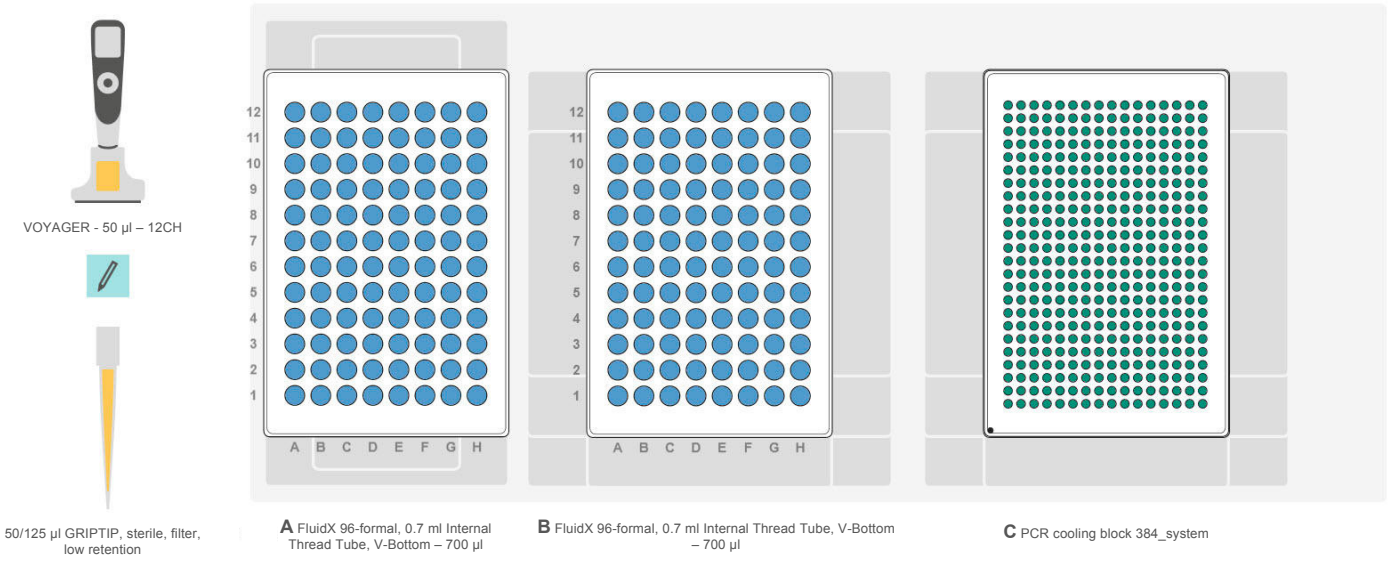


Figure 3: The set-up for program 2-COVID-19.

2. Add the nucleic acid templates

Transfer the samples from four 96-format tube racks to the 384 well plate.

Nucleic acid templates extracted from combined nasopharyngeal/oropharyngeal flocked swabs or sputum samples are stored in FluidX Cluster 0.7 ml tubes placed in a 96-format rack. The VOYAGER pipette transfers 2.5 µl of template from the tubes to the 384 well plate, automatically changing the GRIPTIP pipette tips after each dispense. Both position A and B are used to house the samples on the deck (see **Figure 3**). The pipette prompts the user when it is time to replace the tube racks on the deck. After user confirmation, the VOYAGER pipette continues reformatting the samples from tubes to the plate.

Tips:

- The VOYAGER pipette's tip spacing capability combined with automatic Tip Change ensures easy and rapid sample transfer without risk of contamination or reformatting errors.

- Using an air gap of 1.5 µl when aspirating the viral nucleic acid template eliminates the risk of contamination risk during pipette tip travel.

Note: Automated RT-PCR testing for COVID-19 with the ASSIST PLUS can also be performed using a VOYAGER 8 channel 50 µl electronic pipette (see **Figure 5**).

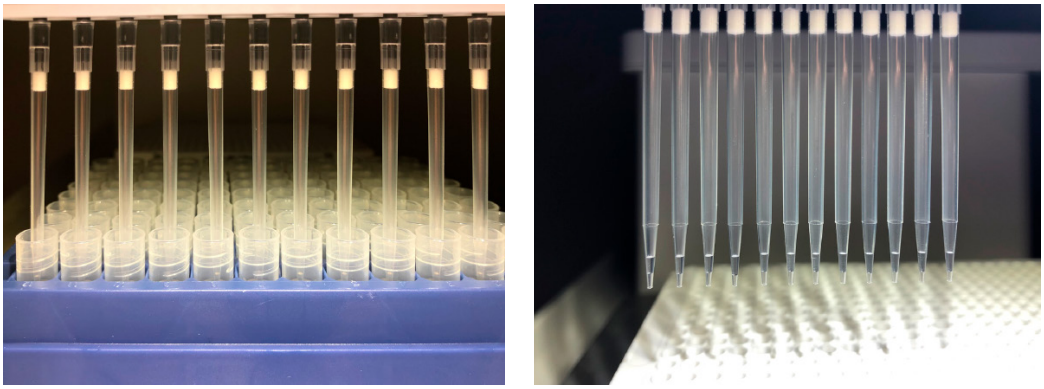


Figure 4: Easy and rapid transfer of patient nucleic acid templates from the tube rack to the 384 well plate using the VOYAGER adjustable tip spacing pipette together with the ASSIST PLUS pipetting robot.

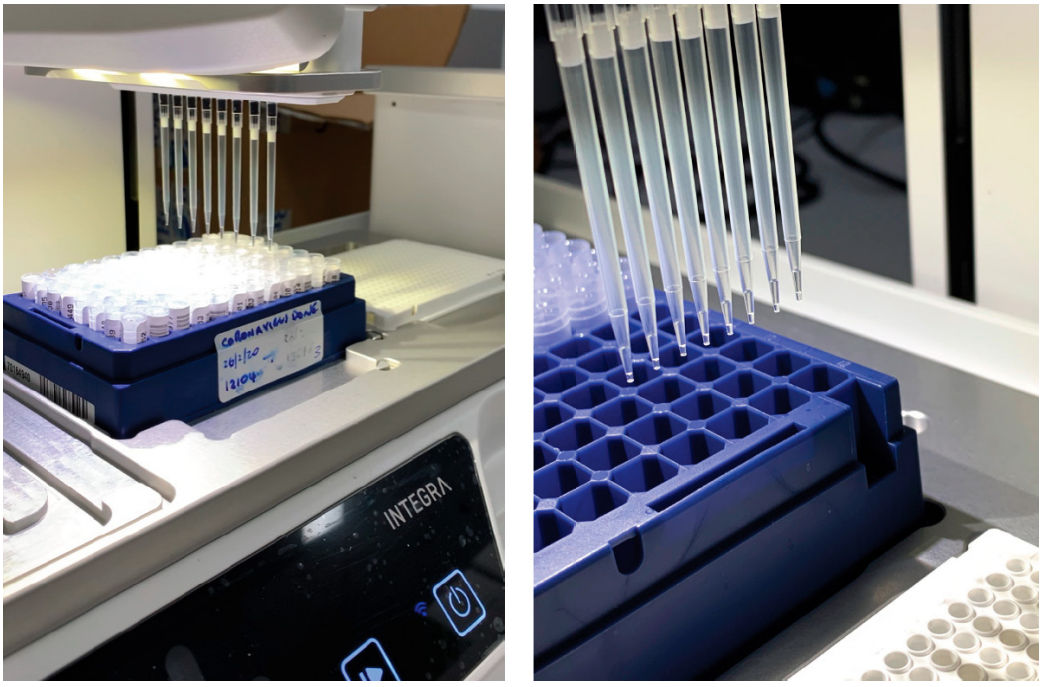


Figure 5: Automated RT-PCR testing for COVID-19 using the ASSIST PLUS pipetting robot together with a VOYAGER 8 channel adjustable tip spacing pipette, as performed in the Microbiology and Molecular Pathology Department at SNP.

Remarks

4 Position Portrait Deck:

If your process allows, the protocol can be compiled into one simple program using the 4 Position Portrait Deck option on the ASSIST PLUS (see **Figure 6**).

96 well plates:

The protocol can be readily adapted to 96 well format.

VIALAB software:

The VIALAB programs can be easily adapted to your specific labware and protocols.

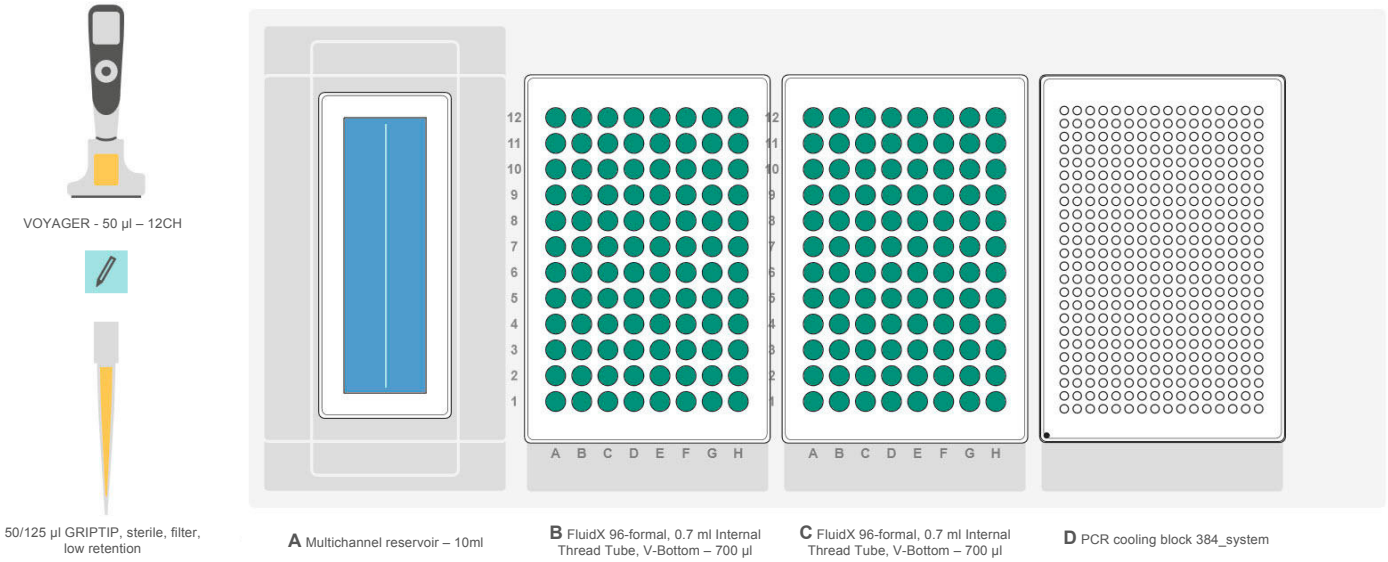


Figure 6: Example set-up of the 4 Position Portrait Deck when combining programs 1-COVID-19 and 2-COVID-19 in one program.

Conclusion

- In the context of a global pandemic where laboratories are under increasing pressure to analyze more and more patient specimens to confirm COVID-19 cases, testing labs can rapidly benefit from the advantages of the ASSIST PLUS pipetting robot, allowing them to increase their sample processing capacity.
- Pipetting results were reproducible, precise and accurate, with no contamination observed in controls or patient samples.
- The ASSIST PLUS pipetting robot, together with the VOYAGER adjustable tip spacing pipette, increases sample processing capacity, reduces the need for manual intervention by laboratory personnel and fast tracks assay development for COVID-19 testing.
- Low retention GRIPTIPS and a low dead volume SureFlo reagent reservoir allow the loss of costly reagents, such as one-step RT-PCR master mix, to be reduced.
- The simple and fast ASSIST PLUS pipetting robot combined with the easy-to-use VIALAB software, offers immediate help for testing labs.
- While the current protocol uses 384 well plates, it can be readily adapted to 96 well format to meet future needs.
- Thanks to the VIALAB software, the pipetting programs can be easily adapted to any specific protocols and labware.

For more information and a list of materials used, please refer to our website.

3.3 Increase your sample screening and genotyping assay throughput with the VOYAGER adjustable tip spacing pipette

Discover the advantages of setting up a genotyping assay or sample screening with the VOYAGER adjustable tip spacing pipette

Laboratories are continually facing the challenge of increasing throughput in the most efficient and economical way, to meet the need to process more and more samples per day. Traditionally, handling and manipulating samples between different labware formats involves the use of single channel pipettes, especially in screening applications and genotyping assays, which is slow, inefficient and error prone.

INTEGRA's VOYAGER adjustable tip spacing pipette has enabled scientists from the Technical University of Munich (TUM) to benefit from the enhanced productivity of a multichannel pipette, reducing tedious liquid handling tasks.

Compared to fully automated solutions, it provides seamless liquid transfers between different standardized and non-standardized microplates, tube and gel chamber formats, and can be used without any special training. Tip spacing can be simply changed one-handedly with the push of a button, eliminating the need for any manual adjustments.

The various operating modes of the VOYAGER adjustable tip spacing pipette help to speed up monotonous pipetting steps, eliminate sample transfer errors between different labware formats, and reduce the risk of developing repetitive strain injuries.

Key benefits

- The VOYAGER's motorized adjustable tip spacing enables the user to benefit from the enhanced productivity of an electronic multichannel pipette throughout the entire genotyping assay, processing samples faster than with traditional single channel pipettes and helping to eliminate sample transfer errors between different labware formats.
- Tip spacing can be adjusted on the fly with the push of a button to match different types of labware, allowing the easy transfer of multiple reaction mix samples from microcentrifuge tubes directly to 96 or 384 well plates, and gel pockets.
- The availability of a range of pipetting modes makes the VOYAGER a very versatile and affordable tool to speed up and standardize pipetting protocols.
- New users quickly get accustomed to the electronic pipette thanks to its intuitive design and easy-to-use pipetting modes.

Experimental set-up

In this protocol, two VOYAGER 8 channel adjustable tip spacing pipettes are used for a genotyping set-up. The genotyping assay is based on a PCR method with a subsequent gel electrophoresis.

The following protocol consists of sample transfers from 1.5 ml microcentrifuge tubes into a 96 well plate, and from a 96 well PCR plate into an agarose gel for electrophoresis.

Overview of the steps:

1. Template transfer
2. Sample transfer into the agarose gel

Step-by-step procedure

Figure 1: Adjust the tip spacing by aligning it against the empty 96 well plate and tube rack.

1. Template transfer

Transfer the templates into a 96 well plate.

Use a VOYAGER 8 channel 300 µl electronic pipette with 300 µl sterile, filter GRIPTIPS. Select ‘Tip spacing’ in the main menu of the pipette to set the required spacing. Choose ‘Positions: 2’ in the tip spacing menu and set the tip spacing according to the 96 well plate and the microcentrifuge tubes in the rack (**Figure 1**). Once saved, the tip spacing is available at any time, for any other pipetting modes.

Figure 2: Sample transfer from a microcentrifuge tube rack to a 96 well plate.

After saving the tip spacing, select ‘Pipet’ mode in the main menu. Set your required sample transfer volume and pipette the templates from the 1.5 ml microcentrifuge tubes into the 96 well plate (**Figure 2**). By pressing left and right on the Touch Wheel interface, the tip spacing can be adjusted on the fly to fit each labware format.

Tips:

- Use the Repeat Dispense mode to dispense several samples successively if duplicate or triplicate samples are required.

- Use the Pipet/Mix mode if samples require mixing in the target wells. Settings like mixing cycles, pipetting speeds and volumes can quickly be adjusted.

2. Sample transfer into the agarose gel

Transfer the PCR product into the agarose gel.

After PCR, use the VOYAGER 8 channel 125 µl electronic pipette with 125 µl sterile, filter GRIPTIPS to transfer the samples from the 96 well PCR plate into the agarose gel for subsequent gel electrophoresis (**Figure 3**). As in step 1, choose 'Positions: 2' in the tip spacing menu and set the tip spacing according to the 96 well PCR plate and the agarose gel.

Set the required sample volume as described in step 1 and transfer the samples from the PCR plate into the agarose gel.

Figure 3: PCR product transfer into the agarose gel.

Tips:

- A low dispensing speed (e.g. 4) helps uniform filling of the wells in the agarose gel.
- If you want a controlled blowin – rather than automatic – keep the run button pressed while dispensing. Blowin will occur when the run button is released.

Conclusion

- The VOYAGER adjustable tip spacing pipette has enabled TUM researchers using different labware formats to benefit greatly from the enhanced productivity of a multichannel pipette, processing assays much faster than using a single channel pipette. The tip spacing can be changed onehandedly at the touch of a button to fit different labware formats, such as PCR plates, tubes and gel pockets.
- Thanks to the intuitive interface, users quickly become accustomed to the electronic pipette. The different pipetting modes make the VOYAGER adjustable tip spacing pipette a versatile yet affordable tool for working with labware of varying sizes and formats.
- The VOYAGER adjustable tip spacing pipette increases the speed of sample testing set-ups, and helps eliminate sample transfer errors between different labware formats and reduce the risk of developing repetitive strain injuries.

For more information and a list of materials used, please refer to our website.

3.4 PCR product purification with QIAquick® 96 PCR Purification Kit and the VIAFLO 96 handheld electronic pipette

Semi-automated PCR product purification on the VIAFLO 96 handheld electronic pipette

QIAquick 96 PCR Purification Kit is suitable for purifying up to 10 µg of material for downstream applications, such as sequencing, cloning, labeling and microarrays. The kit facilitates the removal of impurities like primers, unincorporated nucleotides, buffers, salts, mineral oils, agarose and enzymes. The vacuum-driven process is much faster than centrifugation, and gives high, reproducible yields. It is important to avoid cross-contamination in nucleic acid purification, and QIAGEN's column design is optimized to limit carryover of contaminants. Although QIAquick 96 provides a high throughput solution, the elution, washing and binding steps are very laborious and time consuming if performed manually. With VIAFLO 96 handheld electronic pipette, the hands-on time is reduced, as samples and reagents can be transferred to all 96 wells at once. This enables rapid and efficient, high throughput PCR clean-up.

Key benefits

- VIAFLO 96 and VIAFLO 384 allow simultaneous pipetting of up to 96 or 384 wells, respectively, maximize throughput of PCR purification by allowing transfer samples and reagents in a single step.
- The z-heights can be predefined, choosing the optimal value to prevent accidental scratching of the well membrane for more consistent results.
- Custom programming of the PCR product clean-up steps allows pipetting parameters, such as aspiration or dispensing speeds, to be predefined. Prompt messages guide the user through the entire pipetting protocol, which is especially useful when several pre-wetting steps are included.
- The VIAFLO 96 or VIAFLO 384's hands-free automatic mode ensures that the PCR clean up protocols are performed in the same way each time, maximizing reproducibility.

Overview: How to purify PCR products with VIAFLO 96

Experimental set-up

This protocol describes how PCR products are purified using a VIAFLO 96 handheld electronic pipette with a two position stage and the QIAGEN QIAquick® 96 PCR Purification Kit. The following procedure is based on the kit manufacturer's protocol for purification of 96 samples (up to 10 µg PCR products).

A 96 channel pipetting head (50-1250 µl) is used together with 1250 µl short, low retention, sterile, filter GRIPTIPS. Customized VIALINK programs are provided to perform the binding, washing and elution steps. Before starting, ethanol (96-100 %) should be added to the Buffer PE concentrate.

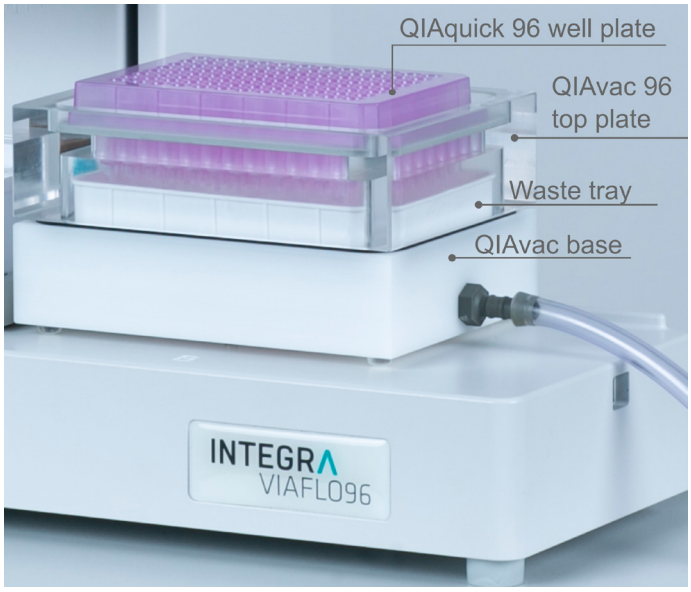


Figure 1: Initial set-up of the vacuum manifold.

Overview of the purification steps:

1. **Step 1:** Binding
2. **Step 2:** Washing
3. **Step 3:** Elution

The initial set-up of the QIAvac 96 Vacuum Manifold consists of a waste tray on top of a QIAvac base, followed by a QIAquick 96 well plate (pink) mounted on a QIAvac 96 top plate, as shown in **Figure 1**.

The QIAvac has to be attached to a vacuum source (house vacuum or vacuum pump) that generates negative pressure between 100 and 600 mbar.

Step-by-step procedure

1. Binding

Binding the DNA to the silica-gel membrane.

Load the 1250 µl short, low retention, sterile, filter GRIPTIPS on the VIAFLO 96. Place a 150 ml automation friendly reagent reservoir in position A. The QIAvac 96 Vacuum Manifold should be placed on position B of the VIAFLO 96 in landscape orientation. No plateholder is needed on position B where the manifold is placed.

Important: The vacuum manifold should be aligned before each run **(Figure 2**).

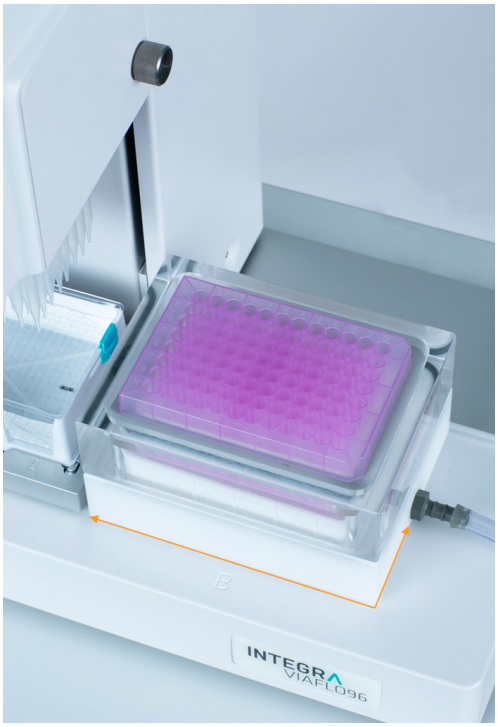


Figure 2: Alignment of the QIAvac 96 Vacuum Manifold.

Begin by launching the custom VIALINK program 'Qiaquick_ purification_M'. The pipette will prompt the user to place Buffer PM on position A, then air is aspirated. This ensures that every single drop of the liquid can be dispensed later. The VIAFLO 96 will then guide the user through the two pre-wetting steps, starting with aspiration and dispensing 200 µl of Buffer PM. After a second aspiration, the pipette will display the prompt 'Move the head out of buffer', before dispensing the final 200 µl of Buffer PM. This is followed by a 20 second wait, giving the buffer residues time to flow down to the tip and be dispensed.

After pre-wetting, the pipette aspirates 75 µl Buffer PM (three times the volume of the PCR product). The instrument then tells the user to remove the reservoir from position A, and replace it with the 96 well plate containing the 25 µl of PCR products. After dispensing, and four mixing steps, the resulting mixture is transferred to the QIAquick plate wells in two steps. It is then time to switch on the vacuum source, as indicated by the pipette.

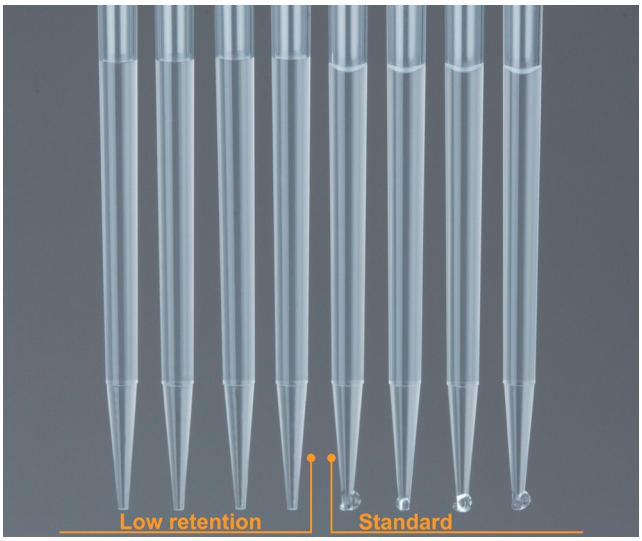


Figure 3: Low retention versus standard tips.

Tips:

- Pre-wetting the tips prior to pipetting prevents droplets and dripping when pipetting volatile liquids, such as isopropanol, which is one of the constituents in Buffer PM.
- Low retention GRIPTIPS (**Figure 3**) are used for these pipetting steps to avoid dripping.

2. Washing

Two-step purification of the PCR product.

Eject the used tips and load new 1250 µl short, low retention, sterile, filter GRIPTIPS on the VIAFLO 96. Place a new 300 ml automation friendly reagent reservoir in position A. The VIAFLO 96 will then prompt the user to pour Buffer PE into the reservoir, followed by a pre-wetting step, which is necessary since the buffer contains ethanol. After pre-wetting, the pipette will aspirate 900 µl of Buffer PE, and dispense it into QIAquick plate wells. The instrument will then notify the user that is it time to turn on the vacuum pump. With the pump turned on, another dose of the buffer is dispensed into the wells, followed by a 10 minute wait to dry the membrane and remove all residual ethanol.

Important: The final drying step is crucial to remove residual ethanol prior to elution. Residual ethanol in the elution buffer could inhibit downstream applications (e.g. PCR).

Tip: After this step, the manufacturer suggests tapping the plate on a stack of absorbent paper to ensure that all residual buffer is removed.

3. Elution

Elution of DNA from the silica-gel membrane.

When prompted, start by replacing the waste tray with the blue collection microtube rack provided, which contains 1.2 ml vessels (**Figure 4a**). Load new 1250 µl short, low retention, sterile, filter GRIPTIPS, and place a new 150 ml automation friendly reagent reservoir in position A. The instrument will then prompt the user to place Buffer EB into the reservoir, aspirate 80 µl, and dispense it into the QIAquick plate wells. After a 1 minute incubation, the pipette tells the user to switch on the vacuum source for 5 minutes.

Tips:

- The purified PCR product could also be eluted in a 96 well microplate. In this case, when replacing the waste tray, the 96 well microplate has to be placed on the empty blue collection tube rack (**Figure 4b**).

- For increased DNA concentration, decrease the elution volume to 60 µl, as per QIAGEN's recommendations, in the VIALINK software.

A)

B)

Figure 4: Elution into a) provided collection microtubes or b) a 96 well microplate.

Remarks

Vacuum manifold:

Alignment of the vacuum manifold is very important in this process. Adding marks on the deck helps to reposition the manifold whenever needed. To check the position of the well plate on top of the vacuum manifold, attach the tips manually to the pipette. The pipette tips should be in the middle of the wells. If not, adjust the position of the vacuum manifold on the deck.

Automatic mode:

The VIAFLO 96 can also operate in hands-free automatic mode, allowing the user to have more walk-away time and less interaction, which is highly beneficial when using the instrument in a laminar flow cabinet. The customized automatic VIALINK program can be found on the INTEGRA website.

Conclusion

- The VIAFLO 96 electronic handheld pipette allows fast and simple liquid transfers for high throughput PCR product purification.
- Optimized pipette settings enable accurate sample and reagent transfer, without the tip touching and scratching the QIAquick membrane.
- The VIAFLO 96 electronic handheld pipette's compact design takes up minimal space and fits on any lab bench.
- The unique operating concept makes the VIAFLO 96 and VIAFLO 384 as easy to use as a conventional electronic pipette.
- The QIAvac 96 manifold is easily placed on the instrument and allows the processing of other kits using 96 well silica-membrane or filter plates.
- Another option for this application is the MINI 96, which is the most affordable 96 channel option on the market.

For more information and a list of materials used, please refer to our website.

3.5 PCR purification with Beckman Coulter AMPure XP magnetic beads and the VIAFLO 96

Automatic magnetic bead purification with the VIAFLO 96 handheld electronic pipette

Agencourt AMPure XP magnetic beads (Beckman Coulter) are an efficient way to clean up samples for PCR, NGS, cloning and microarrays. The kit provides a solution for medium to high throughput requirements when carried out in a 96 well plate, but the protocol involves many washing and transfer steps that make it tedious to perform manually. With the VIAFLO 96, a handheld 96 channel electronic pipette, multistep protocols such as PCR clean-up and DNA purification can be performed quickly and efficiently, increasing throughput tremendously by transferring samples and reagents to all 96 wells at once. Thanks to its unique operating concept, the VIAFLO 96 remains as easy to use as a traditional handheld pipette and can even provide critical information (user-defined prompts) about the protocol steps.

Key benefits

- The VIAFLO 96 enables transfer of samples, reagents and wash solutions to 96 wells at once, increasing the throughput of magnetic bead-based DNA purification methods.
- The partial tip loading of the VIAFLO 96 allows purification of fewer than 96 DNA samples if necessary; 8, 16, 24, 32, 40 or 48 GRIPTIPS can be loaded for easy purification of different numbers of samples.
- The optimal immersion depth for removing supernatant or adding liquid right onto the samples is guaranteed by defining the z-height of the VIAFLO 96.
- The Tip Align setting of the VIAFLO 96 automatically positions the tips in the center of the wells of a 96 well plate, avoiding any disturbance of the beads.

Overview: How to automate PCR purification steps with VIAFLO 96

The VIAFLO 96 handheld electronic pipette with a three position stage is used to purify DNA with AMPure XP beads from Beckman Coulter. The following protocol is an example of a set-up for 96 samples, where each well of a 96 well plate is filled with 10 µl of DNA sample and 18 µl of AMPure XP beads, then further processed with the VIAFLO 96. The PCR purification can be performed manually or semi-automated using the VIAFLO 96 in automatic mode. Custom-made VIALINK programs are provided. The VIALINK programs are set up according to the manufacturer's protocol (AMPure XP Beckman Coulter).

Step-by-step procedure

1. Dispense AMPure XP beads into PCR tubes

Transfer AMPure XP beads from the stock solution into 12 PCR tubes placed in a cooling block from INTEGRA.

Note: The cooling block is just used as a support in this instance, not for cooling down the samples.

To ensure a homogenous stock solution, beads are thoroughly mixed by shaking/inverting until the solution appears consistent in color. The beads are transferred into 12 PCR tubes using the Repeat Dispense mode of a VIAFLO single channel 1250 µl electronic pipette. A customized VIALINK program (AMP_Transfer1) is available to aid bead transfer.

For optimal pipetting, ensure beads are thoroughly mixed before each transfer. Mixing steps can be defined by the number of cycles and the pipetting speed. Both influence the efficiency of mixing and thus the quality of the clean-up. Saving these parameters in the pipetting program ensures that mixing is always carried out as defined, yielding consistent results. Insert a pre- and post-dispense step to enhance accuracy and precision while pipetting precious reagents, such as AMPure XP beads.

Tip: The use of sterile, filter, low retention GRIPTIPS ensures that every dispense is as accurate as possible, with no loss of beads or sample.

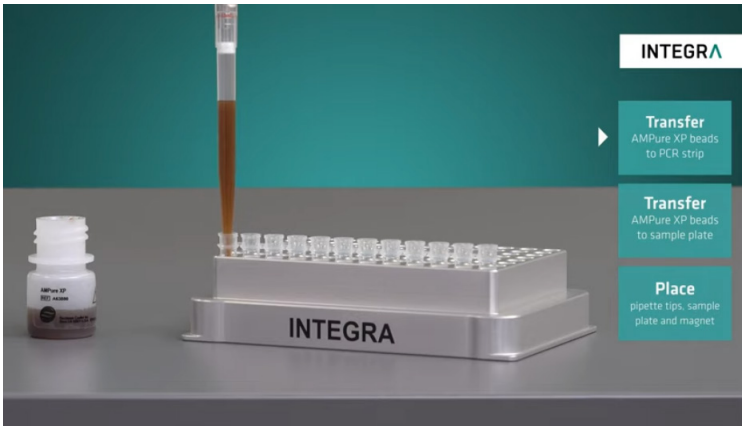


Figure 1: Transfer AMPure XP beads from the stock solution into 12 PCR tubes.

2. Transfer AMPure XP beads into the DNA samples

Transfer AMPure XP beads from the PCR tubes into a 96 well plate preloaded with DNA samples.

Pipette the beads from the PCR tubes into the 96 well plate using a VIAFLO 12 channel 50 µl electronic pipette. For optimal pipetting, make sure the tips are exchanged, and mix the beads thoroughly before each transfer. A customized VIALINK program (AMP_Transfer2) is provided for this step.

Tip: Use low retention GRIPTIPS to minimize loss of beads adhering to the tip wall.

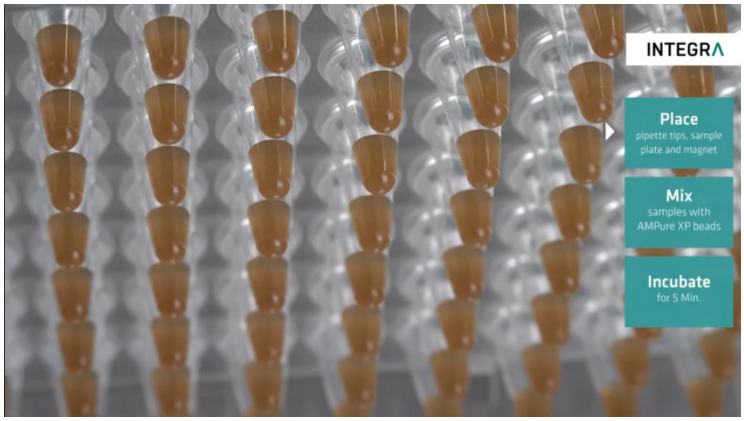


Figure 2: Transfer AMPure XP beads from the PCR tubes into a 96 well plate preloaded with DNA samples.

3. Mixing and binding of the AMPure XP beads

Mixing and binding of the magnetic beads to the PCR samples.

Load GRIPTIPS (position A) then select and run the AMPure_XP_M program on the VIAFLO 96. The samples are now mixed 10 times by pipetting up and down on position B. A five minute wait time follows, timed by the VIAFLO 96, to allow the DNA to bind to the beads.

Tip: Use the z-height setting of the VIAFLO 96 to define the optimal tip immersion depth. This prevents air entering the tip during mixing and avoids the pipette tip touching the bottom of the plate. Setting the Tip Align support strength to 3 for positions A and B makes it more comfortable to use the VIAFLO 96. These settings can be incorporated into the program so that they are not forgotten.

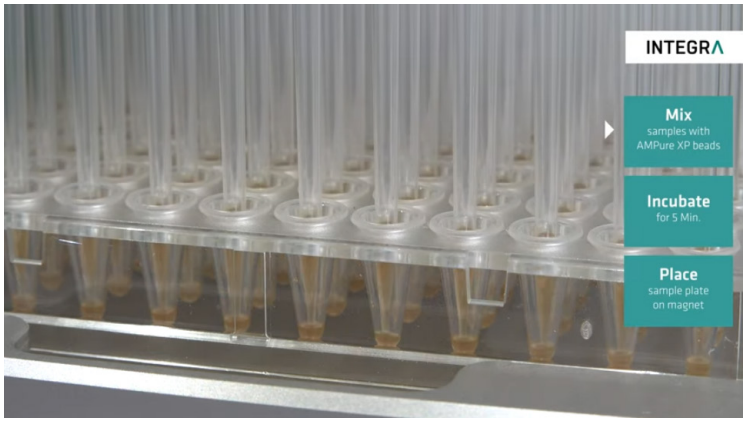


Figure 3: Mixing and binding of the magnetic beads to the PCR samples.

4. Magnetic separation of the AMPure XP beads

Separating the magnetic beads from the PCR samples.

Note: Make sure new GRIPTIPS are loaded before continuing the protocol to ensure removal of the supernatant without bead carryover.

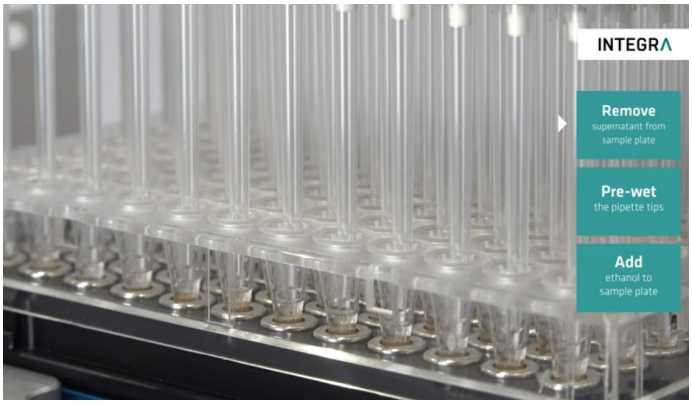


Figure 4: Separating the magnetic beads from the PCR samples.

A prompt on the pipette screen reminds the user to move the sample plate from position AB onto the 96 well magnet (position B) and place an automation friendly reagent reservoir for waste collection on position AB. After a two minute incubation time, the beads form a ring-shaped structure and the solution becomes clear. Load new GRIPTIPS before continuing the procedure to ensure accurate removal of the supernatant without bead carryover. Follow the instructions on the pipette and aspirate the supernatant slowly from the sample, dispensing it into the waste reagent reservoir (position AB).

Tip: To avoid disturbing the ring of beads, the supernatant is aspirated slowly at speed 1. Leave 5 µl of supernatant in the plate to prevent beads being drawn out during aspiration. The z-height limit is again used to ensure that the beads are not disturbed during pipetting.

5. AMPure XP bead clean-up

Wash the magnetic beads twice with 70 % ethanol.

Place an automation friendly reagent reservoir containing 70 % ethanol on position A and change the GRIPTIPS before continuing with the wash step. Follow the prompts on the pipette. Pre-wet the GRIPTIPS with 70 % ethanol. Then wash the samples with 70 % ethanol. Repeat the washing step again as indicated by the pipette.

Figure 5: Wash the magnetic beads twice with 70 % ethanol.

Tip: Pre-wetting the GRIPTIPS with 70 % ethanol ensures equilibration of the humidity and the temperature between the air in the pipette/tips and the sample/liquid. In-house testing has shown that low retention GRIPTIPS prevent ethanol from dripping while traveling from one pipetting position to another.

6. Elute samples from the magnetic beads

Elute the purified samples from the magnetic beads by adding the elution buffer.

As indicated by the pipette, replace the 70 % ethanol reagent reservoir on position A with an elution buffer reagent reservoir and move the sample plate from the magnet (position B) to position AB. Load new GRIPTIPS before continuing with the protocol. After transferring and thoroughly mixing the elution buffer with the beads, the pipette prompts the user to place the sample plate back onto the magnet (position B). During the one minute incubation time, place a new 96 well plate on position AB.

Figure 6: Elute samples from the magnetic beads.

7. Transfer the sample eluates

Transfer the sample eluates into the new 96 well plate.

Note: Load new GRIPTIPS to ensure a clean eluate transfer without bead carryover.

Continue with the same program, slowly and carefully transferring the eluates from position B into the new plate (position AB).

Tip: Optimizing pipette settings (aspiration speed, volume and height) allows the volume of the transferred eluate to be maximized without carryover of beads. These settings can be easily tweaked at any time. Performing a test run with water before implementing any new assay is an ideal way to optimize pipette settings.

Figure 7: Transfer the sample eluates into the new 96 well plate.

Remarks

Automatic mode:

The VIAFLO 96 can also operate on its own, enabling less user interaction, which in turn improves ergonomics and reproducibility. This also makes it even more ideal for use in tight spaces, such as under a laminar flow cabinet.

Partial tip load:

If you are not working with a full set of 96 samples, the VIAFLO 96 is able to work with any number of tips loaded, allowing purification of smaller numbers of samples.

Figure 8: Automatic mode and partial tip load.

Conclusion

- The VIAFLO 96 is perfectly suited to magnetic bead purification in a 96 well format. An entire plate with 96 samples can be purified in a fraction of the time it would take with a traditional pipette.
- Optimized tip immersion and pipette settings in combination with the use of low retention GRIPTIPS allow maximum sample recovery at the end of the purification protocol.
- The VIAFLO 96 can guide the user through the entire protocol step by step, ensuring the correct workflow and enhancing the reproducibility of results.
- The optional automatic mode of the VIAFLO 96 enables the instrument to operate on its own to minimize pipetting errors, making it even more ideal for use under a laminar flow cabinet.

For more information and a list of materials used, please refer to our website.

3.6 PCR purification with Beckman Coulter AMPure XP magnetic beads and the ASSIST PLUS

Automatic magnetic bead purification with ASSIST PLUS pipetting robot

Agencourt AMPure XP beads (Beckman Coulter) are used for DNA purification in a variety of applications, including PCR, NGS, cloning and microarrays. The ASSIST PLUS pipetting robot provides a solution for optimal bead separation and maximized recovery of precious samples. User guidance throughout the entire protocol ensures an error-free pipetting procedure. Careful and accurate handling of the magnetic beads by the ASSIST PLUS leads to superior reproducibility and consistency during the experiment. Taken together, the ASSIST PLUS provides researchers with an easy and highly efficient way to purify DNA from PCR reactions using AMPure XP magnetic beads.

Key benefits

- The VIAFLO and VOYAGER electronic pipettes, in combination with ASSIST PLUS, provide unmatched pipetting ergonomics.
- Optimal pipette settings, including tip immersion depth, pipetting speeds and angles, maximize reproducibility and sample recovery.
- Exact positioning of the pipette tips in the sample wells avoids the risk of disturbing the ring of magnetic beads or bead carryover.
- The ASSIST PLUS automates many steps of a magnetic bead purification protocol and guides the user through the remaining manual operations to ensure an error-free process.

Overview: How to automate PCR purification steps with ASSIST PLUS

The ASSIST PLUS is used to purify DNA samples using AMPure XP beads (Beckman Coulter). The pipetting robot runs a VOYAGER 8 channel 125 µl electronic pipette with 125 µl sterile, filter, low retention GRIPTIPS. The use of low retention GRIPTIPS guarantees optimal liquid handling of viscous (AMPure XP buffer) and volatile (70 % ethanol) solutions.

Below is an example set-up for 24 samples, preparing 10 µl DNA samples (position B) with 18 µl of AMPure XP beads (position A). The pipetting programs were prepared according to the manufacturer's protocol (AMPure XP, Beckman Coulter) using VIALAB software.

The protocol is divided into two programs that guide the user through every step of the PCR purification process.

- **Program 1:** Binding (AMP_BINDING)
- **Program 2:** Washing and elution (AMP_WASH_ELUTE)

Experimental set-up: Program 1

Deck position A:	PCR 8 tube strip containing the AMPure XP beads **(Figure 1**, blue), placed onto a cooling block from INTEGRA. Note: the cooling block is just used as a support in this instance, and not for cooling down the samples.
Deck position B:	96 well plate with 24 DNA samples for purification (**Figure 1**, green).
Deck position C:	96 well ring magnet.

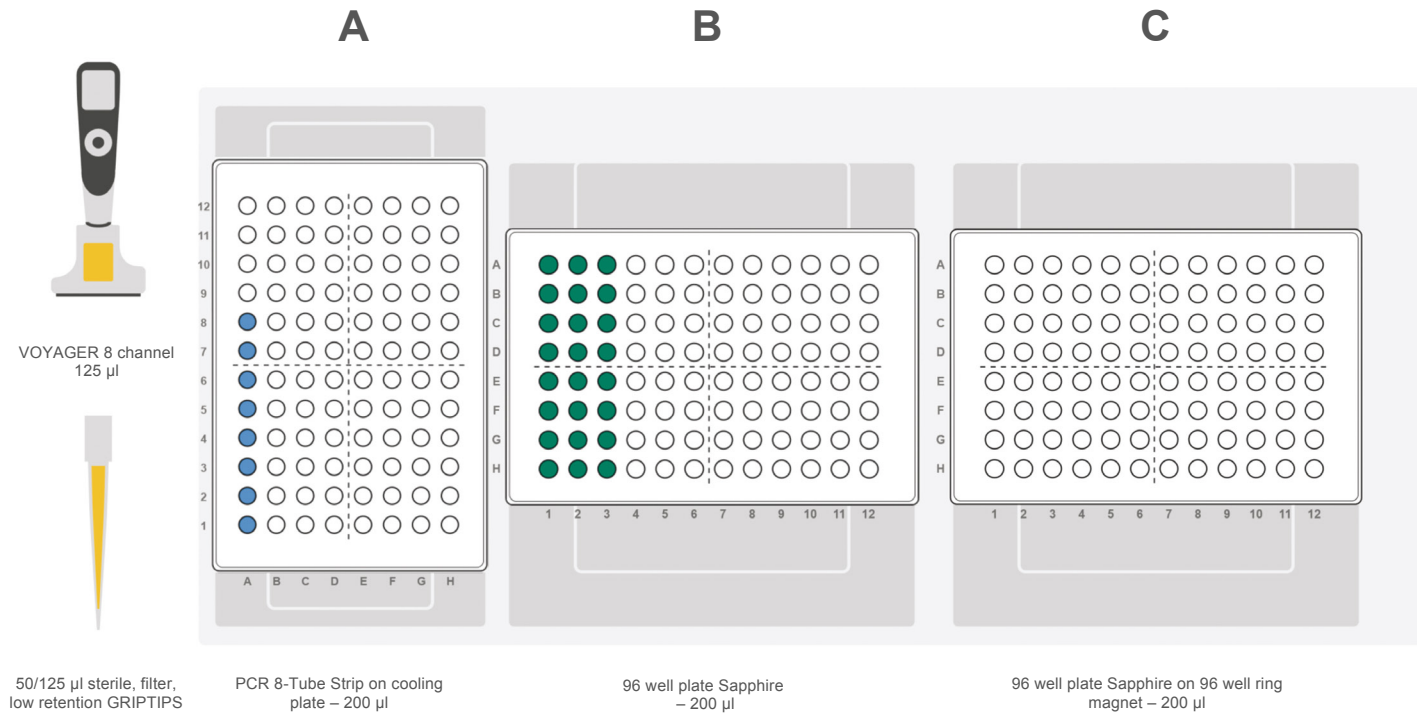


Figure 1: Pipetting schema, set-up for program 1.

Run program 1: transfer & binding

Select and run the AMP_BINDING program on the VOYAGER electronic pipette. The ASSIST PLUS pipetting robot immediately starts the protocol.

1. AMPure XP transfer

Transferring AMPure XP beads from an 8 tube PCR strip to a 96 well plate containing the DNA samples.

To ensure the AMPure XP buffer is homogenous, the beads are resuspended by pipetting up and down 10 times before being transferred to the samples. The beads and DNA fragments are thoroughly mixed together before the pipette automatically starts the timer for a 5 minute incubation, ensuring optimal conditions for the DNA strands to bind onto the magnetic beads.

Tip: Using low retention GRIPTIPS rather than regular GRIPTIPS prevents the loss of AMPure XP beads during the pipetting steps (see **Figure 2**).

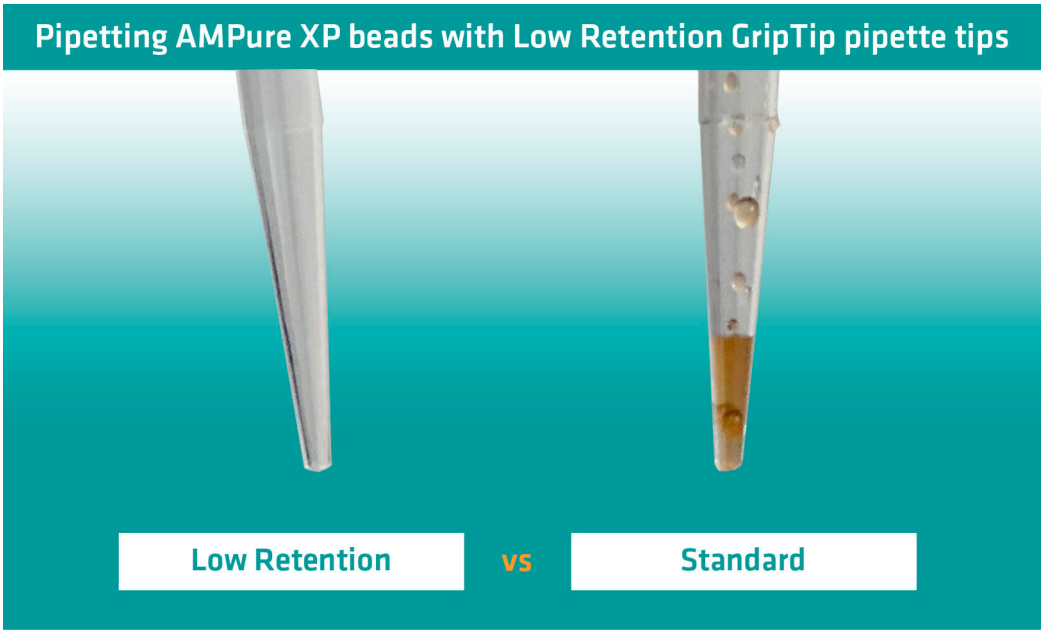


Figure 2: The image highlights the advantages of using low retention GRIPTIPS versus regular GRIPTIPS when pipetting AMPure XP beads.

Figure 3: The beads and DNA fragments are thoroughly mixed together before the incubation.

2. Magnetic separation of the AMPure XP beads

Separating the magnetic beads from the PCR samples.

A message instructs the user to move the plate (position B) onto the magnet (position C). Continue the program to start the timer. After a two minute incubation on the magnet the beads form a ring in the sample well and the solution becomes clear. The program resumes automatically, and the supernatant is removed. On completion of this step, the pipette prompts the user to continue with the AMP_WASH_ELUTE program and to replace the labware on position A with the 8 row polypropylene (PP) reagent reservoir containing the ethanol and elution buffer.

Tip: The supernatant is aspirated slowly using the Tip Travel feature of the ASSIST PLUS to avoid disturbing the ring of beads. The Tip Travel feature keeps the tip immersion depth constant during aspiration and dispensing. 5 µl of supernatant remain in the plate to prevent beads being drawn out during aspiration.

Figure 4: The ASSIST PLUS settings allow removal of the supernatant without any bead carryover.

Experimental set-up: Program 2

Deck position A:	The 96 well PCR cooling block is replaced by an 8 row polypropylene (PP) reagent reservoir filled with 70 % ethanol in row 1 (blue) and elution buffer in row 2 (orange). Row 8 is used for waste (purple).
Deck position B:	Emtpy 96 well plate.
Deck position C:	96 well ring magnet and 96 well plate with 24 DNA samples for purification (green).

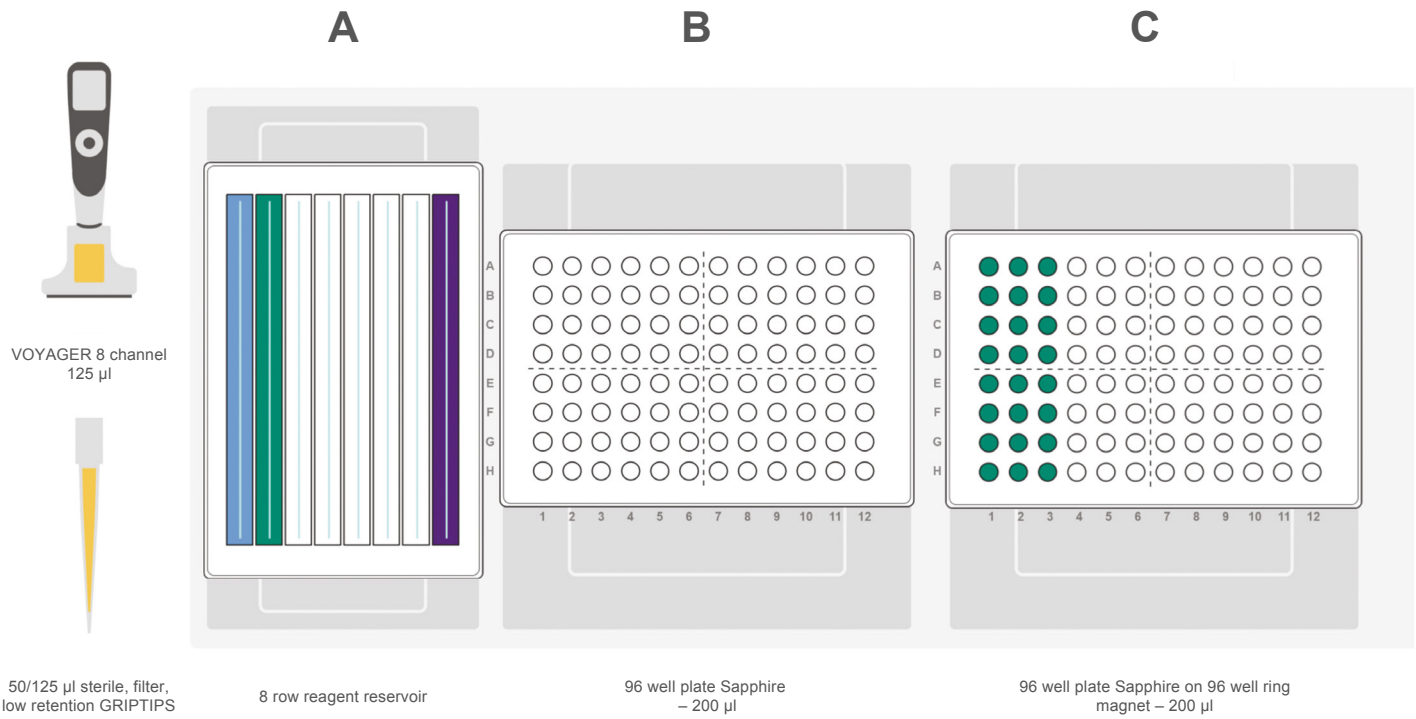


Figure 5: Pipetting schema, set-up for program 2.

Run program 2: Washing & elution

Start the AMP_WASH_ELUTE program on the VOYAGER electronic pipette. The ASSIST PLUS washes the beads twice by automatically adding and removing ethanol.

3. Magnetic bead clean-up

Washing the magnetic beads twice with 70 % ethanol.

The programmed pipette settings allow the beads to be washed without disturbing the bead ring. At the end of the second washing step, all the ethanol is removed. If necessary, an additional drying time can easily be added using VIALAB software.

Tip: The use of low retention GRIPTIPS prevents ethanol from dripping while traveling from position A to position C (see **Figure 6**).

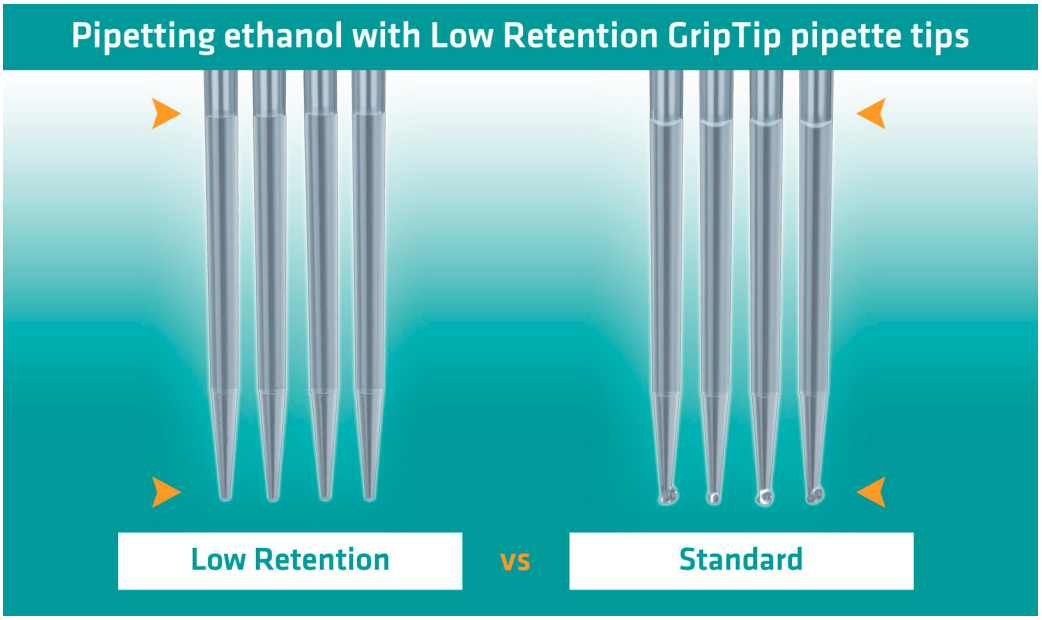


Figure 6: The image highlights the advantages of using low retention GRIPTIPS (left) versus regular GRIPTIPS (right) when pipetting ethanol.

4. Elute samples from the magnetic beads

Eluting the samples from the magnetic beads by adding an elution buffer.

The pipette prompts the user to move the reaction plate from the magnet (position C) to position B. Continuing the protocol, the ASSIST PLUS transfers the elution buffer to the DNA samples bound to the magnetic beads (position B, orange). After mixing carefully and thoroughly 10 times, the pipette prompts the user to place the 96 well plate on the magnet (position C).

5. Transfer the sample eluates

Transferring the sample eluates into a new 96 well plate.

As indicated by the pipette, place a new 96 well plate onto position B and continue the program. The sample eluates are then transferred into the new plate automatically.

Tip: Optimized pipette settings (aspiration speed, volume, height, tip travel and tip touch) allow the volume of eluate transferred to be maximized without carryover of beads (see **Figure 6**). A tip touch after the transfer removes droplets that may still cling to the end of the pipette tips. Pipetting heights on the ASSIST PLUS can be fine-tuned at any time. Performing a test run with water before implementing any new assay is an ideal way to optimize pipette settings.

Results

Figure 7: Magnetic beads are clearly visible in the 96 well plate with no supernatant remaining.

Figure 8: No carryover of beads is observed in the eluate.

Conclusion

- Magnetic bead purifications can be easily automated on the ASSIST PLUS pipetting robot.
- Optimized tip immersion and pipette settings together with the use of low retention GRIPTIPS allow maximum sample recovery at the end of the purification protocol.
- The pipette loaded onto the ASSIST PLUS prompts the user when needed, eliminating the risk of human errors.
- VIALAB programs can be easily adapted to specific labware.
- Prolonged pipetting tasks lead to repetitive strain injury. This can be avoided by automating these steps with the ASSIST PLUS.

For more information and a list of materials used, please refer to our website.

CHAPTER 4: Customer testimonials

Our range of innovative liquid handling products has helped countless laboratories to achieve PCR success, improve their throughput and further their ground-breaking research. But don't just take our word for it! Here are a few stories from our satisfied customers, demonstrating why INTEGRA Biosciences is the right choice for PCR pipetting solutions and labware.

4.1 INTEGRA pipettes – the obvious choice for start-up PCR labs

The gradual reopening of the world following the pandemic has led to an unprecedented demand for COVID-19 testing, with schools, universities and workplaces relying on negative PCR tests to continue operating. Matrix Diagnostics – a dedicated COVID-19 testing lab in California – is helping to fulfill this critical need, relying on INTEGRA's EVOLVE and MINI 96 pipettes to streamline and accelerate PCR workflows.

PCR-based diagnostic testing is a well-established technique in clinical labs around the world, and this method has been brought to the attention of every household as the gold standard for COVID-19 testing. However, the public is less aware that the sensitivity of this technique makes it time-consuming and troublesome to perform without the right tools, as it is very sensitive to pipetting errors and cross-contamination.

Founded in January 2021, Matrix Diagnostics was established to meet the growing demand for PCR testing in the San Francisco Bay Area, and the newly formed team understood the need for effective pipetting solutions from the outset. Fady Ettnas, Lab Manager at Matrix Diagnostics, explained: "We realized that, to meet the anticipated demand for testing, we would have to turnover between 2000 and 5000 samples every day. This seemed like an impossible task for a new lab with limited resources but, after implementing INTEGRA's pipettes in our lab, we quickly alleviated the pipetting bottlenecks, putting us on track to achieve our targets."

Photo courtesy of Matrix Diagnostics

Evolving workflows

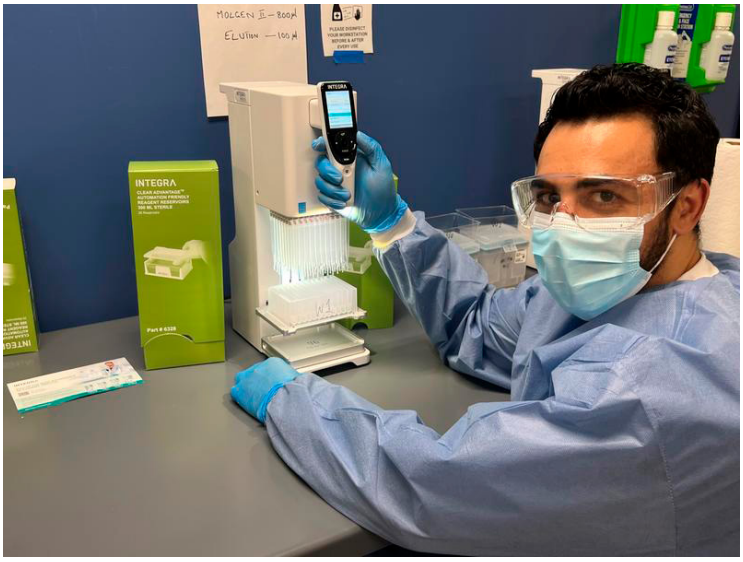


Photo courtesy of Matrix Diagnostics

"Our protocols involve a range of repetitive pipetting steps – including mixing reagents and serial dilutions – for thousands of samples a day, which has the potential to be a cumbersome and error-prone task," Fady continued. "We therefore chose INTEGRA's EVOLVE manual pipettes and MINI 96 portable electronic pipettes to improve the reproducibility and productivity of our workflows. We have a number of single channel EVOLVE pipettes, covering volumes ranging from 0.2 to 5000 µl, as well as 8, 12, and 16 channel models. What I like most about EVOLVE is its ergonomic design and ability to set volumes in a flash. The unique design of INTEGRA's GRIPTIPS also means that they never leak or fall off, avoiding cross-contamination and maintaining sterility. We also use the compact MINI 96 extensively, which is especially well suited to PCR set-up. It saves a lot of time and effort – around 15 minutes per cycle – when performing the wash steps. And because we run more than 25 cycles every day, this is a huge saving, allowing us to process a much higher number of samples. It is a perfect and affordable solution for our needs."

A long-term investment

The benefits of these pipettes to users, particularly in terms of preventing physical strain caused by repeated pipetting actions, are a priceless advantage. "I think the pipettes are a great investment with huge returns, allowing the team to process more samples and improving their pipetting experience. The company's customer service is quick, responsive and helpful and, crucially, the team was able to advise us on the right choice of pipettes to meet our workload and objectives."

Planning future with INTEGRA

"Currently, we are only offering COVID-19 tests, but we plan to expand to include other tests including sexually transmitted diseases, urinary tract infections and flu, and we know that we will need to automate our workflow. We will need something flexible and incredibly efficient and, therefore, we are planning to acquire an ASSIST PLUS pipetting robot. I like all the INTEGRA products that I've used, and have rarely encountered even minor technical issues. I think they are the most obvious pipetting choice for both for start-ups and established lab set-ups, and are well worth the investment," Fady concluded.

4.2 A better qPCR pipetting experience

Manual pipetting can be a major bottleneck for research laboratories, especially when they face the challenge of combining accurate results with high throughput. Like all repetitive tasks that require precise actions, filling multiwell plates by hand is time consuming, and physically and mentally draining, which can lead to errors. When Daisy Shu joined the Saint-Geniez laboratory at Harvard Medical School, her experience was quite different, thanks to the INTEGRA VIAFLO electronic pipettes.

From patients to pipettes

After graduating in optometry from the University of New South Wales in Sydney, Daisy worked as an optometrist for two years before deciding to pursue a PhD in cataract research at the University of Sydney. She explained: “The move from my usual clinical work with patients to research was a big change for me, as I had to dive deep into molecular biology. I didn't even know how to use a pipette back then! Cataracts – clouding of the eye’s lens – are a leading cause of blindness worldwide, and I studied their formation and ways to prevent that happening. My focus was on transforming growth factor beta (TGF-β), which has an important role in cancer metastasis, but is also relevant for certain types of cataracts. I looked at the different signaling pathways it activates and how those pathways interlink.”

Photo courtesy of Harvard Medical School

Daisy completed her PhD in January 2019, and straight afterwards flew to Boston to work as postdoctoral fellow in the Saint-Geniez laboratory, continuing her research into eye health. Here, she was able to apply her knowledge of TGF-β to age-related macular degeneration (AMD). Daisy continued: “I'm now looking at how TGF-β causes the retinal mitochondria to change morphology and become dysfunctional, altering cellular metabolism. The research is still at an early stage, so we're mainly trying to understand how to prevent AMD, but the end goal is to find a cure.”

A better pipetting experience

At Harvard, Daisy was introduced to VIAFLO electronic pipettes, which were a complete contrast to the large, fully automated pipetting workstation she had used during her PhD research. The laboratory was already using two VIAFLO pipettes – a 125 µl eight channel pipette and a 12.5 µl single channel version – and their flexibility compared to the automated workstation dramatically improved her pipetting experience. “Complete automation on a large workstation has its place, but there are downsides,” said Daisy. “You have to program every

single step perfectly before you can click one button and run the protocol, and the process of fine-tuning takes a long time."

Photo courtesy of Harvard Medical School

"I found the VIAFLO pipettes amazing. A lot of our work is PCR-based, performed in 384 well plates, and the VIAFLO pipettes are real lifesavers. I use the 8 channel VIAFLO for most qPCR liquid transfers, and the single channel pipette to add the primers. Once you've made your master mixes and programmed the pipette, it's really fast; it only takes me 20 minutes to do a complete 384 well plate. When I was using the robotic workstation in Sydney, I used to think that doing a qPCR was really a big deal. Now, with the INTEGRA pipettes, it's just so easy."

VIAFLO pipettes provide a choice of pipetting modes and allow easy adjustment of parameters such as volume and speed, as well as providing pre-set programs and the option for custom workflows. This helps laboratories to reduce errors and increase throughput and reproducibility regardless of the users' pipetting experience. For Daisy, VIAFLO electronic pipettes have become the standard for how pipetting should be: "In any pipetting workflow, you have to get every step right first time, otherwise you'd end up having to troubleshoot the assay and do it again. I'm really surprised when I hear people from other labs say they pipette each well individually with manual single channel pipettes. I'm sure that would take forever compared to electronic pipetting, and my eyes would really suffer. The VIAFLOs make everything easy. I love the color coding – it makes it so simple to match the right tip to the right pipette – and the instrument can even be set to alert you when you need to pipette again."

4.3 COVID-19 – Accelerate your PCR set-up

The emergence and outbreak of the novel coronavirus SARS-CoV-2 (COVID-19) has placed unprecedented demands on laboratories testing patient samples for COVID-19, leaving scientific staff to contend with a spiraling influx of COVID-19 samples and a rapid, continuous growth in workload. Among the challenges faced by the Microbiology and Molecular Pathology Department at Sullivan Nicolaides Pathology (SNP) – part of the Sonic Healthcare Group – in Brisbane, Australia, is the increased pressure on laboratory automation used for both coronavirus and pre-existing respiratory virus panel testing.

As a result of the coronavirus pandemic, SNP found itself analyzing extreme numbers of samples, which exhausted the capacity of its automation platforms. At the same time, staff were faced with a need to spend more time working up new virus testing protocols, which were often performed manually or using semi-automated methods to fast track test response times, leaving them prone to increased ergonomic strain. There was a clear need for additional automated liquid handling instruments to increase sample processing capacity, reduce manual intervention by laboratory analysts and fast track assay development for COVID-19 sample testing.

Working together

In early March 2020, Kelly Magin and James Sundholm from INTEGRA's Australian distributor, BioTools Pty Ltd, partnered with Shane Byrne, Scientific Department Head, Microbiology and Molecular Pathology Department, SNP, to support COVID-19 testing of patient samples using the ASSIST PLUS pipetting robot. An ASSIST PLUS automated pipetting protocol was developed and validated, enabling samples to be prepared in low volume, 384 well plates for subsequent processing on a rapid, high throughput, plate-based, real-time PCR amplification and detection instrument. A VOYAGER adjustable tip spacing pipette and low retention GRIPTIPS were used to transfer one-step RT-PCR master mix from a low dead volume (<20 µl) SureFlo 10 ml reagent reservoir into a 384 well plate. The VOYAGER pipette also allowed automatic transfer and reformatting of nucleic acid template extracted from combined nasopharyngeal/oropharyngeal flocked swab(s) or sputum samples, from 4 x FluidX™ 1.0 ml 96 format tube racks into the 384 well plate. The total PCR reaction volume was reduced to 10 µl; 7.5 µl one-step RT-PCR master mix and 2.5 µl of nucleic acid template. This miniaturization doubled the available testing capacity and simultaneously reduced consumption of expensive one-step RT-PCR reagents of dwindling availability, with associated cost savings.

Photo courtesy of Sullivan Nicolaides Pathology

Defining success

SNP successfully validated the automated protocol against its existing manual processing method, performed using a handheld electronic pipette. The results were shown to be reproducible, precise and accurate, with no contamination observed in either the control or patient samples. The compact, easy-to-use ASSIST PLUS pipetting robot, complete with validated protocol, was fully deployed within five working days. While the current protocol uses 384 well plates, it can be readily adapted to 96 well format to meet future needs.

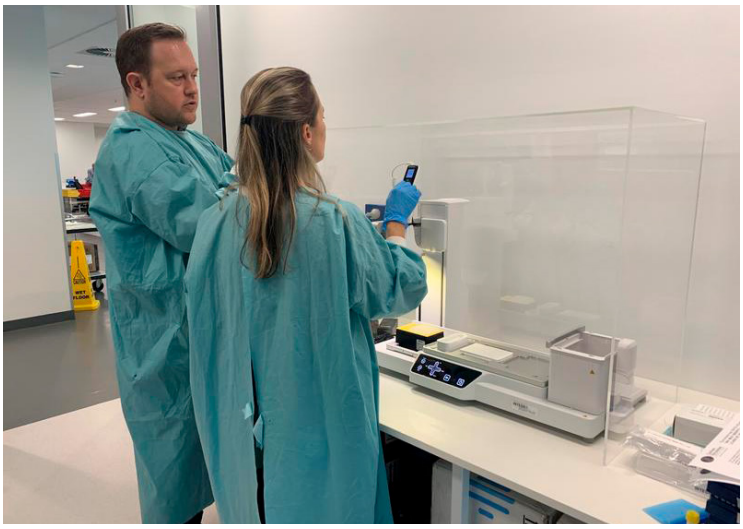


Photo courtesy of Sullivan Nicolaides Pathology

4.4 Reducing protocol time for PCR using 96 channel pipette

Implementing an INTEGRA VIAFLO 96 electronic pipette has enabled the Virus- and Prion Validation (VPV) Department at Octapharma Biopharmaceuticals GmbH, (Frankfurt, Germany) to reduce the time taken to undertake PCR assays by greater than 60 %.

Since its foundation in 1983, Octapharma has been committed to patient care and medical innovation. Its core business is the development and production of human proteins from human plasma and human cell-lines.

The VPV Department has been set-up to investigate pathogen inactivation and removal steps along the manufacturing processes. Among other techniques, multi-step 96 well format PCR assays were developed, which involve three washing steps twice in the protocol. To undertake their PCR assay more efficiently, Octapharma sought a system that enabled reproducible and accurate liquid handling in the 96 well format and was able to completely remove residual liquid as well as avoid well-to-well contamination.

Dr. Andreas Volk, a research scientist at Octapharma Biopharmaceuticals commented: "The classical liquid handling solutions, fully automated robots or ELISA plate washers were either too costly or prone to cross contamination in a PCR assay." He added: "When we tested the INTEGRA VIAFLO 96 channel pipette, it fully met our requirements as it enabled medium-throughput liquid handling while minimizing cross-contamination. Additionally, the

VIAFLO 96 electronic pipette provided all the adjustment options, which we had been used to with manual pipettes, plus a specified tip immersion depth for each pipetting step. With our PCR protocol, which involves ten full liquid transfers per plate, we now only use half the amount of pipette tips as we can use the same tips for liquid addition and aspiration in each washing step. VPV Department staff has found using the VIAFLO 96 benchtop pipette highly intuitive and the overall time required for our PCR washing procedures has been reduced to approximately one third of the original time."

Dr. Andreas Volk, Octapharma Biopharmaceuticals

The INTEGRA VIAFLO 96 is a handheld 96 channel electronic pipette that has struck a chord with scientists looking for fast, precise and easy simultaneous transfer of 96 samples from microplates without the cost of a fully automated system. The VIAFLO 96 was designed to be handled just like a standard handheld pipette – a fact borne out by consistent end user feedback that no special skills or training are required to operate it. Users immediately benefit from the increased productivity delivered by their VIAFLO 96. Fast replication or reformatting of 96 and 384 well plates and high precision transferring of reagents, compounds and solutions to or from microplates with the VIAFLO 96 is as easy as pipetting with a standard electronic pipette into a single tube. Four pipetting heads with pipetting volumes up to 12.5 µl, 125 µl, 300 µl or 1250 µl are available for the VIAFLO 96. These pipetting heads are interchangeable within seconds enabling optimal matching of the available volume range to the application performed. For 384 well pipetting, an enhanced version is available with VIAFLO 384. It features 384 channel pipetting heads in the volume range of 12.5 µl and 125 µl and is compatible with 96 channel pipetting heads.

CHAPTER 5: Conclusion

So, there you have it, a full run down of PCR. By now, you should have all the information you need to become a PCR pro, but if you'd still like to learn more about this interesting topic, we have a wealth of articles on our website. Whatever your PCR requirements, we at INTEGRA Biosciences are always available to answer your questions and provide you with the best workflow solutions.

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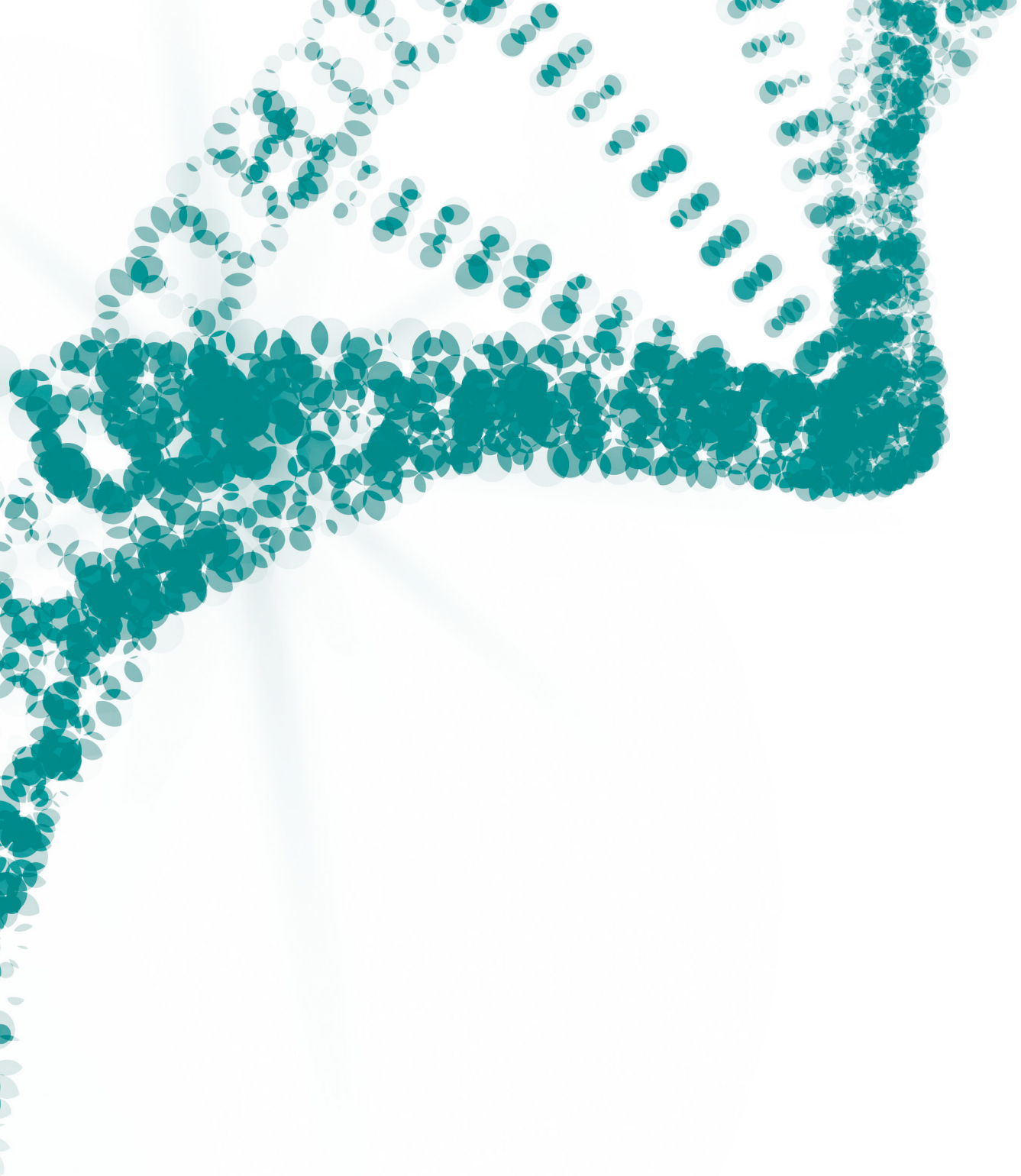

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