Chapter 2

General Guidelines

2

2

2	General Guidelines	Page

2. General Guidelines

2.1 Preventing Contamination in the PCR Laboratory

PCR generates large numbers of amplicons. Therefore, it carries a high risk of contamination. Even infinitesimal amounts of contaminating DNA may lead to significant concentrations of amplicons and false results.

Clearly, contaminant-free assays are critical for identifying pathogenic organisms or "fingerprinting" DNA. Any PCR lab involved in developing such assays must avoid contamination at all costs since their assay samples may be hazardous to the health of laboratory personnel. In addition, these assays, if ultimately approved for clinical or forensic use, will affect the lives of many people.

Similarly, labs that use PCR for exploring the behavior and function of genes must take steps to avoid contamination, since no one wants to waste enormous amounts of time and money pursuing research based on contaminated assays.

Sources of Contamination

Contamination may be defined as "accidental contact or mixing of the sample with exogenous material (nucleic acids or microbial organisms) that make the sample impure or corrupt." As stated above, any source of contamination, no matter how small, can lead to false positive results.

Contamination usually arises from two sources:

- Cross-contamination, or contamination of the sample with non-amplified material from the environment (*e.g.*, aerosols, cloned DNA molecules that carry the target gene) or other samples. This may be due to parallel storage, handling or processing of intermediate samples.

- End-product (*i.e.*, carryover) contamination, or contamination of the sample with amplicons from earlier PCRs, leading to subsequent amplification of both target and contaminant amplicons. This may be due to storage, handling or processing of samples between PCRs.

In sections 2.1.1 and 2.1.2 below, we will describe laboratory practices designed to exclude cross-contamination from exogenous microbial (*i.e.*, bacterial, fungal or viral) DNA in routine diagnostic laboratories. These practices are currently used in our Application Laboratory for the LightCycler® 2.0 System (Penzberg, Germany). Although developed for real-time PCR assays on a LightCycler® 2.0 Instrument, these guidelines are also generally suitable for (or can be adapted to) other applications, such as qualitative PCR analysis on a block cycler in a research laboratory.

These practices are based on working procedures that routine diagnostic laboratories must follow to comply with German regulations. These regulations cover, among other things, the physical separation of areas for extraction, PCR setup and amplification/detection, and the use of filtered tips. For a more detailed discussion, see Mauch et al. (2001).

A technique for preventing carryover contamination will be discussed in section 2.1.3 below.

2

2.1.1 Setting Up the PCR Laboratory Space Correctly

Contaminant DNA usually finds its way into PCR assays through:

- Working environment (*e.g.*, rooms and equipment, lab benches and other work surfaces).
- Consumable reagents and supplies (*e.g.*, oligonucleotides, media for sample collection and transport, plasticware).
- Laboratory staff and their work habits (*e.g.*, contaminants from skin, hair, gloves, production of aerosols during pipetting).

Therefore, preventing microbial cross-contamination has two equally important components: setting up laboratory space correctly (discussed below) and cultivating laboratory habits that prevent cross-contamination (discussed in section 2.1.2). Once the lab is correctly configured and good laboratory habits are followed by all personnel, preventing contamination become almost automatic and the laboratory can focus on producing meaningful results.

Usually, cross-contamination and environmental contamination occur during sample manipulations. So, from the very beginning, the PCR laboratory should be set up to accomplish two goals:

- Physically separating the lab areas used for sample preparation, DNA extraction, amplification and post-PCR analysis, and
- Minimizing the number of sample manipulations.

Physical Separation of Work Areas

In our laboratory, separate areas are provided for preparation of samples for nucleic acid isolation, automated nucleic acid isolation/processing in the MagNA Pure LC Instrument and amplification/analysis in the LightCycler® Instrument.

Even if all steps are performed manually, a properly set-up PCR lab will contain physically separate areas for sample preparation, DNA extraction, amplification and post-PCR analysis. Ideally, each of these takes place in a separate room and each of these rooms has:

- A dedicated set of reagents, pipettes, and disposables that are kept and used exclusively in that room.

For analysis of microbiological samples, we recommend storing bags that contain disposables (tubes, tips, reagent tubs) in a separate, suitable room. (Presence of UV light fixtures in this storage room is recommended.)

- A full set of equipment, such as refrigerator, centrifuges and safety cabinets.
- A dedicated set of lab coats that are kept and used exclusively in that room.
- A safety cabinet, if samples are potentially hazardous or infectious.

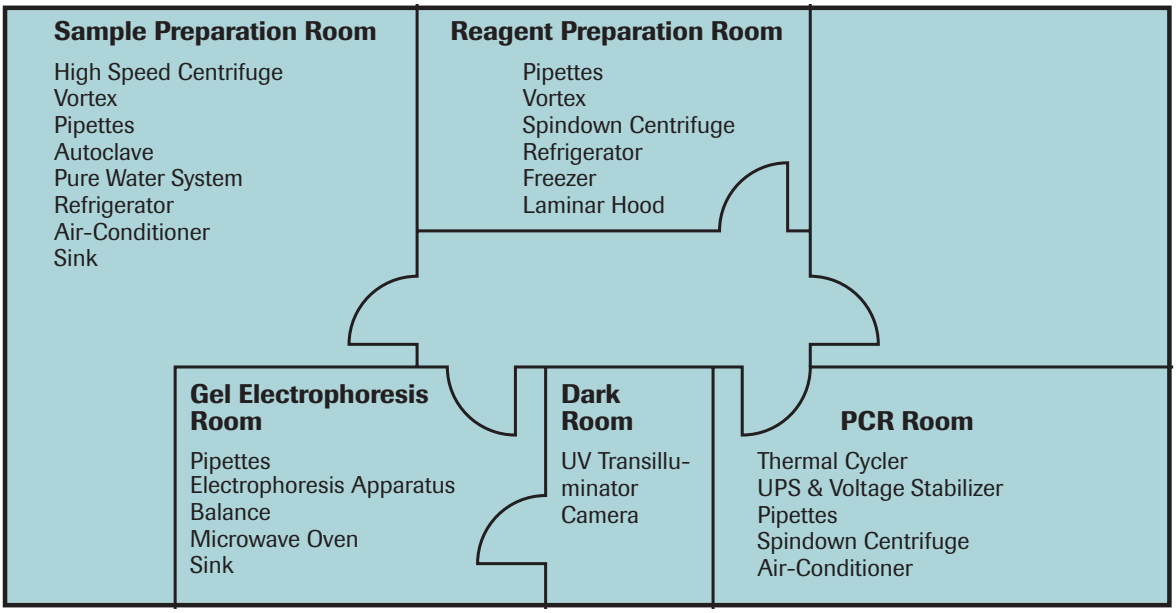


Figure 2.1.1 Organization of a PCR laboratory with separate pre- and post-PCR rooms.

Minimizing Sample Manipulation

The best way to minimize sample manipulation (and the corresponding risk of contamination) is to automate as many steps in PCR analysis as possible. See "Guidelines for Preventing Cross-Contamination" in section 2.1.2 below for more information on what steps can be automated.

2.1.2 Cultivating Laboratory Habits That Prevent Cross-Contamination

By itself, a properly set-up PCR laboratory space will not prevent cross-contamination. Once people start working in that space, their actions introduce significant risks of contaminating the PCR samples.

Therefore, preventing cross-contamination requires the cooperation of all laboratory personnel. Only if all the people in the laboratory follow good work habits, will the laboratory be free of contamination.

This may seem unduly harsh. We all know laboratory personalities that seem to lead a charmed life, working with total disregard of good laboratory practices yet seeming to always get good results. However, once those persons join a laboratory group, their actions have the potential to ruin the results of all their co-workers. That means even if you are tempted to take short cuts in a PCR procedure, or to ignore the precautions outlined below, don't do it!

Example: Never reverse the direction of the workflow (*e.g.*, by transporting amplified material into the DNA extraction room). The steps in the PCR workflow are always unidirectional, from DNA extraction to amplification. This principle holds for working procedures as well as for reagents and consumables.

Below we list a general strategy for identifying sources of contamination and developing guidelines to avoid them.

2

Developing Laboratory Work Guidelines: Assessing and Avoiding Risks in PCR Workflow

Even in the best physical workspaces, microbial DNA contamination can still get into PCR samples. Therefore, in developing good laboratory habits, start by attempting to quantify the hazards and risks a worker may encounter during the entire experimental procedure. A contamination hazard may be defined as the introduction of contaminating nucleic acid from any possible source; risk is the probability that the hazard will occur.

The goal of a risk assessment is to carefully consider all hazards and risks associated with the whole process, so that the risk can be minimized and preventive actions can be implemented. Any change in the process (*e.g.*, new lab personnel, new equipment, a new supplier of consumables or a change in assay design) requires a new risk assessment.

For risk assessment, consider sample handling steps, materials (reagents and disposables) used, and storage of intermediate products (such as extracted DNA) (Figure 2.1.2).

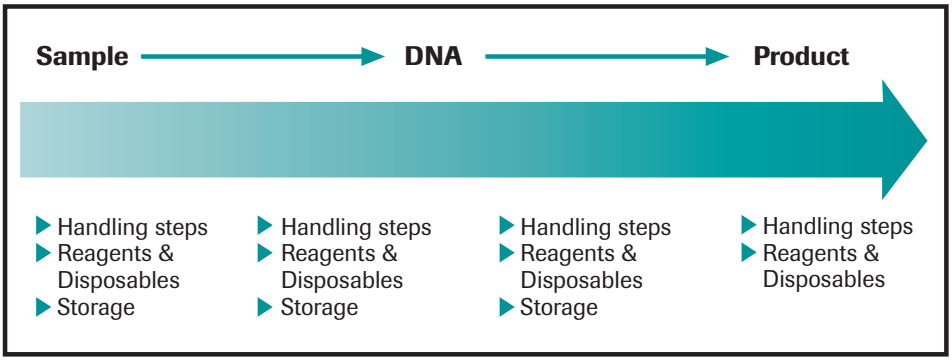


Figure 2.1.2. Critical steps in PCR workflow.

Once risks have been identified and quantified, the laboratory can adapt strategies to minimize or avoid these risks. These strategies can be formalized as laboratory work guidelines.

For example, we list below one possible source of contamination and the strategies we adapted to avoid these risks.

Because each laboratory has unique practices and environments, no single strategy for risk assessment and avoidance can be appropriate for all laboratories. Each laboratory should perform its own risk assessment and adapt avoidance strategies that are appropriate for the risk.

Strategies for Avoiding One Possible Source of Contamination

Most manufacturers of consumables and reagents do not guarantee that their products are DNA-free, although most would guarantee them to be sterile. Consequently, these products may constitute a risk of contamination.

The problem arises because in microbiological terms, "sterile" means "absence of viable, replicable bacteria or other microorganism." Thus, "sterile" does not imply absence of bacterial or fungal DNA in any form. In fact, commercially available "sterile" instruments and culture media may frequently contain microbial DNA. The instruments and media are still "sterile" because the source of this DNA (= the organism) cannot be cultivated. The amount of such contamination depends on the supplier as well as the production lot.

Generally, any reagent, device or consumable has the potential to cause false positive results because it may be contaminated with microbial DNA. Specifically, most molecular biology enzymes contain some amount of bacterial DNA since they were derived from different strains of bacteria.

One strategy for preventing such false positives is to screen individual reagents and consumables in PCR before they are used to assay unknowns. This is especially true for oligonucleotides (primers and probes).

In addition, you should include a negative control in the PCR run for every step that uses fresh reagents and disposables. It is also helpful to include a reliable positive control to demonstrate true gene expression.

Guidelines for Preventing Cross-Contamination

The tables below summarize the guidelines that our laboratory follows to prevent PCR contamination.

If each member of the laboratory converts these guidelines into personal work habits, most contamination can be avoided and, if contamination occurs, its effects can be minimized by identification and elimination of the contamination source.

2

Area of Concern	Steps To Take
All stages	
Disposables and reagents	▶ Use of reagents and disposables is room-specific. Always store a unique set of reagents and disposables in each of the procedure-specific rooms (DNA isolation, PCR, etc.) and use that set only in that room. ▶ Always use the highest quality of reagents available for each step of the procedure. ▶ To guarantee that bags of plastic disposables remain free of DNase and RNase, reseal these bags immediately after removing the disposables needed for each procedure. ▶ Use each plastic disposable only once. ▶ Divide reagents into aliquots that will be consumed in a single procedure (DNA isolation, PCR, etc.). Make only enough reagents for the number of samples you are preparing (*e.g.*, if you usually run 10 samples, make aliquots with enough reagents for 11 reactions). ▶ Minimize pipetting steps by using reagent premixes (*e.g.*, PCR master mixes available from Roche Applied Science) whenever possible. ▶ Discard any reagents left over from a procedure rather than storing them and reusing them. ▶ Always use pipette tips that prevent aerosol formation. ▶ Regularly wipe pipette tips with ethanol-soaked tissue. *Do not get any ethanol into the pipette tip itself.*
Cleaning/Decontaminating the laboratory environment	▶ Access to the laboratory is controlled and limited. ▶ Develop a formal written plan for monitoring cleaning and decontaminating the laboratory.[a] Make this plan comprehensive (*e.g.*, include such matters as how and where to clean laboratory coats.) ▶ Train all laboratory and cleaning personnel in routine cleaning and decontamination procedures. ▶ Always use cleaning agents that are suitable for and dedicated to decontaminating PCR lab surfaces and instruments. (Several decontaminating reagents are commercially available, *e.g.*, License-to-Kill, DNAZap, DNA Remover, or DNA-ExitusPlus. Alternatively you may also use bleach [10% solution of sodium hypochloride]; before using bleach on instrument surfaces make sure this is allowed for your instrument.) Do not use reagents that are used to clean other parts of the facility. ▶ UV irradiation (at 254 nm) can "inactivate" DNA contamination in disposable, reagents and PCR samples. UV dimerizes the thymidine residues in DNA, thus rendering it incapable of serving as a PCR template. Suitable irradiation devices include those for crosslinking DNA to nylon membranes. *Be sure to UV irradiate PCR samples before adding the template DNA, DNA polymerase, and dNTPs!*
Water	▶ Always use ultrapure water for reagent and sample dilution. ▶ Do not use autoclaved water because it may be contaminated with DNA if the autoclave is also used for sterilization of cultures. ▶ Since DNA is highly soluble in water, water may be used to clean the laboratory environment, dilute DNA (to minimize the risk of contamination) and to transport DNA away from contaminated benches. ▶ Autoclaving facilities must be completely separate from the PCR labs and steam must not be carried into the PCR labs via the air conditioning system. *Since DNA is soluble in water, it is also soluble in steam. Therefore, steam which escapes from an autoclave (e.g., after sterilization of cultures) may contain large amounts of DNA.*
Laboratory clothing	▶ Always wear gloves and lab coats during the procedures. ▶ Always clean lab clothing according to the formal laboratory cleaning plan. (See above.)

Area of Concern	Steps To Take
Preparing reagents and samples for DNA isolation	
General	Follow all guidelines under "All stages" above.
Physical space	Always dedicate separate rooms in the laboratory that are used only for preparing reagents and samples for DNA isolation.
Handling of biohazardous or infectious material	▶ Prepare all sample materials and positive controls in a safety cabinet. ▶ Follow all country-specific guidelines and regulations for handling infectious or biohazardous material.
DNA isolation	
General	Follow all guidelines under "All stages" above.
Physical space	Always dedicate a separate room in the laboratory that is used only for DNA isolation.
Isolation procedure	▶ Always use reagents that are specifically designed for isolation of DNA. ▶ Ideally, automate as much of the nucleic acid isolation procedure as possible by using an automated instrument (*e.g.*, the MagNA Pure LC Instrument* or the MagNA Pure Compact Instrument*) ▶ At the very least, minimize the number of hands-on steps required by using manual nucleic acid isolation kits.* *Starting with the nucleic acid extraction step, manual PCR steps will always carry a higher risk of contamination than the corresponding automated steps.*
Preparation of instrument (*e.g.*, MagNA Pure LC Instrument) for automated DNA isolation (optional)	▶ Decontaminate the instrument with UV light for 30 minutes. ▶ Change all waste collectors (*e.g.*, on the MagNA Pure LC Instrument, the dropcatcher, the waste bag on the waste slide, the waste bottle tray, the waste bottle, and the waste funnel.) ▶ If using biohazardous material, set the instrument to automatically discard waste. ▶ After the isolation run, make the instrument ready for the next run by performing the following cleaning steps: ▶ Seal and autoclave all waste receptacles (*e.g.*, on the MagNA Pure LC Instrument, the waste bags and the waste bottle). ▶ On the MagNA Pure LC Instrument, decontaminate all waste collectors and accessories (*e.g.*, the cooling block) as recommended by the manufacturer.
PCR	
General	Follow all guidelines under "All stages" above.
Physical space	Always dedicate a separate room in the laboratory that is used only for PCR.
PCR set-up	▶ If your thermal cycler requires a mineral oil overlay, make sure the mineral oil does not become contaminated by careless pipetting. For example, pour an aliquot of mineral oil from a stock bottle to a microfuge tube, use the oil in this tube for dispensing to sample tubes, then discard the microfuge tube at the end of the set-up. ▶ Perform all set-up procedures that require open reagents and boxes of disposables in a PCR cabinet with a laminar (nonturbulent) airflow. ▶ Prepare positive controls in a room separate from the sample preparation room. ▶ If possible, to minimize hands-on steps, use an instrument that automatically performs the PCR sample set-up (*e.g.*, the MagNA Pure LC Instrument).
Cooling blocks	▶ Cooling blocks that are used in different parts of the laboratory should only be opened in a PCR cabinet with laminar airflow. ▶ After each use, clean the cooling blocks with approved reagents. ▶ In addition to the above cleaning, completely degrade all nucleic acids on the block by cleaning the block with DNAZap; completely remove RNase contamination by cleaning with RNaseZap.
Controls	▶ Always prepare positive and negative control samples for every PCR run. ▶ Include a negative control in a PCR run for every step that uses fresh reagents and disposables (*e.g.*, sample preparation, DNA extraction and amplification). For example, if you isolate sample material from a culture, include one sample that contains just culture medium. ▶ Prepare positive controls in a room separate from the sample preparation room. ▶ Optionally, include an internal (or endogenous) positive control in the run to help identify PCR inhibitors.

Area of Concern	Steps To Take
PCR continued	
PCR sample tubes	▶ Use sterile, DNA-free, disposable forceps to close the tube lids. ▶ Never touch any surface of the disposable tubes (*e.g.*, interior of lids) that will make contact with the sample.
Automation of PCR	A real-time PCR instrument (*e.g.*, the LightCycler® 2.0 Instrument* or the LightCycler® 480 Instrument) can automate the amplification process.
Post-PCR processing	
General	Follow all guidelines under "All stages" above.
Physical space	Always dedicate a separate room in the laboratory that is used only for post-PCR processing of PCR products.
Automation of product detection	A real-time PCR instrument (*e.g.*, the LightCycler® 2.0 Instrument*) can automate the product detection process.

a) For example, our laboratory follows the country-specific regulations for setting up a laboratory hygiene plan. In Germany, such regulations are available from the Robert Koch Insitut.

* Manual nucleic acid isolation kits, the MagNA Pure LC Instrument and the LightCycler® 2.0 Instrument are available from Roche Applied Science.

2.1.3 Using Uracil N-Glycosylase To Eliminate Carryover Contamination

Another serious source of contamination is carryover of product from a previous PCR. This is a particularly serious problem in laboratories that amplify the same target in many different samples. Even minute amounts of carryover can lead to false positive results. For example, Kwok and Higuchi (1989) calculated that, if 100 µl of a PCR product from a typical PCR were diluted into an olympic-size swimming pool and mixed, a 100 µl sample taken randomly from that pool-sized mixture would contain approximately 400 molecules of DNA.

Obviously, the guidelines listed in section 2.1.2 above can help prevent carryover contamination as well as cross-contamination. However, there is another precaution that can be taken to specifically negate the effects of carryover contaminants (*i.e.*, contamination with previously amplified PCR products).

Including uracil-DNA N-glycosylase (UNG) and deoxyuridine triphosphate (dUTP) in all PCRs can help prevent carryover contamination. The prevention procedure involves incorporating dUTP during PCR so the amplicons contain deoxyuracil (dU), then pretreating subsequent PCR mixtures with UNG. If a dU-containing contaminant from a previous PCR is present in a new PCR, it will be cleaved by a combination of UNG digestion and the high temperature of the initial denaturation step; after treatment, it cannot serve as a PCR template. Since any newly added DNA template (*e.g.*, your target DNA) contains thymidine rather than uridine, it is not affected by this procedure. For more information on the use of UNG, see section 4.6, "Preventing Carryover" in Chapter 4.

Country-specific safety and health regulations also contain many procedures for minimizing carryover contamination. Although this manual does not discuss these regulations, we recommend implementation of such procedures and compliance with all such guidelines.

2.2 Factors To Consider When Setting Up a PCR

2.2.1 Equipment Required

Below is a brief overview of the equipment needed for PCR.

Thermal Cyclers

A thermal cycler must, at a minimum, accurately and reproducibly maintain the three PCR incubation temperatures (denaturation, annealing and elongation), change from one temperature to another ("ramp") over a definable time, reach the selected temperatures without significantly over- or undershoot and cycle between the temperatures repeatedly and reproducibility.

The thermal cycler cited most frequently in the protocols given in Chapters 4 and 5 of this manual is the Applied Biosystems GeneAmp System 2400. If you choose another thermal cycler, you will need to adjust the cycling conditions given in this Manual to obtain optimal results.

When choosing a thermal cycler, consider the following factors to determine which best fits your experimental needs:

- What size (or size range of) sample is accommodated.
- How samples are held (*e.g.*, in a thermal block or other device).
- How samples are heated and cooled (*e.g.*, by air, electrical resistance within a fluid or electrical resistance modulated with a Peltier semiconductor device).
- How sample evaporation is controlled (*i.e.*, by a heated lid on the thermal cycler or by a simple mineral oil overlay of the samples).
- How the reaction steps are programmed (*e.g.*, determination of ramping rates, inserted pauses in the reaction).
- How temperature is monitored.
- Whether the reaction can be fully or partially automated.
- Whether the device simplifies PCR optimization (*e.g.*, if it has a gradient feature that allows testing of several preset temperatures simultaneously on a single thermal block).
- Whether and how product accumulation can be monitored.
- Whether reaction products can be refrigerated and kept in the thermal cycler for short-term (*e.g.*, overnight) storage.

2

Mineral Oil

If the thermal cycler you are using has a heated lid, there is no need to overlay the samples.

However, if the thermal cycler lacks a heated lid, you should place a layer of mineral oil atop the reaction mixture to prevent evaporation during PCR. For best results, use a high quality light mineral oil (available from chemical companies such as Sigma-Aldrich, Inc.)

Sample Containers

A variety of disposable sample containers is available for PCR. Some examples include:

- Reaction tubes: The reaction tubes used for PCR affect the rate at which heat transfers from the thermal cycler to the reaction mixture. Therefore, we recommend that you use thin-walled reaction tubes that are specifically designed for PCR and that fit precisely into the wells of your thermal cycler. Some tubes (and the corresponding caps) are available in convenient strips that fit into a row of the thermal cycler. A variety of such tubes is available from Roche Applied Science.

- Capillaries: For rapid PCR, small-volume capillaries allow much faster heat transfer. However, these only fit in sophisticated PCR instruments (*e.g.*, the LightCycler® 2.0 Instrument from Roche Applied Science).

- Microplates: For medium- and high-throughput applications, the preferred sample carrier is a PCR microplate.

Other Plasticware and Disposables

Tubes for reaction set-up: For aliquoting reagents and preparing master reaction mixes (but not for the PCR itself), the most convenient container is the disposable, conventional 1.5 ml microfuge tube.

Disposable pipette tips: As much as possible during reaction set-up, use aerosol-resistant pipette tips, to prevent contamination of the samples.

Equipment for Product Analysis

Most laboratories already have the equipment needed for routine analysis of PCR products, including:

- Agarose gel electrophoresis, the most common way to detect PCR products qualitatively.

- Acrylamide gel electrophoresis, for analysis of smaller PCR products.

- Southern/slot/dot blots, for quantitation and sequencing of PCR products.

For more information on the use and suitability of various analytical techniques for PCR, see Chapter 6.

For faster, more sophisticated analysis of PCR products (*e.g.*, for high-throughput applications), you may want to consider using real-time PCR instruments. See Chapter 7 for more information on these powerful instruments.

2.2.2 Choosing the Correct Enzymes for PCR and RT-PCR

The choice of a PCR enzyme can profoundly affect the outcome of the PCR. As mentioned in Chapter 1, researchers have purified, modified and commercialized a variety of PCR and RT-PCR enzymes. Below is a brief overview of the major types of enzymes available.

Taq DNA Polymerase and FastStart Taq DNA Polymerase

The primary requirements for a DNA polymerase used in PCR are optimal activity at temperatures around 75°C and the ability to retain that activity after prolonged incubation at even higher temperatures (95°C). The first thermostable DNA polymerase to be widely used for PCR was Taq DNA Polymerasetaq dna polymerase. For many conventional PCRs that do not require extensive optimization, Taq DNA Polymerase is still a good choice. High quality, recombinant Taq DNA Polymerase (such as the preparation available from Roche Applied Science) produces the best results. Nevertheless a major drawback of standard Taq DNA Polymerase is its activity at temperatures below its optimum of 72°C. In non-optimized systems, this will lead to formation of primer-dimers due to elongation of primers annealed to each other before the first DNA denaturation step has occurred.

More recently, modifications of the Taq enzyme were developed which make it more useful for PCR. These so-called "hot start" preparations of Taq DNA Polymerase (*e.g.*, FastStart Taq DNA Polymerase available from Roche Applied Science) are inactive at low temperatures, but readily activated at DNA denaturing temperatures. Thus, hot start polymerases minimize the formation of troublesome primer-dimers during reaction set-up.

FastStart Taq DNA Polymerase is a chemically modified form of thermostable recombinant Taq DNA Polymerase that shows no activity up to 75°C. The enzyme is active only at high temperatures, where primers no longer bind non-specifically. The enzyme is completely activated (by removal of blocking groups) in a single pre-incubation step (95°C, 2-4 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot-start techniques.

Today, therefore, FastStart Taq DNA Polymerase is the best enzyme for most basic PCRs (*i.e.*, those that amplify normal or GC-rich templates up to 3 kb in length).

Proofreading DNA Polymerases

Standard Taq DNA Polymerase and its chemically modified form FastStart Taq DNA Polymerase lack the ability to detect the incorporation of a wrong nucleotide during polymerization and cannot remove such misincorporated nucleotides. Thus, these DNA polymerases have a relatively high PCR error rate. Furthermore, misincorporation during replication may lead to stalling of the DNA polymerase and thus to shortened PCR products. If your application requires a better fidelity than provided by (FastStart) Taq DNA Polymerase (*e.g.*, if you want to clone or sequence a PCR product), you need to use a thermostable DNA polymerase with "editing" or "proofreading" activity. Proofreading DNA polymerases possess an additional 3´-5´ exonuclease activity which removes misincorporated nucleotides.

For details of a lacI-based assay that can determine the transcriptional accuracy (fidelity) of a PCR enzyme, see Frey and Suppmann (1995).

Pwo SuperYield DNA Polymerase (available from Roche Applied Science in most countries, but not available in the U.S.) has a much (up to 18-fold) lower error rate than Taq DNA Polymerase, which makes this enzyme ideal for high fidelity amplification of targets with length up to 3 kb.

Information Note: A drawback of all known proofreading DNA polymerases is, that they cannot be used for carryover prevention using the Uracil-DNA N-glycosylase (UNG) method. Proofreading DNA polymerases are of archaeal origin and belong to the Family B-type DNA polymerases. All B-type DNA polymerases possess a proofreading (3'->5' exonclease) activity but lack a 5'->3' exonuclease activity. In contrast to Taq DNA Polymerase, archaeal DNA polymerases cannot copy DNA strands containing uracil residues: they possess a "read-ahead" function that detects dU residues in the template strand and stalls DNA synthesis (Martin A. Greagg *et al.*, 1999). Thus, exchanging dTTP by dUTP would lead to inhibtion of PCR when using a B-type DNA polymerase.

PCR Enzyme Mixtures and Blends

As PCR became more sophisticated, polymerase mixtures and blends began to be used. For specific purposes, these blends actually outperformed the individual DNA polymerases. Several examples are given below.

One approach to overcoming the limited accuracy of Taq DNA Polymerase alone was to combine the Taq DNA Polymerase with a thermostable, proofreading polymerase or another protein that has proofreading activity. Such a blend (*e.g.*, the Expand High Fidelity PCR System from Roche Applied Science) transcribes DNA approximately threefold more accurately than the Taq DNA Polymerase alone and can be used for high fidelity amplification of moderately long (up to 5 kb) targets.

For amplification of GC-rich sequences with high yield, the GC-RICH PCR System* from Roche Applied Science is the best mix to choose.

For amplification of multiple targets in a single reaction (multiplex PCR), FastStart High Fidelity PCR System offers several advantages. This enzyme mixture transcribes more accurately (up to fourfold higher fidelity) than Taq DNA Polymerase alone and is better able to amplify sequences with high (40 – 60%) GC content. Also, the blend is very sensitive, producing good yields of amplicon from small amounts of target DNA.

Certain blends of enzymes, buffers and additives allow accurate amplification of very long templates (*e.g.*, up to 25 kb targets with the Roche Applied Science Expand Long Range dNTPack).

To allow high-fidelity PCR in combination with prevention of carryover contamination using the Uracil DNA Glycosylase method, Roche Applied Science introduced the Expand High Fidelity^{PLUS} PCR System and the FastStart High Fidelity PCR System: these enzyme blends consist of Taq DNA Polymerase and a novel proofreading protein, isolated and characterized by Roche Applied Science. This protein mediates proofreading activity but has no polymerase activity itself and thus PCR is not inhibited by dU-containing DNA.

For a complete listing of PCR enzymes available from Roche Applied Science, see the Ordering Information in the Appendix. For more information on the properties of PCR enzymes, see the table, "Comparison of PCR Enzymes," below. For more information on the appropriateness of the different PCR enzymes for specific applications, see the PCR Protocol Selection Guide (in Chapter 4) or the PCR Selection Guide (in the Appendix).

Reverse Transcriptases

RT-PCR extends the power of PCR to the amplification of RNA by using an RNA-dependent DNA polymerase, commonly called a reverse transcriptase, to convert an RNA into a cDNA, then using a thermostable DNA polymerase to amplify the cDNA to detectable levels. This combination of reactions can be performed as either a one-step (consecutively, in a single tube) or a two-step (consecutively, in separate tubes) process. There are a variety of reverse transcriptases available for both one-step and two-step RT-PCR.

Transcriptor Reverse Transcriptase (available from Roche Applied Science) can be used in two-step RT-PCR to amplify RNA targets up to 14 kb long.

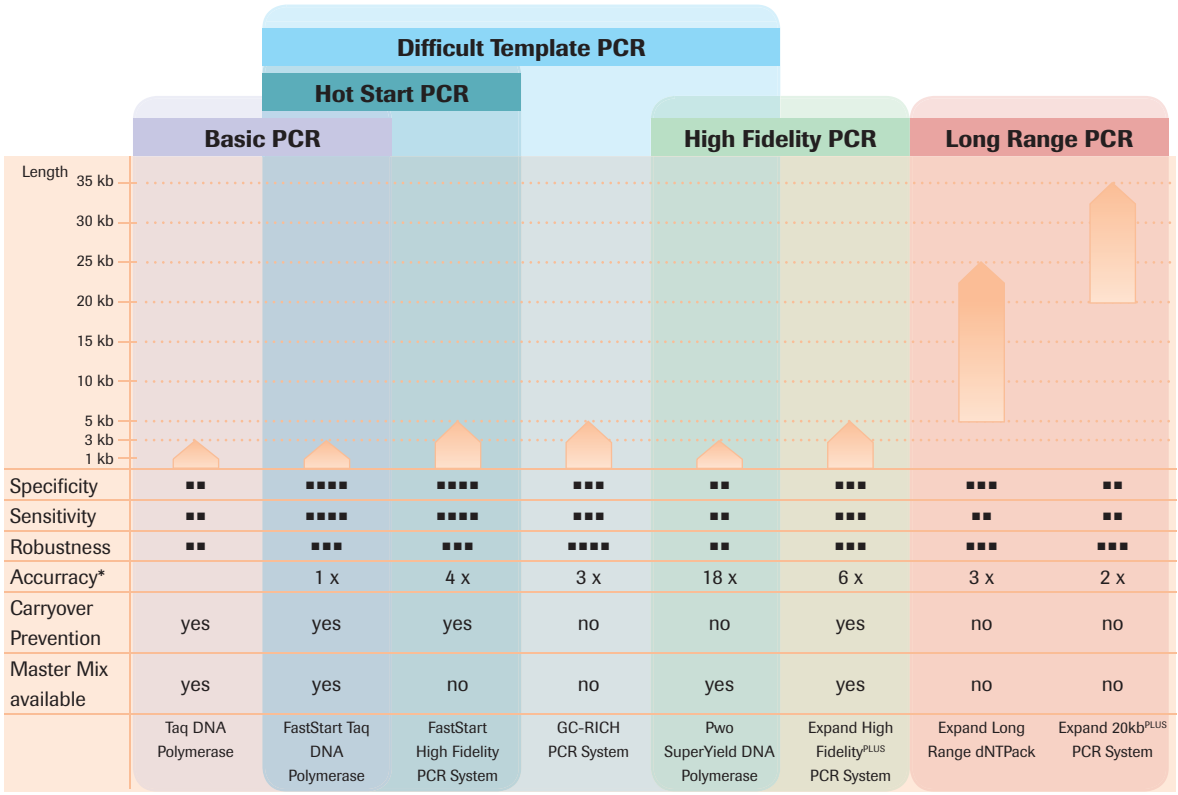
You can choose from several one-step RT-PCR Systems, depending on the requirements of your RT-PCR assay (*e.g.*, length of amplicon, GC content, error rate). The Titan One Tube RT-PCR System is best for amplicons up to 6 kb length but moderate GC content. The C. *therm.* Polymerase (both available from Roche Applied Science) is an efficient enzyme for one-step RT-PCR of RNA targets up to 3 kb long.

Tth DNA Polymerase (available from Roche Applied Science) will reversely transcribe RNA templates (in the presence of Mn^{2+} ions) and thus may be used for one-step, one-tube RT-PCR of short (up to 1 kb) RNA templates.

For a complete listing of RT-PCR enzymes available from Roche Applied Science, see the Ordering Information in the Appendix. For more information on the properties of these enzymes and their appropriateness for specific applications, see "Choosing RT-PCR Enzymes" (in Chapter 5, section 5.1) and the RT-PCR Selection Guide (in the Appendix).

2

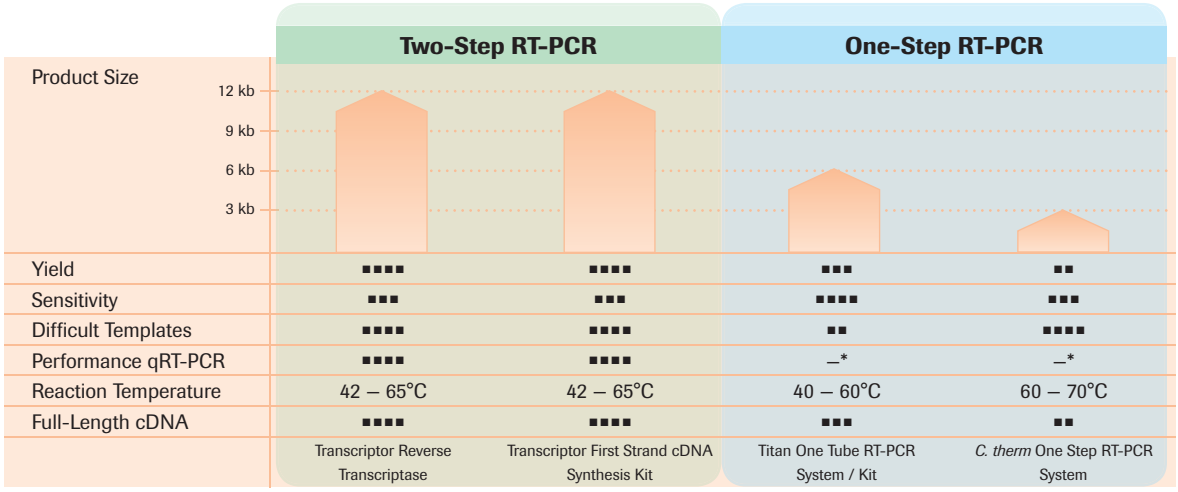
Table 2.1: Comparison of PCR- and RT-PCR Enzymes.



	Basic PCR	Hot Start PCR		Difficult Template PCR	High Fidelity PCR		Long Range PCR	
Specificity	■■	■■■■	■■■■	■■■	■■	■■■	■■■	■■
Sensitivity	■■	■■■■	■■■■	■■■	■■	■■■	■■	■■
Robustness	■■	■■■	■■■	■■■■	■■	■■■	■■■	■■■
Accurracy*		1 x	4 x	3 x	18 x	6 x	3 x	2 x
Carryover Prevention	yes	yes	yes	no	no	yes	no	no
Master Mix available	yes	yes	no	no	yes	yes	no	no
	Taq DNA Polymerase	FastStart Taq DNA Polymerase	FastStart High Fidelity PCR System	GC-RICH PCR System	Pwo SuperYield DNA Polymerase	Expand High Fidelity^{PLUS} PCR System	Expand Long Range dNTPack	Expand 20kb^{PLUS} PCR System

* compared to Taq DNA Polymerase

Comparison of PCR Enzymes.



	Two-Step RT-PCR		One-Step RT-PCR	
Yield	■■■■	■■■■	■■■	■■
Sensitivity	■■■	■■■	■■■■	■■■
Difficult Templates	■■■■	■■■■	■■	■■■■
Performance qRT-PCR	■■■■	■■■■	–*	–*
Reaction Temperature	42 – 65°C	42 – 65°C	40 – 60°C	60 – 70°C
Full-Length cDNA	■■■■	■■■■	■■■	■■
	Transcriptor Reverse Transcriptase	Transcriptor First Strand cDNA Synthesis Kit	Titan One Tube RT-PCR System / Kit	*C. therm* One Step RT-PCR System

* for One-Step RT-PC we recommend to use our LightCycler® kits

Comparison of RT-PCR Enzymes.

Abbreviations: kb, kilobase pairs
Explanation of terms:
Processivity: Number of bp that can be added to a copy by one molecule of polymerase before it falls off the template.
Length: Maximum PCR target length that can be amplified from a human genomic DNA template with good yield.
Specificity: Amplifies only the target of interest.
Yield: Produces large amounts of products in a given number of PCR cycles.
Reproducibility: Gives the same results from reaction to reaction.
Sensitivity: Amplifies template present at a low copy number (fewer than 500 copies0
Robustness: Amplifies template even in the presence of contaminating agents or high GC content.
Accuracy: Amplifies template without introducing excessive transcriptional errors.

2.2.3 Other Important Reaction Components

2.2.3.1 Templates and Primers

Quality and Amount of DNA Template

Obviously, the purity and quality of the template are critical to the success of the PCR. For details on how pure the template should be and how to get the best quality template, see Chapter 3.

The amount of template in a reaction also strongly influences performance in PCR. For standard PCR, follow these recommendations:

- The maximum amount of human genomic DNA should be 200 ng. Use less if possible.

Low amounts of genomic template (e.g., <10 ng human genomic DNA) will require specific reaction modifications, such as increases in cycle number, redesign of primers, use of a "Hot Start" reaction, etc.

- For initial experiments, use:
- 1–10 ng bacterial DNA
- 0.1–1 ng plasmid DNA
- 2 µl cDNA (as template for a 50 µl PCR)

When using cDNA as template, do not let the volume of the cDNA exceed 10% of the volume of the PCR mixture (e.g., for a 50 µl PCR mixture, use no more than 5 µl cDNA from a reverse transcriptase reaction). Greater amounts of cDNA may inhibit the PCR.

Suitable Primers

In most PCR applications, it is the sequence and the concentration of the primers that determine the overall assay success.

For convenience, several primer design software programs are available. These can be used to ensure that the primer sequences are suitable for the reaction.

For details on primer design and concentration, see Chapter 3.

2.2.3.2 Nucleotides and Magnesium Ions

High Quality Nucleotides

Nucleotides are vital components in amplification reactions and the purity of these reagents significantly influences PCR results. Be aware that not all preparations of nucleotides are acceptable for PCR.

Specifically, many nucleotide preparations contain trace amounts of contaminants (pyrophosphate, mono-, di- and tetraphosphate nucleotides and organic solvents) that can inhibit amplification reactions. Such nucleotide preparations are suboptimal for PCR.

In contrast, Roche Applied Science uses a process to synthesize nucleotides that drastically reduces the amount of contaminants. These high quality ("PCR Grade") nucleotides are virtually free of all contaminants and are now accepted in the scientific community as the standard nucleotides that should be used for PCR.

Since nucleotides only contribute approx. 1—2% to the total cost of a PCR reaction, buying nucleotides from low cost suppliers will not save very much money. However, considerable costs can be incurred if PCRs are inhibited by impure nucleotides and have to be repeated. For best results, use only PCR Grade nucleotides from Roche Applied Science.

Concentration of Deoxynucleoside Triphosphate (dNTPs)

During PCR, always use balanced solutions of all four dNTPs to minimize polymerase error rate. Imbalanced (*i.e.*, unequal) concentrations of dNTP will reduce the fidelity of the thermostable DNA polymerase.

An exception to this rule is the use of dUTP for carryover prevention. A higher concentration of dUTP is usually used in place of dTTP. This is due to differences in the rates at which dTTP and dUTP are incorporated in DNA. For more information, see "Preventing Carryover" in Chapter 4 of this manual.

The usual concentration of dNTP in standard PCR is 200 µM (each nucleotide). For some applications, concentrations ranging from 50 to 500 µM may be acceptable.

If you increase the concentration of dNTPs, you must also increase the concentration of Mg^{2+} ion in the reaction. Increases in dNTP concentration reduce free Mg^{2+}, thus interfering with polymerase activity and decreasing primer annealing.

Concentration of Magnesium Ions

Most thermostable DNA polymerases require a source of divalent cations to function. In most cases, the divalent cation required is Mg^{2+}. Mg^{2+} influences enzyme activity and increases the T_m of double-stranded DNA. Mg^{2+} forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes.

In general, lower Mg^{2+} concentrations lead to specific amplification and higher concentrations produce more nonspecific amplification.

A few DNA polymerases (such as Tth DNA Polymerase) use Mn^{2+} rather than Mg^{2+}. However, in general, DNA polymerase reactions in the presence of Mn^{2+} make DNA copies with significantly lower fidelity than in reactions in the presence of Mg^{2+}.

The concentration of free Mg^{2+} depends on the concentrations of compounds that bind the ion, including dNTP, template DNA, primers, free pyrophosphate (PP_i) and EDTA. Therefore, determining the correct Mg^{2+} concentration in a PCR is not easy.

The most commonly used concentration of Mg^{2+} used in standard PCR with Taq DNA Polymerase is 1.5 mM (with 200 µM dNTPs). However, for best results, always determine the optimal Mg^{2+} concentration for each reaction system empirically. Try a range of Mg^{2+} concentrations, from 1 mM to 10 mM.

If you optimize no other reaction component, at least optimize the Mg^{2+} concentration. For details on optimizing the Mg^{2+} concentration, see "Optimizing the Mg^{2+} Concentration for the Standard PCR Protocol" (Chapter 4, section 4.7).

2.2.3.3 Other Reaction Components

Appropriate Buffers

Always use only the purest buffers in PCR. These buffers should be designated "PCR Grade."

In most cases, the reaction buffer supplied with the PCR enzyme(s) will give optimal results with that enzyme. The pH of that buffer will generally be between pH 8.3 and 9.0.

An easy way to test the effect of varying pH on the PCR is to use the Roche Applied Science PCR Optimization Kit. For details, see Chapter 4, section 4.7.

Reaction Additives and Enhancers

In some cases, adding the following compounds can enhance the efficiency, specificity or yield of standard PCR with Taq DNA Polymerase:

Table 2.2: Effect of Additives on a Standard PCR.

Additive/	Recommended Concentration in Reaction [a]	Effect of Additive [a]
Ammonium sulfate [$(NH_4)_2SO_4$] [b]	5 - 30 mM	Facilitates DNA strand separation
Bovine serum albumin *	50 - 500 ng per 50 µl reaction	Binds many PCR inhibitors found in tissue samples
Dimethylsulfoxide (DMSO)[b]	2 - 10% v/v	Lowers T_m of target DNA to enhance annealing
Dimethylformamide (DMF)	<10% v/v [c]	Lowers T_m of target DNA to enhance annealing
Betain		Lowers T_m of target DNA to enhance annealing
Formamide	1.25 - 10% v/v [c]	▶ Changes T_m of primer-template hybridization to increase specificity and yield ▶ Stabilizes Taq DNA Polymerase
Gelatin [b]	0.01- 0.10% w/v	Stabilizes Taq DNA Polymerase
Glycerol [b]	5 - 15% v/v	Stabilizes Taq DNA Polymerase
PEG 6000	5 - 15% v/v	Stabilizes Taq DNA Polymerase
SDS	less than 0.01% w/v [c]	Prevents aggregation of polymerase
Spermidine		Reduces nonspecific binding of polymerase to template DNA
T4 Gene 32 protein *	0.05 - 0.1 nmol per 50 µl reaction	Changes T_m of primer-template hybridization to increase specificity and yield
Triton X-100	0.01% v/v	Prevents aggregation of polymerase
Urea	1 - 1.5 M [c]	Lowers T_m of target DNA to enhance annealing

a) Part of the information in this table is from Aoyagi (2001). The effects of the additives were determined only for reactions with Taq DNA Polymerase.
b) Component of the Roche Applied Science PCR Optimization Kit. For details on optimizing PCR with the kit, see Chapter 4, section 4.7.
c) Higher concentrations are inhibitory.
* Available from Roche Applied Science.

Further Reading

For more information on choosing the correct PCR equipment and reagents, see Aoyagi (2001), Cohen (1995), Gelfand (1992a, 1992b), McPherson and Møller (2000b).

2

2.2.4 How Cycling Parameters Affect a PCR

Sections 2.2.2 and 2.2.3 above show how different reaction components affect the PCR. Not surprisingly, the thermal cycling program also greatly affects the chances for a successful PCR. For example:

- The denaturation time must be long enough to fully denature the template, but short enough not to inactivate the thermostable DNA polymerase.
- The optimal annealing temperature depends on the melting temperature of the primers.
- The optimal elongation time depends on the length of the target to be amplified.
- The optimal number of cycles depends on the abundance of the target in the starting sample (*e.g.*, rarer targets require more cycles to amplify).

Because the optimal cycling parameters vary with each experimental system, the thermal cycling program given in any publication (including the protocols of this manual) should be considered guidelines only. For optimal results, these parameters should be optimized empirically for your particular experimental system and equipment.

The table below summarizes the effects of under- or overshooting the optimal time and temperature for each stage of the thermal cycle. You can use that information to help you determine the optimal PCR parameters for your system.

Table 2.3: How Cycling Parameters Affect PCR.

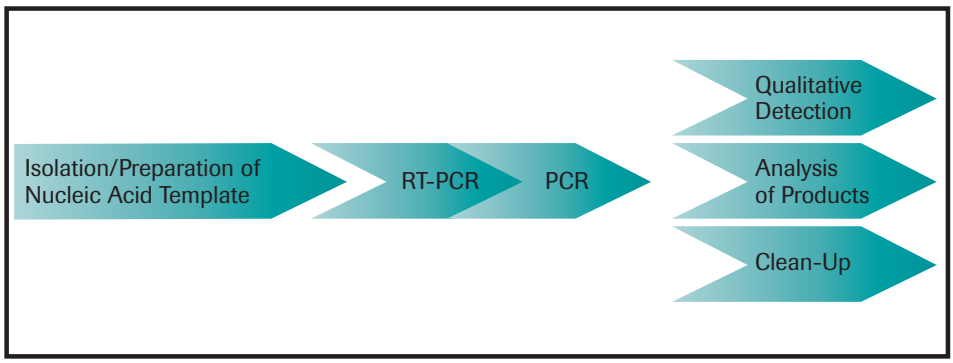
Cycling Parameter	Value Used in Standard PCR [a]	Effect if Parameter Value Is Lower than Optimal [b]	Effect if Parameter Value Is Higher than Optimal [b]
Initial Denaturation	94°C 2 min	Few or no PCR products	Premature denaturation of polymerase, leading to reduced yield
Denaturation during Cycling	94°C 15 – 30 s	Reduced yield	Reduced yield
Primer Annealing	50 to 65°C [c] 30 – 60 s	Reduced yield	Formation of nonspecific products
Elongation	72°C 45 s – 2 min [d]	Reduced yield	▶ Reduced yield ▶ Increased error rate
Total Number of Cycles	25 – 30	Reduced yield	Formation of nonspecific products
Final Elongation	72°C 7 min	Products are not fully double-stranded	Formation of nonspecific products

a) With Taq DNA Polymerase; copied from standard PCR protocol (Chapter 4, section 4.1). Other enzyme systems will require different parameter values.
b) From Aoyagi (2001). Effects are generally independent of the enzyme system used.
c) Optimal temperature depends on primer melting temperature.
d) Elongation time depends on fragment length; for Taq DNA Polymerase, the recommended times are 45 seconds for targets that are 1 kb or shorter, 1 minute for 1.0 – 1.5 kb, and 2 minutes for 1.5 – 3.0 kb.

2.3 Typical Workflow for PCR/RT-PCR

Now that you have learned how to prevent contamination in your laboratory (section 2.1) and set up a PCR (section 2.2), you are ready to apply the power of PCR to your research.

Even though there are many different ways to use PCR, the overall workflow for almost all PCR and RT-PCR experiments involves the same general steps:



You can think of this workflow as links in a chain. As everyone knows, a chain is only as strong as its weakest link. Therefore, Roche Applied Science designs and tests its reagents, kits, instruments and experimental protocols as elements of a chain. By using the Roche Applied Science products and protocols described in this manual, you can rest assured that:

- The information in this manual will help you choose the right products and protocols for your specific application.

For a quick overview of how the information in this manual can help you choose the right products and protocols to forge the strongest "PCR workflow chain," see the table below.

- Each product and protocol is optimized for its designated applications.
- All elements of the Roche Applied Science product/protocol chain will work smoothly together.
- Using Roche Applied Science products and protocols will reduce the complexity of your PCR experiments, saving you both time and money.

There are sophisticated, real-time PCR instruments available (e.g., the LightCycler® 1.5 Instrument, the LightCycler® 2.0 Instrument and the LightCycler® 480 System, all from Roche Applied Science) that perform some of the workflow steps concurrently. (If you want a brief look at the power of these real-time PCR instruments, see Chapter 7.)

2

Table 2.4: Overview of How This Manual Can Help You Choose the Right Products and Protocols.

Isolation of Nucleic Acids	Amplification of Nucleic Acids	Qualitative Detection	Clean-up and Analysis (optional)
Manual or automated isolation of RNA (See Chapter 3) ▶ Total RNA ▶ Messenger RNA ▶ Viral RNA	**Reverse Transcription and Amplification of RNA Templates** Before you start: ▶ Prevent degradation of RNA (See section 5.2 in Chapter 5) ▶ Choose the correct enzymes, kits, etc. (See section 5.1 in Chapter 5 and the RT-PCR Product Selection Guide in the Appendix) Via: separate reverse transcription and amplification steps (Two Step RT-PCR; see section 5.4 in Chapter 5) or coupled reverse transcription and amplification (One Step RT-PCR; see section 5.3 in Chapter 5) Applications: ▶ RT-PCR of normal templates (See sections 5.3 and 5.4 in Chapter 5) ▶ RT-PCR of GC rich templates (See sections 5.3 and 5.4 in Chapter 5) ▶ RT-PCR of long templates (See section 5.4 in Chapter 5) ▶ Special RT-PCR applications (See Chapter 8)	Agarose gel electrophoresis (See Chapter 6)	Post PCR purification (See Chapter 6) Cloning of PCR products (See Chapter 6)
Manual or automated isolation of DNA (See Chapter 3) ▶ Genomic DNA ▶ Plasmid DNA ▶ Viral DNA	**Amplification of DNA Templates** Before you start: Choose the correct enzymes, nucleotides, kits, etc. (See section 4.6 in Chapter 4 and the PCR Product Selection Guide in the Appendix) Applications: ▶ Overview: PCR Protocol Selection Guide (See Chapter 4) ▶ Amplification of normal templates (See sections 4.1 through 4.2 in Chapter 4) ▶ Amplification of long templates (See section 4.3 in Chapter 4) ▶ Amplification of difficult templates (See section 4.4 in Chapter 4) ▶ Prevention of carryover contamination (See section 4.5 in Chapter 4) ▶ Multiplex reactions (See Chapter 8) ▶ High throughput analysis (See Chapter 7) ▶ Special PCR applications (See Chapter 8)		
	Quantitative, real-time PCR Analysis (See Chapter 7)		

Disclaimer

For Titan One Tube RT-PCR System, Cat. Nos. 11 888 382 001, 11 855 476 001; Titan One Tube RT-PCR Kit, Cat. No. 11 939 823 001; C.*therm.* Polymerase One-Step RT-PCR System, Cat. Nos. 12 016 338 001, 12 016 346 001; Tth DNA Polymerase, Cat. Nos. 11 480 014 001, 11 480 022 001, and Protector RNase Inhibitor, Cat. Nos. 11 480 014 001, 11 480 022 001 see Disclaimer No. 1.

For Pwo DNA Polymerase, Cat. Nos. 11 644 947 001, 22 644 955 001; Pwo SuperYield DNA Polymerase, Cat. Nos. 04 340 868 001, 04 340 850 001, Pwo SuperYield DNA Polymerase, dNTPack, Cat. Nos. 04 743 750 001, 04 743 776 001, and Pwo Master, Cat. No. 03 789 403 001, see Disclaimer No. 2.
For the PCR Master, Cat. No. 11 636 103 001 see Disclaimer No. 3.
For the PCR Core Kit ^{PLUS}, Cat. No. 11 578 553 001 see Disclaimer No. 4.