

# POLYMERASE CHAIN REACTION AND THERMALCYCLING

## Introduction

This week begins the second major experiment we will perform. We will collect bacteria from the environment and identify them by using DNA sequencing of a particular segment of the bacterial genome, the DNA that produces the ribosomal RNA for the 16S subunit of the bacterial ribosome.

Goals:

1. learn the principles of DNA isolation on a silica matrix.
2. Understand the process of Polymerase Chain Reaction and how the thermalcycler helps carry out that process.
3. Know the basic thermalcycler files.
4. Be able to set up a PCR reaction.

## Topic I: ISOLATION OF GENOMIC DNA

The first step after the bacteria are collected is to isolate genomic DNA. The miniprep procedure, even though it is easy and fairly fast, specifically removes genomic DNA. We will use a kit designed for genomic DNA isolation from a variety of organisms – bacteria, eukaryotic tissues, cells in culture, blood cells or yeast.

### 1. Principles of the Isolation Procedure.

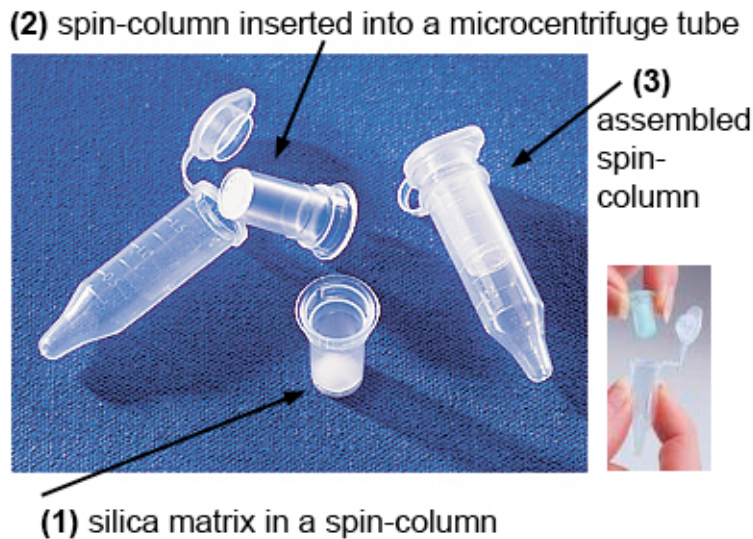
This isolation procedure uses a silica matrix to bind DNA under certain chemical conditions. The impurities are washed through the matrix, and then the wash solution is changed to one that will release the DNA from the silica. The silica matrix is on a membrane suspended in a “spin column” (see Figure 6.1). The basic procedure has been expanded to include the isolation of DNA from a gel slice, and even plasmid DNA isolation.

#### A) Silica

In the presence of high chaotropic salts (like guanidinium chloride) , nucleic acids will bind to silica particles with great affinity. The chaotropic

salts denature the proteins and break down the polymeric structure of agarose. Denatured proteins and agarose monomers can be selectively washed from the silica with ethanol containing wash buffers. The pH of the binding and wash solutions and the concentration of the ethanol are critical conditions for isolating different size and strand nucleic acids. The nucleic acid can then be eluted with low-salt buffer (TE or water). Using a warmed elution buffer (50-60°C) may improve nucleic acid recovery. <http://www.piercenet.com/Objects/View.cfm?type=Page&ID=C6F0F5F2-8424-4B68-9EFC-CD6EACEC46F1>

Figure 6.1: A spin-column for DNA isolation.



[www.htslabs.com/.../Spin%202mL.htm](http://www.htslabs.com/.../Spin%202mL.htm)  
[www.fishersci.com/.../ITEMDETAIL?catnum=42530](http://www.fishersci.com/.../ITEMDETAIL?catnum=42530)

## B) Chaotropic agent

An agent that causes molecular structure to be disrupted; in particular, those formed by nonbonding forces such as hydrogen bonding, Van der Waals interactions, and the hydrophobic effect. Often structural features, as detected by means such as circular dichroism can be titrated in a chaotrope concentration-dependent fashion.

The most commonly used chaotropes are 6~8M urea and 6M guanidinium chloride, with urea being an uncharged molecule and guanidinium chloride being a hydrochloride salt. A kit for DNA purification contains a reagent with a chaotropic agent (a guanidinium salt) for inactivating nucleases and an alcohol (ethanol or isopropanol) for the simultaneous inactivation of the nucleases and the precipitation of the nucleic acids without the need for protease digestion or organic extraction.

<http://www.nationmaster.com/encyclopedia/Chaotropic-agent>

### C) Silica column-based kits

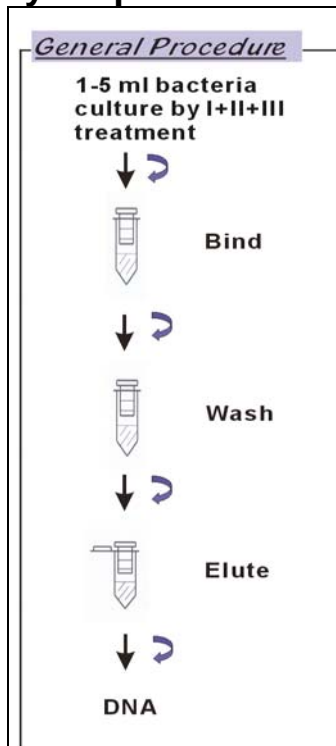
Column-based kits offer a convenient approach to DNA cleanup. The principle is that chaotropic salts are added to the sample to denature the DNA by disrupting its hydrogen bonding. Under these conditions, the DNA will selectively bind to the silica resin in the column, allowing it to be separated from the rest of the sample. After washing the DNA is eluted from the column with a low salt solution that allows the renaturing of the DNA, causing it to lose affinity for the silica. Kits are available for a range of applications including agarose gel extraction, enzymatic reaction, nucleotide and PCR clean-up.

*Advantages:* Convenient, relatively fast and the user can process large number of samples using the vacuum manifold option.

*Disadvantages:* Fairly expensive, may experience low yields (as low as 25%) and chaotropic salt carry-over are common.

<http://bitesizebio.com/2007/10/25/5-ways-to-clean-up-a-dna-sample/>

## 2. Key Steps of the Protocol



**A)** For bacterial and human cells, the cells must first be lysed in a lysis buffer. This may also involve addition of protease or Proteinase K, heating in the heat block, and repeated vortexing. The point is to open up the cells so we can get to the DNA. For gel extraction, the combination of melting buffer and heat solubilized the gel and released the PCR product. The buffers are the proper pH (<7.5) and contain a chaotropic salt that is necessary for the DNA to bind to silica.

**B)** The solution with the DNA is applied to the silica membrane on the spin-column. The solution is put through the spin-column, flow-thru is discarded, and process continued until all solution has been passed through the spin-column and the DNA bound to the silica.

**C)** During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, agarose, oils, ethidium bromide,

[http://www.hopegenbio.com/pro\\_detail\\_kits\\_01.asp?id=535](http://www.hopegenbio.com/pro_detail_kits_01.asp?id=535)

dyes, unincorporated nucleotides, and detergents (e.g., DMSO, Tween® 20) do not bind to the silica membrane but flow through the column. Salts are quantitatively washed away by the ethanol-containing buffer. This wash may be performed one or two times. All residual buffer (which may

interfere with subsequent reactions) is removed by an additional centrifugation step.

- D)** Elution is performed in low-salt solutions. Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer. *Contrary to DNA adsorption, elution is most efficient under basic conditions and low salt concentrations.* DNA is eluted with 50 or 30  $\mu\text{L}$  of the provided buffer (10 mM Tris-Cl, pH 8.5), or water. The maximum elution efficiency is achieved between pH 7.0 and 8.5.
- E)** When using water to elute, the pH must be within this pH 7.0 - 8.5 range and DNA must be stored at  $-20^{\circ}\text{C}$  when eluted with water since DNA may degrade in the absence of a buffering agent. Elution with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) is possible, but not recommended because EDTA may inhibit subsequent enzymatic reactions such as PCR or restriction enzyme digestion.

## Topic II: THERMALCYCLING

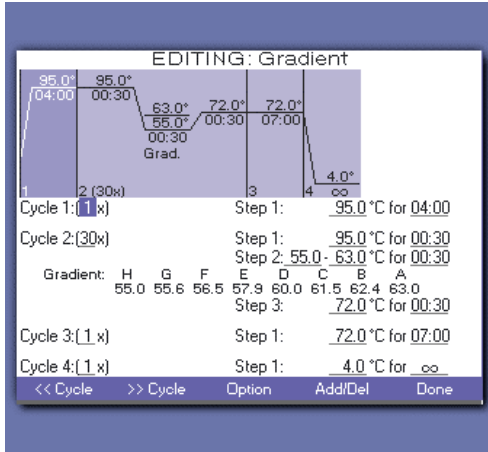
Polymerase Chain Reaction (PCR) has had a tremendous impact on the field of Molecular Biology, as significant as the initial use of restriction enzymes. The *Taq* Polymerase (and DNA Polymerase from other thermophilic bacteria) allows rapid amplification of a specific DNA sequence.

However, the thermalcycler (or thermocycler) is as important to this process as the *Taq* Polymerase. This machine produces rapid, sharp temperature changes for each of the three steps in the PCR cycle. The thermalcycler can be programmed to suit the needs of the experimenter.



Figure 6.2: (right) The Bio-Rad® iCycler. (below left) programming steps of a PCR reaction. (below right) The heat block is capable of rapid temperature changes.

<http://www.bio-rad.com/B2B/BioRad/product/>

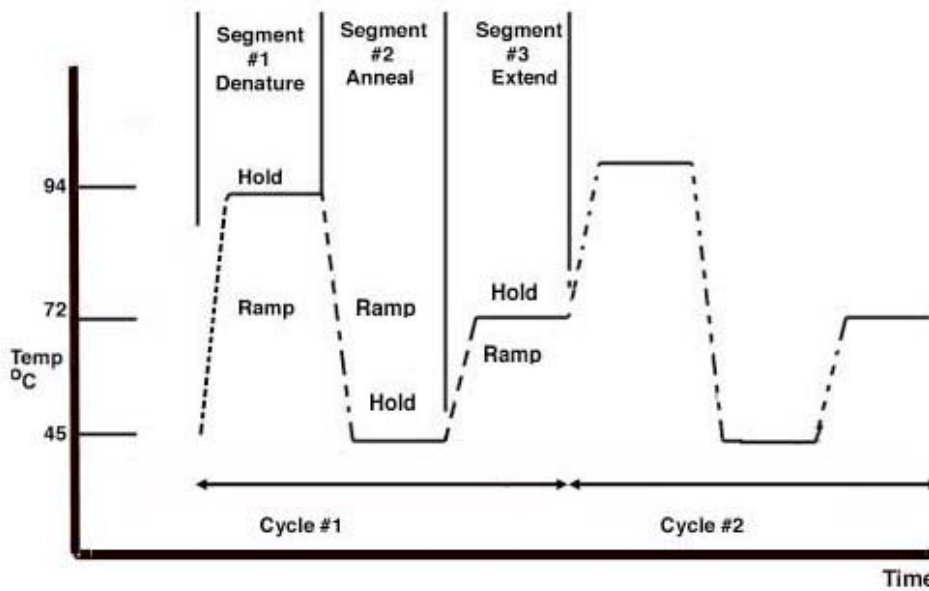


## 1. Thermalcycle Files

### A) The Step-Cycle File:

The step-cycle file is used for DNA amplification. The typical step-cycle file has three segments. Usually, the *first* segment is used for **template denaturation or melting** (separation of double-stranded DNA). The *second* segment is used for **primer annealing**, and the *third* step is used for **primer extension** (DNA synthesis). Each cycle in the step-cycle file can be repeated up to 99 times. Usually, PCR is run for 20-35 cycles.

Figure 6.3 shows two cycles of a step-cycle file that has three segments:



Each segment in a step-cycle file has a **target temperature** and a **hold time**, which is the length of time that the block will be held at the target

temperature. The **ramp time** is the length of time that it takes the machine to move from one hold temperature to another. You cannot program ramp times; the machine heats and cools as quickly as possible. Thermalcycler manufacturers vie for the quickest ramp times in their machines.

For the three steps in the cycle, the usual target temperatures are:

- Denaturation = 94-96°C (strand separation).
- Annealing = 45-60°C (the most variable; depends on the conditions of your experiment. As a general rule, the annealing temp should be 5°C below the  $T_m$  or melting temperature of the DNA duplex).
- Synthesis = 72°C (the optimal synthesis temp for *Taq*).

### **B) The Time-Delay File:**

A time-delay file will take the heat block to a specific temperature and hold it at that temperature. The heat block is programmed for a certain delay time, which includes both the transition time and the incubation time.

There are two common uses for the time-delay file:

- Before thermal-cycling. The complete denaturation of the DNA template at the start of the PCR reaction is of key importance. Incomplete denaturation of DNA causes inefficient utilization of template in the first amplification cycle and in a poor yield of PCR product. Initial denaturation should be performed for 1-3 min at 95°C. These files can also be used to destroy unwanted nucleases and proteases present in the DNA sample.
- After thermal-cycling. The time-delay file can be used as an additional extension or synthesis time for the *Taq* Polymerase. As the step-cycle file nears completion, there are often partially-completed synthesis products present. An additional 5-15 min at 72°C enables the *Taq* to complete synthesis on these partial products.

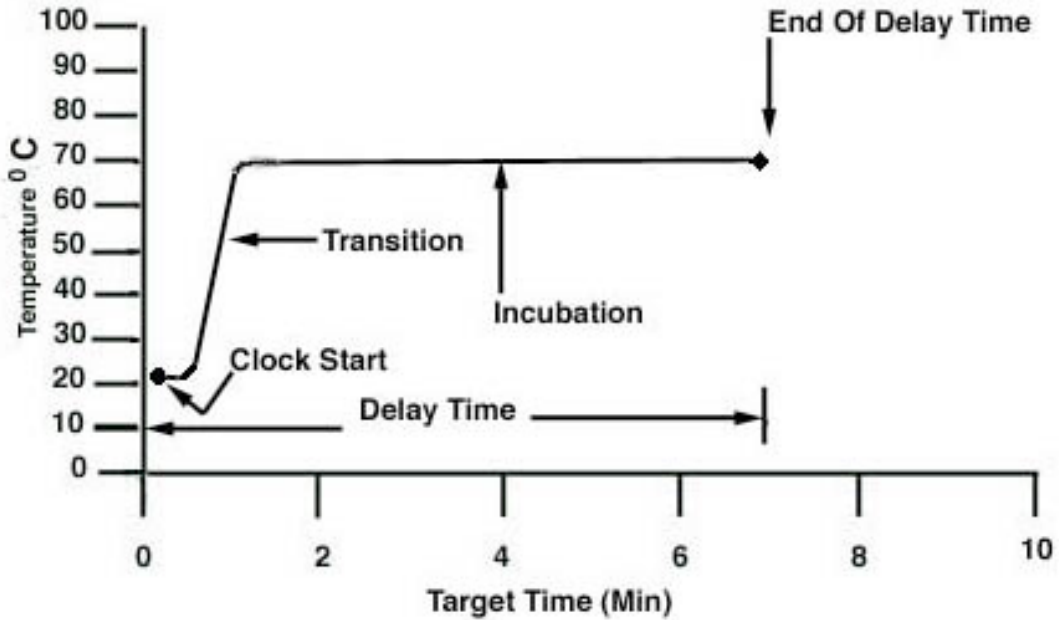
### **C) The Soak File:**

A soak file is an incubation file in which the target temperature is reached as soon as possible and held indefinitely, until you stop the run by pressing the STOP button. Soak files are often used at the end of the run. The block is held at a cool temperature (5-15°C) to protect the samples from degradation, especially when the run goes overnight. It is the equivalent of putting the PCR machine in the refrigerator and coming in the next morning to get them.

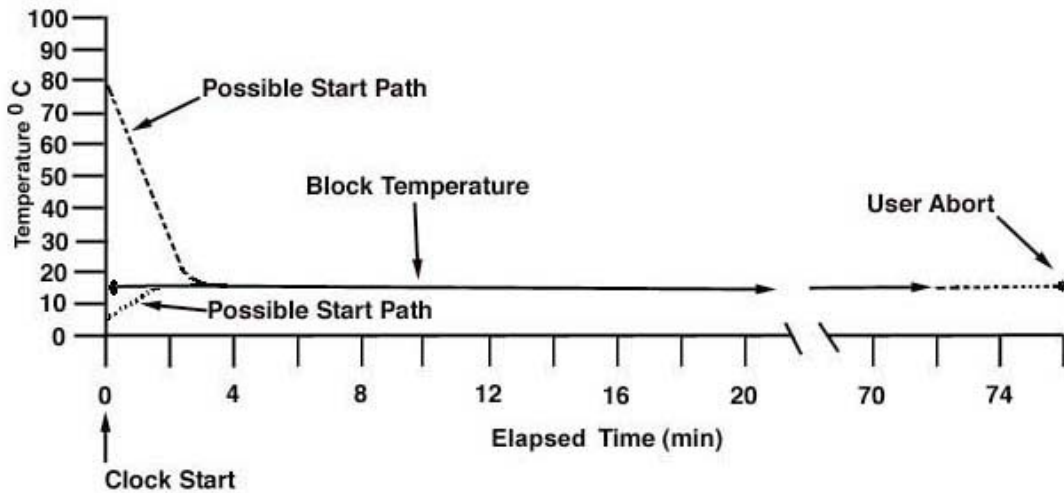
These files are LINKED to produce a PCR program that suits the needs of the researcher. For example, the program may start with a time-delay file of 5 minutes at 95°C for initial denaturation (and inactivation of all proteins but the *Taq* Polymerase), then linked to 30 cycles of the step-cycle file (denaturation at 95°C, annealing at 55°C, synthesis at 72°C) then linked to another time-delay file

of 10 minutes at 72°C (this gives time for any partially- completed PCR products to finish elongation), and finally linked to a soak file (indefinitely at 5-15°C) to hold the PCR products till you can put them in the freezer or load your gel.

The time-delay file is shown in Figure 6.4:



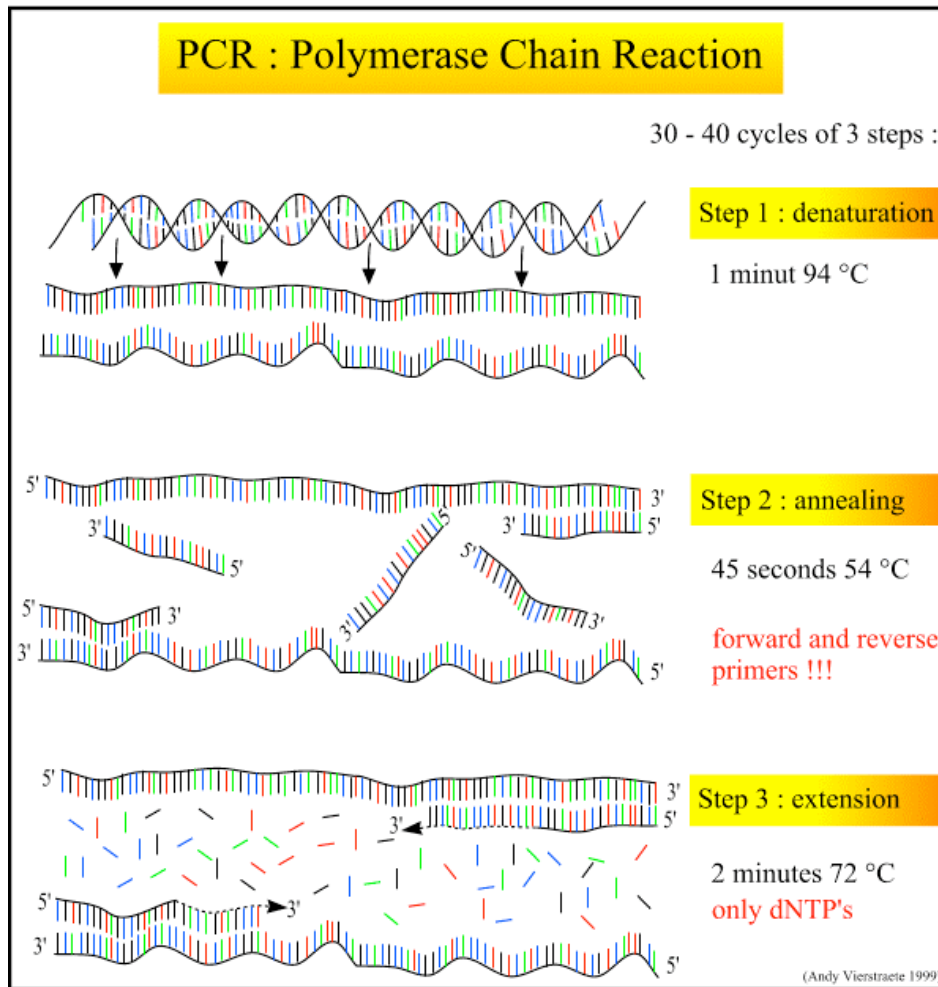
A soak file is shown in the Figure 6.5:

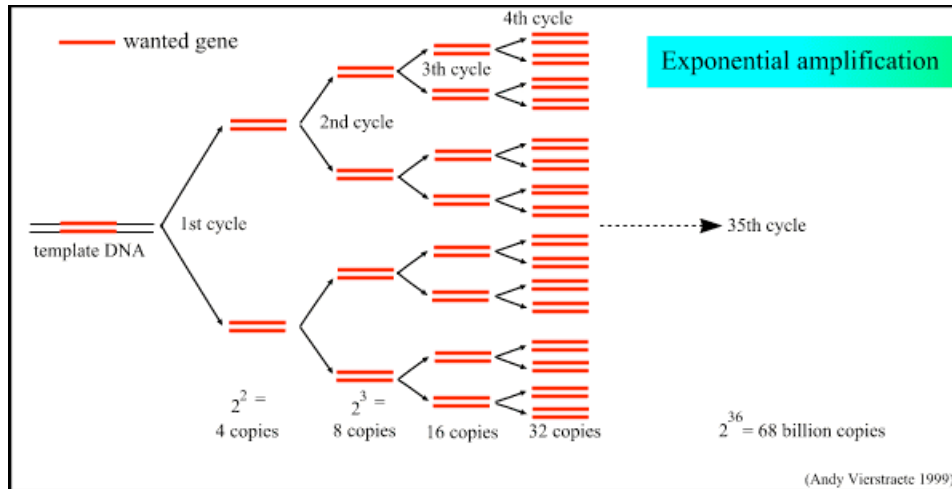


### Topic III: POLYMERASE CHAIN REACTION

Following the introduction of PCR, the technique spread through the community of molecular biologists like - well, a chain reaction. Almost overnight, PCR became a standard research technique and the practical applications soon followed. PCR changed the way many classic molecular techniques were performed, and introduced entirely new ways to study molecular biology (such as RT-PCR and cycle sequencing). In 1993 the Nobel Prize in Chemistry was awarded to Kary B. Mullis (a South Carolina native) and Michael Smith for their invention.

Figure 6.6: (top) The three steps in one PCR cycle. (bottom) The exponential amplification of the PCR product.





<http://users.ugent.be/~avierstr/principles/pcr.html>

Some aspects of PCR reactions are similar to a restriction enzyme digestion but there are some significant differences. Because the optimum reaction temperature for the *Taq* enzyme is 72°C, it can be stored on ice or mixed with other reaction components and not lose activity like restriction enzymes. For PCR, a **master mix** is usually prepared that contains all but one of the important components of the PCR reaction (template DNA or primers). Enough master mix is prepared for all the PCR reactions, plus some extra, and aliquotted into the PCR tubes containing the missing component.

The ingredients of a PCR reaction include:

- forward and reverse primers
- template DNA
- dNTP's
- Taq* Polymerase
- buffer
- MgCL<sub>2</sub> (may be in the buffer)
- water

Criteria for designing oligonucleotide primers for 16s rDNA analysis:

1. Sequences are highly conserved among prokaryotes.
2. Segments of amplified fragment show significant variation.
3. Fragment size between 350 and 600 bp is optimal for PCR and sequencing from both ends



Example of sequencing primers that have been used for this experiment:

Primer A - 28 nts - coding strand (310 ~340)	Primer B - 26 nts - non-coding strand (770~740)
5'CGGCCAGACTCCTACGGGAGGCAGCA-3'	5'-GCGTGGACTACCAGGGTATCTAATCC-3'

Magnesium is a required cofactor for thermo stable DNA polymerases, and magnesium concentration is a crucial factor that can affect the success of the amplification. Magnesium is usually supplied as magnesium chloride and this ion is required for enzyme to bind template. The actual substrate for polymerization is dNTP-Mg<sup>++</sup>. Magnesium co-ordinates between charges on two of the phosphate groups, and stabilizes contacts in the polymerase active site. Increasing Mg<sup>2+</sup> conc. will decrease stringency and can result in additional products besides the desired specific product. Often you have to optimize the Mg<sup>2+</sup> concentration. Optimal concentration is reached when you have specific product, without unspecific bands.

Handy "PCR Master Mix Calculators" are available online to determine how much of each component should be added to the master mix. The reaction volume can range from 30-100 uL, with 50 uL a usual volume.

[http://www.protocol-online.org/tools/sms2/pcr\\_mix.html](http://www.protocol-online.org/tools/sms2/pcr_mix.html)

REAGENT	Stock Concentrations	Desired Values	Reaction Mix (uL)
Reaction buffer	10 X	X	
Magnesium	15 mM	mM	
dNTP's (total)	10 mM	mM	
Forward primer	50 uM	uM	
Reverse primer	50 uM	uM	
DNA template	100 ng/uL	ng	
Taq Pol.	2.5 U/uL	U	
Water			
<i>Total rxn Vol:</i>			<i>50 uL</i>

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