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# Amplification of ribosomal RNA sequences

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## Introduction

Comparisons of rRNA sequences, pioneered by Woese and his colleagues, defined the main lineages in the evolution of microorganisms [42]. An advantage of rRNA sequence comparisons is the generation of an increasingly expanding data base against which newly determined sequences may be compared [5,23]. Nearly 60,000 16S rRNA sequences are currently available in the Ribosomal Database Project II [23]. Initially, sequences were obtained from well described pure cultures for phylogenetic research. Pace et al. [31] recognized that as the rRNA tree filled in, the data base would serve not only for continued comparison of sequences obtained from pure cultures, but also for comparison of sequences obtained directly from natural microbial communities without needing to grow the representative members in the laboratory. The concept of comparing gene sequences from microbial communities revolutionized microbial ecology. Subsequently, a suite of molecular methods was developed that employ rRNA sequences [1,11,37].

Divergence of the primary lines of bacterial descent occurred early in biotic history so highly conserved molecular chronometers are best suited to the task of reconstructing bacterial phylogeny [42]. Ribosomal RNAs are integral elements of the protein synthesizing apparatus, the basic components of which are present in all primary kingdoms, and are among the most highly conserved cellular molecules. Yet, rRNAs also contain sufficient sequence variability so that relationships between closely related groups can be determined. The abundance of rRNAs in actively growing cells made them readily obtained in the purified form needed for the earliest methodologies which determined their sequences directly. At first, rRNAs were compared by oligonucleotide cataloguing [42]. This entailed the digestion of purified rRNA with a ribonuclease to generate fragments that were then electrophorectically separated and individually sequenced. Comparison of rRNA oligonucleotide catalogs, and contiguous sequences determined from cloned genes, led to identification of highly conserved nucleotide tracts in dispersed regions of the 16S rRNA. The conserved tracts, serving as priming sites, made it possible to rapidly determine nearly complete sequences using rRNA as the template for reverse transcriptase in dideoxynucleotide terminated sequencing reactions [18,19]. Highly conserved nucleotide tracts have also now been identified in the 23S rRNA [18].

The advent of PCR [35] made rRNA genes even more accessible for sequencing. In any PCR it is necessary to first have knowledge of sequence at the distal and proximal ends of the DNA to be amplified. The conserved nature of 16S rRNA enabled the design of primers that amplify nearly full length 16S rRNA sequences. As the template is the rRNA gene, the amplification target or product is sometimes referred to as rRNA. Medlin et al. [25] first described amplification of 16S-like rRNA from algae, fungi, and protozoa, and reports using 16S rRNA of bacteria and other eukaryotes soon followed [9,10,39,40]. The small amount of DNA needed for a PCR enables amplification from minute amounts of material to obtain phylogenetic information when only a few cells are available. The amplifications provide a simple, rapid approach (without the need to screen a large genomic DNA shotgun library) to obtain rRNA that may either be sequenced directly when working with pure cultures [6,7,9,11], or cloned and sorted through a recombinant library when working with natural communities.

## Experimental Approach

### *Precautions*

The exceptional sensitivity of PCR makes the possibility of amplifying contaminating DNA a real concern [38]. Care should be taken to avoid the introduction of extraneous DNA into PCR reagents or reaction mixtures. This is particularly necessary when performing amplification of rRNA genes; rRNA genes are ubiquitous, and the primers used in rRNA gene amplifications are often designed for broad phylogenetic groups. Therefore, appropriate negative control reactions must be employed. The potential to amplify contaminant DNA is even greater when using broad specificity primers in nested PCRs. Products from contaminant DNA in a negative control not visible during the first round of amplification could become apparent after the second round of amplification.

Contaminant DNA can arise from the carry over of previous amplifications of homologous targets, from DNA purifications carried out in the laboratory and from microbial contamination of reagents. DNA contamination can be effectively avoided with careful laboratory practice. Guidelines include physically separating areas where PCR reagents are used from areas where finished reactions are used, autoclaving solutions and PCR tubes, briefly centrifuging reaction tubes to avoid splashes when they are opened, changing gloves frequently, always using positive (previously verified template) and negative (no template) controls, and using positive displacement pipettes [16,17]. Aerosol resistant tips are a convenient alternative to positive displacement pipettes. Pipette tips that have a shaft guard (Rainin; Oakland, CA; #GPS-10G) are available to protect PCR reagents or reactions against contamination from the pipette shaft. Additionally, never use the same pipette tip to withdraw from a tube more than once. Small aliquots of reagents can be stored at −20 °C until needed, with a "working PCR Box" kept at 4 °C that contains one aliquot of each reagent. DNA contamination in reagents, including that introduced during their manufacture, can be actively controlled by UV or DNase treatment [30, 33]. It is important to work in an area with limited air disturbance, such as might occur near a fume hood or beneath an air duct, when preparing reactions. DNase and RNase free tubes, tips and reagents are widely available and are highly recommended. The investigator should become familiar with these guidelines and use them habitually when performing PCRs.

### *Template*

The first section of this manual (see Section 1) describes procedures for the isolation of microbial nucleic acids from pure cultures and environmental samples. The sensitivity of PCR also makes it possible to amplify rRNA genes directly from small amounts of cells [12,36], a portion of a lyophilized cell pellet [41], or with DNA obtained using rapid nucleic acid purification protocols [11].

DNA templates must be of high-molecular weight. The size and quality DNA is assessed by electrophoresis on a 0.8% agarose gel. Often nucleic acid samples purified from the environment are highly degraded and sheared which can lead to the formation of chimeric rRNA gene PCR products. During the annealing cycle, amplified rRNA genes from different templates may re-associate leading to heterogeneous products in proceeding cycles [20,32].

DNA purified from an environmental sample often has contaminating inhibitors in the final preparation. DNA purity is assayed by determining the ratio between wavelength measurements at 260 nm and 280 nm. Pure DNA has an A260/A280 ratio between 1.8 and 2.0. An absorbance ratio of less than 1.8 will indicate the presence of potential inhibitors such as humic acid, protein or phenol. These inhibitors may be eliminated by re-extraction, ethanol precipitation and/or centrifical ultrafiltration through a purification cartridge (such as available from Mo Bio Laboratories, Inc.; Carlsbad, CA). However, it is unlikely that DNA extracted from an environmental sample will be purified to an A260/A280 ratio of 1.8. Typically one can expect the DNA to have an A260/A280 ratio of around 1.6 with a brownish color due to humic substances. The presence of inhibitors in the final DNA preparation, which may lead to false negative results, can be assessed by preparing control reactions spiked with template DNA known to amplify under the PCR conditions being used.

It is also possible to perform amplification beginning with a rRNA template. In the first step of the process a cDNA is generated through reverse transcription (RT) [28]. However, secondary structures and modified nucleotides in an rRNA template can interfere with polymerization causing early termination of the cDNA. This could skew conclusions of studies on *in situ* microbial diversity. Interference from secondary structure can sometimes be overcome by using a DNA polymerase such as *Tth* (*Thermus thermophilus*) that can transcribe RNA at a higher temperature in the presence of $Mn^{2+}$. Next, the RT product is amplified in a PCR (*Tth* in

*Table 1.* Web Sites Useful for Primer Design.

| Software | Sponsor | Web address |
|---|---|---|
| CODEHOP | Fred Hutchinson Cancer Research Center | http://www.blocks.fhcrc.org/codehop.html |
| Primer3 | Whitehead Institute for Biomedical Research | http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi |
| NetPrimer | PREMIER Biosoft International | http://bioinf.bmi.ac.cn/mirror/NetPrimer/netprimer.html |
| Primer Premier 5 | PREMIER Biosoft International | http://www.PremierBiosoft.com/primerdesign/primerdesign.html |
| GeneWalker | CyberGene | http://www.cybergene.se/primerdesign/ |
| Web Primer | Saccharomyces Genome Database | http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer |
| PRIMROSE | School of Biosciences, Cardiff University | http://www.cf.ac.uk/biosi/research/biosoft/ |

the presence of $Mg^{2+}$ will accomplish this as well). Examination of the RT-PCR product on an agarose gel often reveals a very faint band or no band at all. It is often necessary to do a second round of PCR here, using the RT-PCR product as the second round template.

### *Primers*

The goal in designing primers is developing a pair that will effectively hybridize to the target DNA and achieve the desired amplification. Beyond that, a well designed primer can enhance the efficiency and yield of the reaction. The finer points of primer design have been detailed by Innis and Gelfand [14] and by Saiki [34]. General primer characteristics are: a length of 18–24 bases, noncomplementary 3′ ends to avoid primer-dimer formation, no internal secondary structure and a 40–60% G-C content. The calculated $T_m$ of the primers should be closely matched and in the range of 55 to 65 °C. There are many software programs available on the internet that will aid in primer design and primer analysis. Some of the programs available are listed in Table 1.

Primers of broad specificity that amplify nearly full-length 16S-like, small subunit rRNA genes are listed in Table 2. The primers in Table 2 generally target the same region of the gene with minor variations in length or sequence used by different investigators. Restriction endonuclease sites engineered at the 5′ ends are recognized by enzymes that rarely cut within a small subunit rRNA gene. A primer pair, one forward and one reverse, selected for the broadest specificity among the organisms of interest (i.e., *Bacteria*, *Archaea*, or *Eukarya*) should enable amplification of *most* small subunit rRNA genes. It has become evident in recent years that domain level primers are *not* all encompassing and this should be considered when selecting a primer set and interpreting the results [13,24,29]. Additional primers of broad specificity that will amplify shorter lengths can be adapted from those used for rRNA sequencing [7,18].

Primers can also be used that selectively amplify rRNA genes of phylogenetically defined groups [2]. Selection of primers can often be guided by comparison of sequences in a data base, or by what has proven successful in previous amplifications from closely related groups or strains. Many selective rRNA gene primers have been described in the literature and it is beyond the scope of this chapter to tabulate them. PRIMROSE is a program that uses sequences from the Ribosomal Database Project to identify and determine the phylogenetic range of oligonucleotides that may be used as rRNA probes or primers [3]. The PRIMROSE website is provided in Table 1. ProbeBase [22] is a database of published rRNA probes with information on target site and specificity. The Ribosomal Database Project II [23] and the ARB website (Strunk,O. and Ludwig,W., 1993–2002, ARB—a software environment for sequence data, http://www.arb-home.de) have programs for checking intended specificities of probes and primers, including those for large subunit 23S-like rRNAs, against rRNA sequences. It is highly recommended that any primer pair, whether newly designed or previously published, be evaluated for its intended specificity prior to beginning any study.

### *Reaction conditions*

"Basic PCR" in the procedures section below describes a good starting reaction. It is always advisable to optimize around a standardized reaction and begin by varying annealing temperatures and $Mg^{2+}$ concentrations [15]. In general, a reaction component at too high a concentration can promote misincorporation of nucleotides and non-specific priming. A less than optimal concentration can reduce the yield of the reaction. The products obtained from a reaction are analyzed on an agarose gel (0.8–2 %, depending on expected product size) where under the optimal conditions a single, bright band of the expected size will be observed.

Thermally stable polymerases from different suppliers may vary in definition of units and optimum magnesium concentration. Reaction conditions may therefore vary depending on the particular enzyme used. Enzyme storage buffers may contain gelatin, bovine serum albumin or nonionic detergents to stabilize the polymerase.

In standard 50 µl reactions, 2.5 units of *Taq* polymerase are routinely used, with a recommended range of 0.5–2.5 units. It is important to choose the DNA polymerase based on the application and need for accuracy [15,32]. Characteristics of DNA polymerases that can be used in PCRs are provided in Table 3.

Stock dNTP solutions come already prepared as part of a PCR kit or may be obtained from suppliers of molecular biology reagents. It is highly recommended that dNTP solutions are of the highest quality available and obtained from suppliers specializing in molecular biology. dNTPs are present in equimolor amounts of 20 to 200 µM each. Magnesium, critical to primer annealing and extension, should be optimized and present in the range of 0.5–2.5 mM. KCl promotes primer annealing

*Table 2.* PCR primers useful for the amplification of nearly full length small subunit rRNA genes.

| Primer[a] | Sequence[b] | Priming site[c] | Designed Specificity | Reference and comments[d] |
|---|---|---|---|---|
| 63f | CAGGCCTAACACATGCAAGTC | 43–63 | most *Bacteria* | [24] |
| T63F | CAGGCCTAACACATGCAAGTT | 43–63 | most *Bacteria* | [13] |
| 1387r | GGGCGGWGTGTACAAGGC | 1404–1387 | most *Bacteria* | [24] |
| 1389r | ACGGGCGGTGTGTACAAG | 1406–1389 | most *Bacteria* | [29] |
| fD1 | ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG | 8–27 | most *Bacteria* | [39] for, *Eco*RI, *Sal*I |
| fD2 | ccgaattcgtcgacaacAGAGTTTGATCATGGCTCAG | 8–27 | enterics and relatives | [39] for, *Eco*RI, *Sal*I |
| fD3 | ccgaattcgtcgacaacAGAGTTTGATCCTGGCTTAG | 8–27 | *Borrelia* spirochetes | [39] for, *Eco*RI, *Sal*I |
| fD4 | ccgaattcgtcgacaacAGAATTTGATCCTGGCTTAG | 8–27 | Chlamydiae | [39] for, *Eco*RI, *Sal*I |
| rD1 | cccgggatccaagcttAAGGAGGTGATCCAGCC | 1541–1525 | many *Bacteria* | [39] rev, *Hind*III, *Bam*HI, *Xma*I |
| rP1 | cccgggatccaagcttACGGTTACCTTGTTACGACTT | 1512–1492 | enterics and most *Bacteria* | [39] rev, *Hind*III, *Bam*HI, *Xma*I |
| rP2 | cccgggatccaagcttACGGCTACCTTGTTACGACTT | 1512–1492 | most *Bacteria* | [39] rev, *Hind*III, *Bam*HI, *Xma*I |
| rP3 | cccgggatccaagcttACGGATACCTTGTTACGACTT | 1512–1492 | Fusobacteria and most *Bacteria* | [39] rev, *Hind*III, *Bam*HI, *Xma*I |
| 5′ | gcgggatccGAGTTTGATCCTGGCTCAG | 9–27 | most *Bacteria* | [20] for, *Bam*HI |
| 3′ | cgcggatccAGAAAGGAGGTGATCCAGCC | 1542–1525 | most *Bacteria* | [20] rev, *Bam*HI |
| pA | AGAGTTTGATCCTGGCTCAG | 8–27 | most *Bacteria* | [8,9] for |
| pH | AAGGAGGTGATCCAGCCGCA | 1541–1522 | most *Bacteria* | [9] rev |

| | | | | |
|---|---|---|---|---|
| reverse | GGTTACCTTGTTACGACTT | 1510–1492 | *Bacteria* | [8] rev |
| Primer A | ccgaattcgtcgacAACCTGGTTGACCTGCCAGT | 1–21 | *Eukarya* | [25] for, *Eco*RI, *Sal*I |
| Primer B | cccgggatccaagctTGATCCTTCTGCAGGTTCACCTAC | 1795–1772 | *Eukarya* | [25] rev, *Sma*I, *Bam*HI, *Hind*III, *Pst*I |
| Primer A | ccgtcgacgagctcAGAGTTTGATCMTGGCTCAG | 8–27 | most *Bacteria* | [11] for, *Sal*I, *Sac*I |
| Primer B | cccgggtaccaagcttAAGGAGGTGATCCANCCRCA | 1541–1518 | most *Bacteria* | [11] rev, *Sma*I, *Kpn*I, *Hind*III |
| forward | CTCCGGTTGATCCTGCC | 7–23 | used with a hyperthemophilic methanogen | [4] for |
| reverse | GGAGGTGATCCAGCCG | 1539–1524 | used with a hyperthemophilic methanogen | [4] rev |
| 27f | AGAGTTTGATCMTGGCTCAG | 9–27 | most *Bacteria* | [18] for, adapted from a sequencing primer |
| 1492r | TACGGYTACCTTGTTACGACTT | 1513–1492 | most *Bacteria*, *Archaea* | [18] rev, sequencing primer adaptable to PCR |
| 1525r | AGGAGGTGWTCCARCC | 1541–1525 | most *Bacteria*, *Archaea* | [18] rev, sequencing primer adaptable to PCR |

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[a] Some primers were not specifically named by the authors. Note the names “Primer A” and “Primer B” were used by different authors for different primers.

[b] Sequences written in the 5′ to 3′ direction. Lower case indicates linker sequence that contains restriction endonuclease recognition sites, upper case indicates region hybridizing with the rRNA gene. The *Pst*I site in eukaryotic Primer B isn within the region hybridizing to the rRNA gene. M = A and C; R = G and A; Y = C and T; W = A and T; N = C,A,T and G; positions where the synthesized primer contains equimolar amounts of more than one nucleotide.

[c] Priming sites indicated by E. coli 16S rRNA numbering for Bacterial- and Archaeal-specific primers, and by the numbering of Saccharomyces cerevisiae 18S rRNA for the eukaryote primers.

[d] for; forward primer with the same sequence as the rRNA site, with extension towards the 3′ end of the rRNA. rev; primer is complementary to the rRNA sequence, with extension towards the 5′ end of the rRNA.

*Table 3.* Thermally Stable DNA Polymerases.

| Thermophilic DNA Polymerase | Features and Applications | Exonuclease Activity | Optimum Extension Temp. |
|---|---|---|---|
| *Taq* (*Thermus aquaticus*) | PCR for amplifying, cloning or labeling. Has a relatively high error rate. | 5′–3′ | 70–75 °C |
| *Tth* (*Thermus thermophilus)* | Efficiently reverse transcribe RNA in presence of $Mn^{2+}$ at higher temp. and synthesize DNA from DNA template in presence of $Mg^{2+}$. Primer extension. | 5′–3′ | 70–75 °C |
| *Pfu* (*Pyrococcus furiosus* ) | High fidelity enzyme, low error rate. Results in blunt ended PCR products. Use in PCR, primer extension, cloning, DNA expression, mutation analysis. | 3′–5′ | 72–74 °C |
| *Tli* (*Thermococcus litoralis)* | High fidelity PCR, Primer extension. | 3′–5′ | 70–75 °C |
| *Tfl* (*Thermus flavus*) | PCR, RT-PCR, 3′ A-tailing of blunt ends, Primer extension, DNA sequencing | 5′–3′ | 70–75 °C |

and should not exceed 50 mM. The reactions are buffered with Tris-HCl (10–50 mM, pH 9.0). Primer concentrations used in rRNA gene amplifications range from 0.1 to 0.6 μM with 0.1 ng to 10.0 ng DNA template added to a 50 μl reaction.

*Enhancers*

It is often beneficial to add additional components to the PCR to "enhance" the specificity and efficiency of the amplification reaction [15]. Enhancer benefit should be determined experimentally during the PCR optimization of each specific template/primer combination. Some commonly used enhancers are described in Table 4.

*Post amplification*

The products of rRNA gene amplification are often further analyzed. Direct analysis, as might occur during a study of microbial community composition, can be accomplished with denaturing gradient gel electrophoresis (DGGE) [27] or identification of terminal restriction fragment length polymorphisms (T-RFLP) [21]. Clone libraries can be conveniently constructed with commercially available kits designed for PCR products (e.g., TA Cloning System; Invitrogen Corp., San Diego, CA). rRNA inserts in clone libraries are readily screened by digestion with restriction enzymes that recognize four base pairs [26]. Conserved rRNA sequencing primers [7,18] enable rapid sequencing of plasmid inserts or PCR products. These procedures are described in detail elsewhere in this manual.

*Table 4.* Common PCR Enhancers.

| Enhancer | Function | Concentration range |
|---|---|---|
| DMSO (dimethyl-sulfoxide) | $\Delta T_m$ of primer-template hybridization reaction to enchance specificity. | 1–10% (v/v) |
| Formamide | $\Delta T_m$ of primer-template hybridization reaction to enchance specificity. | 1.25–10% |
| Glycerol | $\Delta T_m$ of primer-template hybridization reaction to enchance specificity. Stabilizes polymerase. | 5–20% (v/v) |
| BSA (bovine serum albumin) | Binds deleterious factors that might otherwise bind to the polymerase. | 10–100μg/ml, 0.01–0.1% (w/v) |
| Non-ionic detergents | neutralizes charges of ionic detergents used in template preparation and other inhibitors | Tween 20: 0.05% (v/v) Triton-X-100: 0.01% (v/v) Nonidet P40 0.5% (v/v) |

**Procedures**

Work at a clean area covered with fresh bench paper and observe the precautions given above. Include the appropriate positive and negative controls. DNA is added next to last to limit the potential for carry over and the DNA polymerase is added last.

*Template DNA*

High-molecular weight DNA (around 25 kb) of the highest purity obtainable should be used. We use about 100 ng of pure culture DNA in a 50 μl reaction. Cells from pure cultures may also serve to provide template (see Note).

Note:
Suspend a small amount of cells from a plate colony (about 1 μl) in 1.0 ml 1/10 TE, vortex, and alternatively freeze (−70 °C) and thaw (65 °C) the suspension three times. About 2 μl of this lysate is added to the reaction. This may also be adapted to small amounts of cells in liquid cultures.

*Basic PCR*

Add the following components to a sterile, DNAse/RNase free, thin walled, 0.2 ml tube:

| PCR Reagent | Voulme for standard 50μl reaction | Final concentration | Optimization range | Commonly used range |
|---|---|---|---|---|
| 10X PCR buffer | 5.0 μl | 1 X | 1 X | 1 X |
| 25mM $MgCl_2$ | 5.0 μl | 2.5 mM | 0.5–5.0 mM | 1.5–2.5 mM |
| 10mM (each) dNTP | 1.0 μl | 200 μM | 200 μM each | 200 μM each |
| Forward primer (25μM) | 0.8 μl | 0.4 μM | 0.1–0.6 μM | 0.1–0.6 μM |
| Reverse primer (25μM) | 0.8 μl | 0.4 μM | 0.1–0.6 μM | 0.1–0.6 μM |
| DNA template | varies depending on DNA concentration | 0.1–10 ng | | 1–10ng bacterial DNA<br>0.1–1ng plasmid DNA |
| $H_2O$ | bring to 50μl volume | | | |
| 5 Units/μl DNA polymerase | 0.5μl | 2.5 Units | | 0.5–2.5 Units/50μl reaction |

Mix gently, quick spin in a microcentrifuge to bring the solution to the bottom of the tube and place in the temperature controller.

Notes:

The 10 X PCR buffer indicated does not contain $Mg^{2+}$. The final concentration of magnesium used in this reaction is 2.5 mM. Alternatively, a PCR buffer which already contains magnesium may be purchased and the magnesium concentration optimized if needed.

When performing multiple PCRs with the same primer pair a "master mix" that combines components can be prepared and aliquoted to the reaction tubes.

A mineral oil overlay is often placed on the reaction mixture if the thermal cycler used does not have a top heater.

Thermal cycle parameters:

| Cycle | Temperature | Temp Range | Time | # of Cycles |
|---|---|---|---|---|
| Initial Denaturation | 94 °C | 94–95 °C | 2 minutes | 1 |
| Denaturation | 94 °C | 94–95 °C | 30 seconds–1 minute | 25–30 |
| Annealing | 5 °C below primer $T_m$ | 55–72 °C | 1 minute | 25–30 |
| Extension | 72 °C | 72 °C | 30 seconds–1 minute | 25–30 |
| Final Extension | 72 °C | 72 °C | 15 minutes | 1 |
| Soak and Hold | 4 °C | 4 °C | 99 hours | 99 |

Note:

Lowering the annealing temperature diminishes primer specificity and allows for mismatches in the template/primer hybrid. This is useful when the primer sequence may vary from the target site, but it could also allow for amplification of non-specific sequences.

### *Enzyme addition*

Start the thermal cycler temperature program. When the initial denaturing temperature has been reached, pause the machine and add:

2.5 units of DNA polymerase to each reaction tube

Complete the thermal cycle program.

Note:
This "hot start" increases specificity of priming by thoroughly denaturing the template.

### *Agarose gel analysis*

Analyze 15 µl of the completed reaction on an agarose gel with size markers (e.g., 1 kb ladder; Bethesda Research Laboratories, Gaithersburg, MD).

Solutions (Stored in small aliquots at −20 °C.)

TE
- 10 mM Tris-HCl, pH 8.0
- 1.0 mM EDTA.

10X PCR buffer for Taq DNA polymerase (supplied with enzyme)
- 100 mM Tris-HCl, pH 9.0.
- 500 mM KCl.

25 mM $MgCl_2$ supplied with enzyme

dNTP mixture (with a final concentration of 10 mM each dNTP)
- Available commercially

Primers are diluted in sterile distilled water to a stock concentration of 25 µM.

Double distilled water, autoclave to sterilize and aliquot through a 0.2 µm syringe filter.

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