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ON FOCUS

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# PCR Amplification

PCR has grown into a workhorse technique in many labs. Life Technologies is licensed to sell *Taq* DNA polymerase and other products for PCR. In keeping with our commitment for technical support, this special issue of *Focus* contains a series of articles related to PCR.

PCR is increasingly being used for site-directed mutagenesis. Owen *et al.* describe a mutagenesis system that combines PCR and UDG cloning for mutagenesis of a double-stranded DNA template.

PCR is also being used more to generate labeled probes for Southern and Northern blots. Mertz and Rashtchian have optimized the nucleotide concentration in PCR to improve the production of radioactively labeled DNA probes. These probes can be generated from much less starting material and result in a higher specific activity than is possible with random priming or nick translation. There are different concerns when generating biotinylated DNA probes for nonradioactive detection. Mertz *et al.* describe a system that results in production of microgram amounts of biotinylated probe by PCR using low amounts of starting template. Biotinylated probes as large as 4.4 kb were used successfully in a Southern blot of genomic DNA. Westfall and Mertz also examine buffers used with column chromatography to remove free nucleotides from biotinylated DNA to optimize the recovery of biotinylated probes.

The optimization of PCR conditions becomes more critical when the amount of template DNA is small and as the size of the PCR fragment increases over several kilobases. Sitaraman and Rashtchian have examined several reaction parameters to improve the efficiency of PCR under these stringent conditions. Hartley and Xu describe a new molecular size standard that can be used to estimate the amount of PCR product in ethidium bromide stained gels.

PCR can also facilitate screening of colonies or plaques for various applications. Natarajan and Cizdziel use PCR to screen cDNA libraries for inserts. Wilson and Schuller have used PCR to screen plaques generated after a mutagenesis experiment to find the mutated products.

These articles demonstrate the variety of applications that are possible with PCR technology.

Doreen Cupo

# A Highly Efficient Method of Site-Directed Mutagenesis Using PCR and UDG Cloning

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## Abstract

**PCR and ligase-free UDG cloning were used for the development of a site-directed mutagenesis system. The method has been optimized to generate the desired mutants after 8 cycles of PCR followed by cloning using the CloneAmp system. PCR was applied to the mutagenesis of a model system based on *lac*Zα complementation and also to the generation of site-specific mutants in a human *c-raf* oncogene. The efficiency of mutagenesis is >90%. The method is applicable to both double- and single-stranded templates and can be used with virtually any plasmid DNAs. The production of single-stranded or modified templates is not required. Using this method, base substitutions, deletions, and insertions can be generated.**

Site-directed mutagenesis of genes is a widely used approach for studying the structure and function of genes and their products. In the last few years, numerous approaches have been described for site-specific mutagenesis (1–4). Regardless of the procedure used, the efficiency of mutagenesis and differentiation between mutants and wild type templates is most important when the sequence changes result in silent, unknown, and nonselectable phenotypes (5).

The efficient uracil DNA glycosylase (UDG) cloning method for DNA amplified using the polymerase chain reaction (PCR) has been described (6–9) and further modified for generation of site-specific mutations (10). This paper describes the PCR site-directed mutagenesis method for rapid and convenient generation of mutants. Application of the system to the mutagenesis of the *lacZ*α complementation gene and the human c-*raf*-1 oncogene is presented.

*Principles of the method.* A site-specific mutation can be introduced into a cloned gene by amplifying the gene using PCR with specifically designed mutagenic oligonucleotide primers (MP1, MP2) that contain the desired mutation (figure 1). The targeted base change can reside in one or both of the mutagenic primers. Cloned inserts are amplified in two fragments. Fragment 1 is amplified using MP1 and the dU-LacZ forward primer (dU-LacFWD), while fragment 2 is amplified using MP2 and the dU-LacZ reverse primer (dU-LacREV). The primers have been designed to anneal to *lac*Zα sequences flanking the multiple cloning site of plasmids and are capable of amplifying the insert along with the multiple cloning site in these plasmids. The dU-LacFWD and dU-LacREV primers are complementary to many vectors (table 1). Thus, a cloned gene can be mutagenized by designing only the two internal primers.

The PCR conditions have been optimized to yield specific products while maintaining conditions recommended for high fidelity DNA polymerization (11). Eight cycles of PCR provide sufficient amplified product for efficient UDG cloning, while minimizing the opportunity for misincorporation of nucleotides by *Taq* DNA polymerase.

Small amounts of intact plasmid (the original template plasmid used for mutagenesis) may survive through the mutagenesis and transformation and will be responsible for the presence of bacterial colonies containing non-mutagenized plasmids. Background caused by

**TABLE 1. Common plasmid and bacteriophage vectors recognized by the dU-LacFWD and dU-LacREV primers**

| | |
|---|---|
| pSPORT series | pBlueBac |
| pAMP series | pCDNA II |
| pUC series* | pCITE™ 2 series |
| M13 series | pCMVEBNA |
| pBlueScript® series | pEMBL 8, 9 |
| pBlueScript M13 series | pALTER™ |
| pGem® series* | pCAT® Control |
| pBC KS, SK | pCAT Promoter |
| pMC1neo, neo Poly(A) | PhageScript |
| pAC360 | pT7/T3 18 and 19 |
| pCaMVCN | pSL 1180 and 1190 |
| pVP16C1, 16C2, 16C3 | |

*Many, but not all, of the series contain sequences complementary to the dU-lacZ primers.

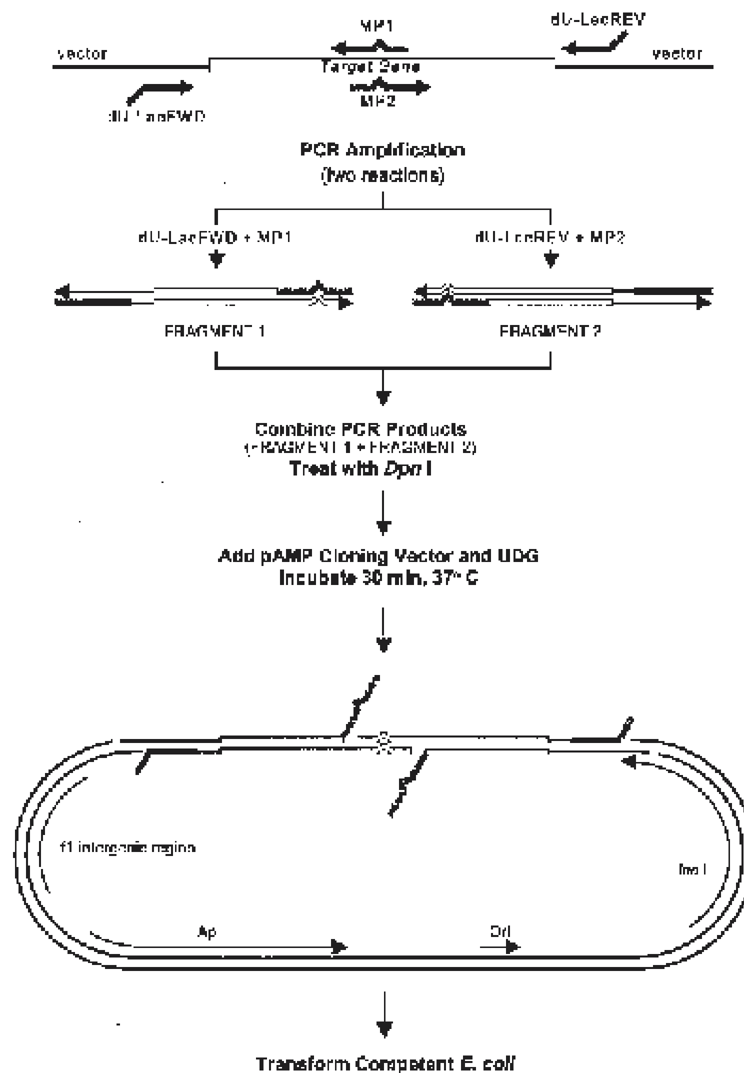


**FIGURE 1. Schematic of the PCR site-directed mutagenesis procedure.**

this template can be eliminated in two ways. First, by linearizing the DNA template before PCR may eliminate interference by nonspecific PCR products. The second method is to specifically eliminate template DNA by treating the reactions with *Dpn* I. *Dpn* I recognizes $G^{m}ATC$. The template DNA is methylated, having been produced in *dam*+ *E. coli*. Conversely, the PCR products are nonmethylated and will remain unaffected by *Dpn* I.

The overlapping, internal primers contain uracil in place of thymidine at their 5' ends (figure 1, table 2). This allows for directional cloning of the amplified DNA by UDG cloning (6). The combined products of amplified fragments 1 and 2 are treated with UDG generating 3' overhangs. The 3' overhangs of the two

**TABLE 2. Sequences of the oligonucleotide primers.**

| Primer / Sequence |
|---|
| **Control Primer Mix 1:** |
| CUA CUA CUA CUA AGC GGG TGT TGG CGG GTG (CP1a) |
| AAC GUC GUG ACU GGG AAA ACC CTG G (CP1b)[a] |
| **Control Primer Mix 2:** |
| AGU CAC GAC GUU GUA AAA CGA CGG C (CP2a) |
| CAU CAU CAU CAU GCG CAA CGC AAT TAA TGT GAG (CP2b) |
| **dU-LacFWD:** |
| CUA CUA CUA CUA CGT TGT AAA ACG ACG GCC AG |
| **dU-LacREV:** |
| CAU CAU CAU CAU AGC GGA TAA CAA TTT CAC ACA GG |
| ***c-raf* mutagenic primer 1 (1303):** |
| CUU UAG GUA CCA UAC UGC AAC ATC TCC GTG |
| ***c-raf* mutagenic primer 2 (1304):** |
| GUA UGG AUC CUA AAG GUU GTC GAC CCA A |

[a]Mutated nucleotides are underlined.

amplification products are complementary and anneal with each other, reconstructing the original sequence without interruption. This same strategy is applied to the amplification primers at the ends of the cloned insert complementary to the vector. Any double- or single-stranded DNA template may be used, and the entire procedure, from PCR amplification to transformation of competent cells, can be accomplished in one day.

## Methods

*Preparation of DNA*. Plasmid pLAC⁻201 was derived from pUC19. Base 89 of the *lac*Zα peptide coding sequence in this plasmid has been changed from G→A by PCR-based site-directed mutagenesis (10,12) resulting in creation of a termination codon (Opal). Plasmid pLAC⁻201 DNA was purified on a CsCl gradient. The plasmid pAMP2 vector was derived from pAMP1 by removing the *lac*OPZ sequence. Plasmid pCMVBXB contains sequences of the human c-*raf*-1 cDNA clone preceded by the immediate early cytomegalovirus (CMV) promoter and followed by the SV40 poly(A) signal (13) and was obtained from G. Heidecker.

*Mutagenesis*. Mutagenesis was performed using the PCR site-directed mutagenesis method as follows (figure 1). The cloned insert was amplified in two fragments, using a combination of mutant and nonmutant primers (10). PCR was performed in 50 µl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.0 mM $MgCl_2$, 50 µM each dNTP, 0.2 µM each primer, 5 ng template DNA, and 0.5 units *Taq* DNA polymerase. Reactions were denatured for 5 min at 94°C, followed by 8 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. 10-µl aliquots from each reaction were mixed

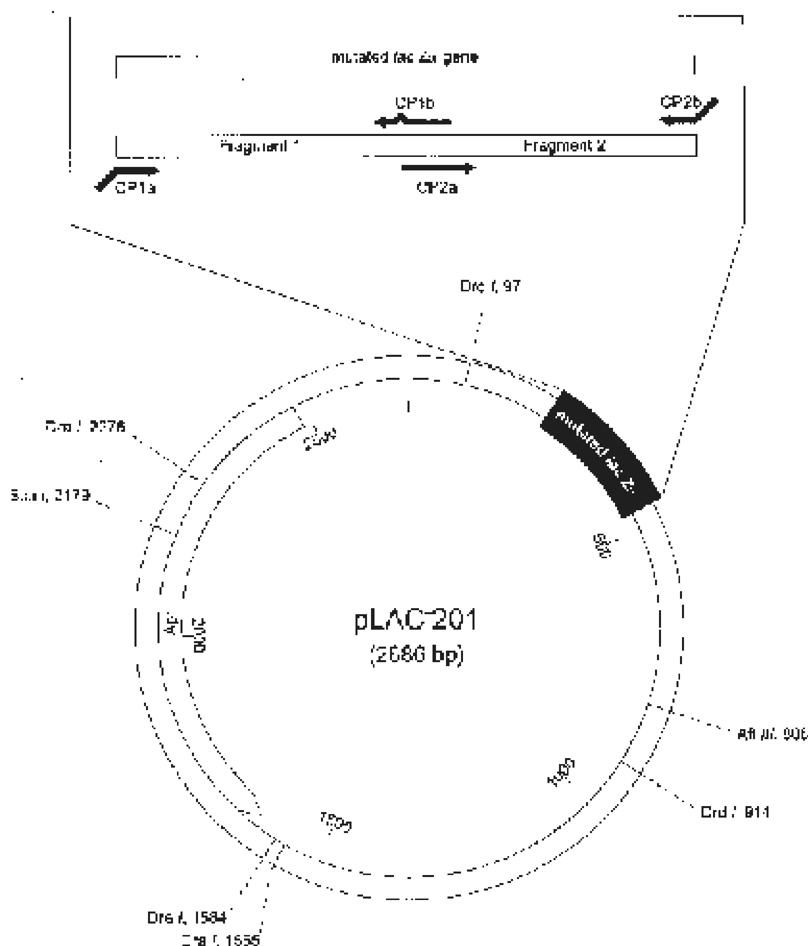


**FIGURE 2. Map of pLAC⁻201.**

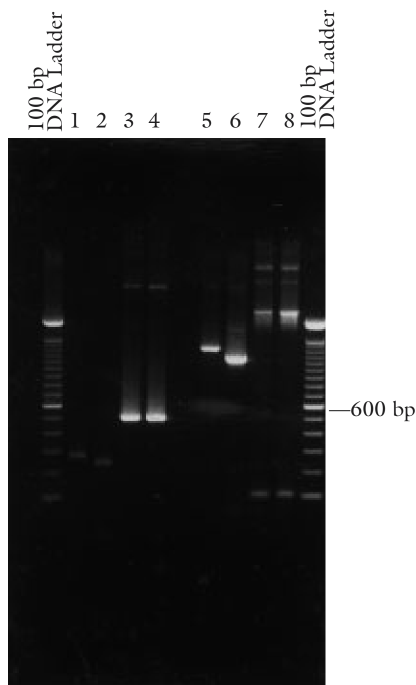


**FIGURE 3. Mutagenesis of the *lacZα* and c-*raf* genes.** Plasmids pLAC⁻201 and pCMVBXB were linearized with *Sca* I. Five nanograms (2 fmol) were used as template for the amplification reaction. Amplification of fragment 1 (lane 1) and fragment 2 (lane 2) of the *lacZα* gene, 277 and 236 bp, respectively. These fragments were cloned into pAMP2. Recombinants were amplified using CP1a and CP2b (lanes 3 and 4, 502 bp). DNA amplification products of the c-*raf* gene were 1,413 bp (lane 5) and 1,180 bp (lane 6). After the fragments were cloned into pAMP2, the recombinants were amplified using dU-LacFWD and dU-LacREV (lanes 7 and 8, 2,534 bp).

and treated with 4 units of *Dpn* I for 30 min at 37°C. *Dpn* I was inactivated at 65°C for 30 min. The reaction mixture was placed on ice, 30 ng of pAMP2 and 1 µl (1 unit) UDG were added, and reactions were incubated for 30 min at 37°C.

*Transformation of cells and analysis of recombinants.* A 10-µl aliquot of the mixture of annealed DNA was mixed with 50 µl GIBCO BRL MAX EFFICIENCY DH5α™ Competent Cells for transformation. 300 µl of the 500 µl of transformed cells were plated onto LB plates containing 100 µg/ml ampicillin, and overlaid with 80 µl of 20 mg/ml X-gal and 8 µl of 20% IPTG.

## RESULTS

*Mutagenesis of lacZα.* The plasmid pLAC⁻201 is a Lac⁻ mutant of plasmid pUC19 due to a mutation in position 89 of *lacZα* structural gene (10). The mutagenesis assay was based on reversion of the base 89 to wild type, resulting in restoration of blue colony phenotype upon transformation.

The *lacZα* sequence of pLAC⁻201 (linearized with *Sca* I) was amplified in two fragments (figure 2). Fragment 1, amplified using control primer mix 1 containing CP1a and CP1b, resulted in a 277-bp DNA product (including the 12-bp dU-containing tails of the primers). Fragment 2, amplified using control primer mix 2 containing CP2a and CP2b, resulted in a 236-bp product (figure 3). CP1b contained the desired A→G mutation at base 89, which restores the *lac*Zα wild type phenotype. The two amplification reactions were mixed, digested with *Dpn* I, treated with UDG, and annealed, reconstructing the original *lacZα* gene without interruption. The primers on the outer edge of the *lacZα* gene included tails of $(CUA)_4$ or $(CAU)_4$ allowing for proper orientation of the insert to complementary sequences in the pAMP2 vector.

After transformation, 91% of the colonies were blue, indicating that the plasmid was reverted to the wild type (table 4). In separate reactions, the plasmid template pLAC⁻201 was digested before PCR with *Drd* I, or a combination of *Drd* I and *Dra* I. The colonies resulting from mutagenesis and transformation using these templates were 91% and 93% blue, respectively. An 817-bp fragment containing the *lacZα* sequences resulted from digestion of pLAC⁻201 with *Drd* I. This fragment was gel purified and also used as a template for the PCR. After mutagenesis and transformation, 93% of the resulting colonies were blue. This strategy may be useful in instances where nonspecific PCR products are generated due to nonspecific priming which would interfere with the cloning of the desired mutagenized fragment. In this case, the result obtained using the purified DNA fragment was similar to that obtained with the *Drd* I-digested plasmid, even when the *lacZ*-containing fragment was not physically separated from the rest of the vector. When the template plasmid is not disabled before the PCR, some intact plasmid survives through the assay and a background of up to 50% nonmutagenized plasmids may be observed. When the DNA templates were included in the assay without the addition of primers, few, if any, white colonies resulted from contamination of the transformation with

template DNA. However, if the *Dpn* I step was neglected as well, a moderate background of colonies (45 in this case) was obtained.

*Mutagenesis of c-raf oncogene.* The plasmid pCMVBXB encodes the kinase domain of the serine/threonine protein kinase *raf*-1 and was used as a template to mutagenize the cloned c-*raf* gene (13). The site targeted for mutagenesis was two adjacent base pairs at codon 375 of full-length c-*raf* (10) which will change the codon of lysine to tryptophan and inactivate the kinase.

The site-directed mutagenesis of pCMVBXB was performed with both of the internal, overlapping primers containing the desired base changes. Fragment 1 was amplified using dU-LacFWD and primer 1303, and fragment 2 was amplified using dU-LacREV and primer 1304, resulting in 1,389 bp and 1,156 bp fragments, respectively (figure 3). The two fragments were mixed with pAMP2, treated with UDG, and transformed into *E. coli*. Twenty of the resulting colonies were screened for the presence of recombinant plasmid containing the full-length *c-raf* by amplification with dU-LacFWD and dU-LacREV. Nineteen contained an insert of ~2,500 bp, the length of the insert in pCMVBXB. When the cloned c-*raf* gene was inserted into pAMP1 in a similar manner, all of the transformants showed the proper size insert. Four of these clones were sequenced in a 200-bp region surrounding the targeted mutation, which confirmed the desired lys→trp substitution with no other divergence from wild type sequence (10).

## Discussion

The site-directed mutagenesis method described is a modification of the original PCR method described by Higuchi (5). This modification simplifies the procedure by eliminating the overlap extension needed by the Higuchi method. The joining of the mutant fragments and cloning of the full-length fragment into an appropriate host are performed simultaneously. If dU substitution is not desired, an alternative overlap extension method (5) combining two overlapping amplification products followed by UDG cloning can be used. This modification allows the user to take advantage of the highly efficient UDG cloning without the need for synthesis of modified primers.

**TABLE 4. Site-directed mutagenesis of pLAC⁻201.**

| DNA Template for Mutagenesis | Mutagenic Primers | *Dpn* I | Blue | White | Efficiency of Mutagenesis |
|---|---|---|---|---|---|
| *Sca* I digested pLAC⁻201 | + | + | 346 | 33 | 91% |
| *Drd* I digested pLAC⁻201 | + | + | 156 | 16 | 91% |
| *Drd* I digested pLAC⁻201 | - | + | 0 | 0 | |
| *Drd* I, *Dra* I digested pLAC⁻201 | + | + | 402 | 30 | 93% |
| *Drd* I, *Dra* I digested pLAC⁻201 | - | + | 0 | 0 | |
| *Drd* I, *Dra* I digested pLAC⁻201 | - | - | 0 | 45 | |
| *lacZ*α-containing fragment | + | + | 291 | 23 | 93% |
| *lacZ*α-containing fragment | - | + | 0 | 4 | |
| pAMP2 vector control | NA | NA | NA | 5 | |

Plasmid pLAC⁻201 was reverted to wild type by site-directed mutagenesis. Before PCR, the double-stranded DNA template was digested by either *Sca* I, *Drd* I, *or Drd* I and *Dra I,* or the *lacZ*α-containing fragment of the *Drd* I digestion was gel purified. After transformation of MAX Efficiency DH5α competent cells, blue and white colonies were scored.

The UDG method has been optimized to provide a high efficiency of mutagenesis while minimizing the number of amplification cycles. The primers used for amplification correspond to the *lacZ*α sequences that flank many pUC-based plasmids. This eliminates subcloning of the insert DNA into other plasmids, as is needed for other mutagenesis methods. Isolation of single-stranded DNA is not necessary to prepare the mutagenesis template, since both double-stranded and single-stranded templates can be used.

When compared to the phagemid method described by Kunkel (4), UDG mutagenesis offers a higher frequency of mutation (>90%) because PCR allows exponential amplification of the mutant molecules. A single-stranded dU-containing template (produced by phagemids grown in a special *dut⁻ ung⁻* strain) is not needed.

In the UDG mutagenesis method, the use of overlapping mutant oligonucleotide primers containing dU allows two PCR products to be joined without further amplification, thereby reducing the number of cycles needed. Furthermore, the high efficiency of mutagenesis combined with the high efficiency of cloning allows cloning after as few as 8 cycles. Besides simplifying the method, this reduces the chance of introducing unwanted mutations in the amplified DNA. Therefore, it is reasonable to propose that UDG mutagenesis in conjunction with use of a DNA polymerase with proofreading capability will result in a very high fidelity mutagenesis method.

Initially, UDG cloning used evenly spaced dU residues (7). The efficiency was not compromised by using naturally occurring sequences in which the position of dU cannot be altered (9,14). In fact, a primer has been successfully used in UDG cloning that had a 9-base stretch of residues at the 5' end that contained no dU. Occasionally, a brief incubation of the DNA at 65°C will facilitate the destabilization of the base pairs in the dU-containing strand, thus improving cloning efficiency (14).

The UDG method has been applied to the mutagenesis of a c-*raf* gene insert that is ~2,500 bp. PCR has been used successfully to amplify a range of targets up to 10 kb (15, 16). Although we have not used such large fragments, it is reasonable to assume that the mutagenesis system described can be performed with any cloned insert which is amenable to amplification in two fragments.

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## Editor's Note:

A system containing du-lacZ primers, appropriate reaction buffers, *Dpn* I, UDG, pAmp2 vector, and a detailed instruction manual is available for field tests. Contact Dr. Judith Owen at Life Technologies. In the U.S. call (800) 828-6686 ext. 7782.

**What competent *E. coli* strains are available for generating ssDNA?**

*E. coli* strains DH11S and DH12S contain the wild-type *end*A gene, which allows for the preparation of highly purified ssDNA from phagemid vectors with M13 helper phage.

# PCR Radioactive Labeling System: A Rapid Method for Synthesis of High Specific Activity DNA Probes

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*Life Technologies, Inc.*
*Gaithersburg, Maryland 20884*

## Abstract

**Polymerase chain reaction (PCR) has been investigated as a convenient and efficient method for synthesis of high specific activity DNA probes. Simple substitution of a radiolabeled nucleotide for one of the unlabeled nucleotides in the reaction results in large dNTP concentration imbalances (>30-fold) and reduced amplification efficiency. However, 2- to 8-fold dNTP concentration imbalances resulted in augmented synthesis of radiolabeled product. With a 2-fold imbalance, this method led to 87% incorporation of added [$\alpha^{32}$P]dCTP into an 800-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification product compared to reduced incorporation with greater nucleotide concentration imbalances. This paper describes a PCR Radioactive Labeling System using reduced dNTP levels for synthesis of high specific activity (>4 × $10^9$ cpm/µg) DNA probes.**

Previously described PCR-radiolabeling methods utilize standard amplification conditions with respect to nucleotide concentration (1). These conditions involve dNTP imbalance, which can lead to nucleotide misincorporation by *Taq* DNA polymerase (2). Due to the lack of 3' exonuclease activity in *Taq* DNA polymerase (3), these misincorporations cannot be corrected. Further, extension from transition and transversion mispairs by *Taq* DNA polymerase is greatly inhibited, resulting in kinetic rates that are $10^4$- to $10^6$-fold lower than those from correctly base paired 3' sequences (4). These reduced rates of extension often result in premature chain termination (2).

Another PCR-radiolabeling method circumvents this problem by addition of a small amount of unlabeled nucleotide counterpart of the radiolabeled dNTP (5). Although this method avoids nucleotide imbalances, the specific activity of the synthesized DNA probe is reduced. In this report, an optimized PCR Radioactive Labeling System for synthesis of high specific activity DNA probes is presented.

## Description of the System

The PCR Radioactive Labeling System (Cat. No. 10199 and table 1) allows synthesis of radiolabeled probes from any insert contained within the multiple cloning site of pUC and pUC-derived vectors. The LacFWD and LacREV primers anneal to *LacZα* DNA sequences present in many vectors (table 2). The control plasmid is composed of a 531-bp human GAPDH (EC 1.2.1.12) insert (nucleotide positions 43–574)(6) in pAMP1 (7,8). Since GAPDH is highly conserved, the probe will recognize GAPDH coding sequences

**TABLE 1. Components of the PCR Radioactive Labeling System.**

| Component | Amount |
|---|---|
| 10X PCR reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] | 500 µl |
| $MgCl_2$ (50 mM) | 200 µl |
| dATP (60 µM) | 100 µl |
| dCTP (60 µM) | 100 µl |
| dGTP (60 µM) | 100 µl |
| dTTP (60 µM) | 100 µl |
| LacFWD primer (5 µM) | 50 µl |
| LacREV primer (5 µM) | 50 µl |
| *Taq* DNA polymerase (5 U/µl) | 25 µl |
| sterile water | 1.25 ml |
| control GAPDH template (100 pg/µl) | 50 µl |
| silicone oil | 4 ml |

**TABLE 2. Plasmids and bacteriophage vectors that contain *LacZα* DNA sequences.**

| | |
|---|---|
| pSPORT series | pBlueBac |
| pAMP series | pCDNA II |
| pUC series* | pCITE™ 2 series |
| M13 series | pCMVEBNA |
| pBLUESCRIPT® series | pEMBL 8, 9 |
| pBLUESCRIPT M13 series | pALTER™ |
| pGEM® series* | pCAT® Control |
| pBC KS, SK | pCAT Promoter |
| pMC1neo, neo Poly(A) | PhageScript |
| pAC360 | pT7/T3 18 and 19 |
| pCaMVCN | pSL 1180 and 1190 |
| pVP16C1, 16C2, 16C3 | |

*Many, but not all, of the series contain sequences complementary to the LacZ primers.

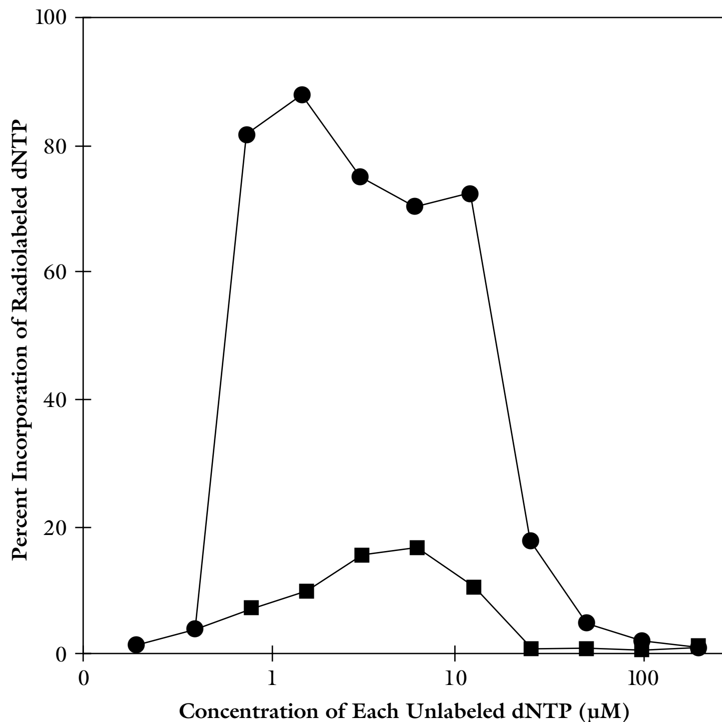


**FIGURE 1. Generation of radiolabeled probe by PCR**. pGAPDH (800 bp, ●) and pCMVBXB (2.6 kb, ■) inserts were amplified using 100 pg of supercoiled plasmid and 0.825 μM [α$^{32}$P]dCTP or [α$^{32}$P]dATP. Concentrations of the other dNTPs were each concurrently varied between 200 and 0.19 μM.

from human and other mammals and can serve as a useful control on blots. This system is not recommended for inserts >2.5 kb. For large inserts, it may be helpful to generate probe from a portion of the insert by using a gene-specific primer in combination with the LacFWD or LacREV primer. Alternatively, a smaller portion of the insert may be subcloned into a pUC or pUC-derived vector for subsequent generation of radiolabeled probe.

## Methods

Target inserts were amplified with the LacFWD (CGT TGT AAA ACG ACG GCC AG) and LacREV (AGC GGA TAA CAA TTT CAC ACA GG) primers. Plasmid pCMVBXB, constructed by insertion of an activated BXB version of the c-raf-1 cDNA clone (9) into a modified pUC19 vector, was provided by Dr. Gisela Heidecker. The pRAF construct was generated by subcloning a 1.4-kb fragment of the pCMVBXB insert into the Gibco BRL pSPORT 1 vector. Plasmid pGAPDH was created by insertion of a 531-bp human GAPDH cDNA sequence into pAMP1 using UDG cloning (7,8) with the Gibco BRL CloneAmp® system. The pβ-globin plasmid was created by insertion of a 4.4-kb *Pst* I insert of a human β-globin genomic clone into pUC18.

PCR-radiolabeling reactions (20 μl) contained: 100 pg of supercoiled plasmid, 250 nM of each forward and reverse primer, 2.5 mM $MgCl_2$, 0.19 to 200 μM each of three nucleotides (dATP, dGTP, and dTTP), and 2.5 units of *Taq* DNA polymerase in PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl]. In addition, 5 μl of [α$^{32}$P]dCTP (3,000 Ci/mmol; 3.3 μM; Amersham) were added for a final concentration of 0.825 μM. In some cases, [α$^{32}$P]dATP (3,000 Ci/mmol; 3.3 μM; Amersham) was added in place of [α$^{32}$P]dCTP, and unlabeled dCTP was substituted for unlabeled dATP. After an initial 5-min denaturation at 95°C, 30 cycles of 94°C for 30 s, 60°C for 75 s, and 72°C for 90 s were performed. For amplification of the longer 2.6-kb pCMVBXB template, the elongation time at 72°C was increased to 2 min, 30 s.

Incorporated radioactivity was determined by TCA precipitation. Aliquots of reactions were spotted on filters and the filters were agitated for 15 min in ice cold 10% TCA (containing 1% sodium pyrophosphate). The filters were then washed in 5% TCA solution (3 washes, 5 min each), followed by a 5-min wash in 100% ethanol. The dried filters were placed in scintillation vials containing Econofluor™-2, and precipitable counts were determined. For use as probes on membranes, the amplification product was purified from unincorporated nucleotides using Sephadex® G-50 spin columns. Prehybridization and hybridizations were at 42°C in Gibco BRL 2X Prehybridization/Hybridization Solution diluted to 1X with an equal volume of formamide. Washed blots were exposed to Kodak X-Omat® RP-XRP film with an intensifying screen at –70°C.

## Results and discussion

*Effect of template DNA on amplification.* Quantitative amplification of pGAPDH, pRAF, pCMVBXB, and pβ-globin inserts in circular or linear plasmids was performed to determine potential differences in product yield. Plasmids were linearized by restriction endonucleases that cleaved the plasmid in the ampicillin resis-

tance gene (*i.e.*, *Sca* I). Unique restriction sites in the polylinker region could not be used because the *LacZα* sequences, complementary to the PCR oligonucleotide primers, must flank the insert for subsequent amplification. No notable differences in product yield were obtained at template levels ≥10 pg/50 µl reaction volume (data not shown). Below this amount, more PCR product was generated from the linearized plasmid. For subsequent experiments, circular plasmid concentrations of ≥100 pg/reaction were used.

*Effect of dNTP concentration on amplification.* The synthesis of radiolabeled DNA probes was examined using the LacFWD and LacREV primers along with pGAPDH, pRAF, and pCMVBXB plasmids that yield 800-bp, 1.4-kb, and 2.6-kb probes, respectively. The probe sizes are larger than the inserts because the amplification primers flank the polylinker region. Therefore, multiple cloning sequences will be copied in the probe. In addition to using 200-µM concentrations of unlabeled nucleotides and low micromolar levels of [$\alpha^{32}$P]dNTP (1), we varied the three nucleotide concentrations concurrently while the concentration of labeled nucleotide was fixed (figure 1). Above nucleotide concentrations of 25 µM, little TCA-precipitable product was obtained. However, when unlabeled nucleotide concentrations were reduced, significant radiolabeled amplification product was synthesized. Optimal amplification of the 800-bp GAPDH probe occurred when the concentrations of dATP, dGTP, and dTTP were approximately 2-fold greater than that of [$\alpha^{32}$P]dCTP. With amplification of the 2.6-kb probe, optimal probe synthesis occurred when the concentrations of the three unlabeled dNTPs were approximately 6 to 8 times the concentration of [$\alpha^{32}$P]dATP. Optimal synthesis of the 1.4-kb pRAF insert occurred with 2- to 5-fold dNTP concentration imbalances (data not shown). Use of [$^{32}$P]dNTP that had decayed to one half life (14 days) led to poor results. PCR-radiolabeling may be especially sensitive to this because as the concentration of [$^{32}$P]dNTP decreases during radioisotope decay, larger dNTP concentration disparities occur.

Under optimal conditions, 87%, 60%, and 18% of the radiolabel were incorporated into the pGAPDH, pRAF, and pCMVBXB probes, respectively. Approximately 19.2 ng (pGAPDH), 13.5 ng (pRAF), and 5 ng (pCMVBXB) of probe were synthesized. The specific activities of these probes were $4.3 \times 10^9$ to $4.4 \times 10^9$ cpm/µg. The decrease in percent incorporation as the size of the template increases likely reflects the difficulty of amplifying larger templates using reduced dNTP concentrations. Although a relatively low percent incorporation was obtained with the 2.6-kb product, $2.6 \times 10^7$ cpm of probe was synthesized, which is sufficient for hybridization to target DNA on blots. Electrophoretic analysis of the 2.6-kb pCMVBXB probe showed a band migrating at that size (data not shown). Additionally, smaller material was observed that represented chain termination products or partial breakdown of the radiolabeled probe.

It appears that small (2- to 8-fold), but not large (>20-fold), imbalances in dNTP concentration are tolerated during amplification and may lead to a greater amplification yield. An increase in amplification yield has been seen with single-copy genes from genomic DNA (Mertz and Rashtchian, unpublished results) under conditions of small dNTP concentration imbalance. When nucleotide concentrations are below the Km (10 to 15 µM) value of *Taq* DNA polymerase (11), elongation rates will be less than half-maximal. Small increases in the three unlabeled dNTP concentrations may aid in augmenting the elongation rate to obtain more amplification product. However, if the nucleotide concentration is increased to a large extent over that of the limiting radiolabeled nucleotide, misincorporation rates may increase and result in increased frequency of premature chain termination. Successful amplification may occur under conditions where the relative dNTP concentrations balance these two factors.

**TABLE 3. Comparison of DNA probes synthesized by random primer-labeling or PCR-radiolabeling.**

| Method | Substrate[1] (ng) | [$\alpha^{32}$P]dNTP (µCi) | Net Synthesis (ng) | Specific Activity[2] (cpm/µg) |
|---|---|---|---|---|
| Random-priming | >25 | 50 | 15–20 | $0.9 \times 10^9$ to $1.8 \times 10^9$ |
| PCR-radiolabeling | 0.1 | 50 | 15–20 | $>4 \times 10^9$ |

[1] For random primer-labeling, gel-purified insert; for PCR-radiolabeling, supercoiled plasmid containing GAPDH.

[2] Includes the unlabeled template.

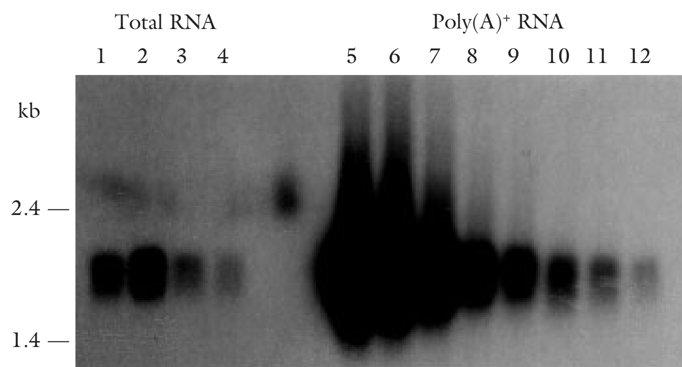


**FIGURE 2. Northern blot analysis of HeLa cell RNA**. HeLa cell total and poly(A)$^+$ RNA were electrophoresed on an agarose gel containing formaldehyde followed by transfer to a PhotoGene™ nylon membrane. The GAPDH probe (S.A. $4.4 \times 10^9$ cpm/µg) was generated by PCR using 3 µM each of the unlabeled nucleotides. Hybridization was performed ($2 \times 10^6$ cpm/ml solution) after a 4-h prehybridization step. GAPDH mRNA on the blot was visualized by autoradiography. Lanes 1–4. 1,250, 2,500, 625, and 313 ng, respectively, of total RNA. Lanes 5–12. 1,250, 625, 313, 156, 78, 39, 20, and 9.7 ng, respectively, of poly(A)$^+$ RNA.

Random primer-labeling generates high specific activity probes (10). However, random primer-labeling requires template amounts that are at least 250-fold greater (>25 ng) than that required by the amplification method (0.1 ng). Probe specific activity must be corrected for the presence of unlabeled starting template, since it will compete for analyte-binding. Therefore, the specific activity of probe generated by amplification is ≥3-fold higher than that synthesized using random primer-labeling (table 3).

The GAPDH probe was used to detect GAPDH mRNA in both total and poly(A)$^+$ preparations of HeLa cell RNA. The probe was able to detect levels of GAPDH mRNA (~2.0 kb) in 313 ng of total HeLa cell RNA, and in 9.7 ng of poly(A)$^+$ RNA (figure 2).

The PCR Radioactive Labeling System has several advantages over random primer-labeling and nick translation. Subnanogram quantities (0.1 to 1 ng) of supercoiled DNA are used to generate high specific activity probes (>$4 \times 10^9$ cpm/µg). Only insert and a small amount of multiple cloning site sequence in pUC-derived plasmids are amplified. To obtain this sequence specificity with random primer-labeling, the insert is cleaved from the vector with a restriction endonuclease and then gel purified. The high specific activity and ease of use make the PCR Radioactive Labeling System a convenient alternative to other labeling methods.

## Acknowledgement

We thank Domenica Simms for the HeLa cell total and poly(A)$^+$ RNA preparations.

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**Does DMSO inhibit *Taq* DNA polymerase?**

Yes. *Taq* DNA polymerase is inhibited 47% in 10% DMSO. 10% DMSO has been used for PCR of GC-rich DNA in the presence of twice the amount of *Taq* DNA polymerase (see *BioTechniques* 15:3, 372).

# PCR Nonradioactive Labeling System for Synthesis of Biotinylated DNA Probes

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## Abstract

**We have developed optimal conditions for generating biotinylated amplification products to be used as DNA probes. Probes are generated from as little as 0.1 ng of template. The optimal concentration of biotinylated nucleotide for probe synthesis varied depending on the size of the template. Chemiluminescent detection of a single-copy gene in a Southern blot from as little as 1.25 µg of genomic DNA is demonstrated with these biotinylated probes.**

Many nonradioactive detection systems use biotinylated probes synthesized by nick translation and random primer-labeling. Besides DNA polymerase I (1) and the Klenow fragment (2), *Taq* DNA polymerase can incorporate biotinylated nucleotides (3,4). In this report, incorporation of biotin-14-dCTP into amplification products by *Taq* DNA polymerase and the chemiluminescent detection of these probes after hybridization to target DNA on membranes is investigated.

## Description of the System

The PCR Nonradioactive Labeling System (Cat. No. 10200, table 1) synthesizes biotinylated probes from any insert contained within the multiple cloning site of pUC and pUC-derived vectors (see page 45 for a list). The primers are directed to plasmid sequences, allowing the same PCR conditions to be used for a variety of inserts. The control DNA is a 531-bp human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) insert (nucleotide positions 43–574) (5) in pAMP1 (6,7). Since the primer-binding sites flank the polylinker region, the size of the GAPDH probe will be 800 bp. GAPDH is highly conserved, allowing the probe to recognize GAPDH coding sequences from human and other mammals, and it can serve as a useful control probe on blots.

## Methods

The oligonucleotide primers and plasmid constructs (pGAPDH, pCMVBXB, pRAF, and pβ-globin) were generated as described in the previous article (page 45). PCR reactions (50 µl) contained supercoiled plasmid template (0.1 to 1 ng), 250 nM each primer, 200 µM each dNTP including the sum of dCTP and biotin-14-dCTP concentrations, 2.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase in PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl]. After an initial 5-min denaturation at 95°C, 30 cycles of 94°C for 30 s, 60°C for 75 s, and 72°C for 90 s were performed. For amplification of the 2.6-kb pCMVBXB and 4.4-kb pβ-globin templates, elongation times were increased to 2 min 30 s and 5 min, respectively.

Following purification, the probes were hybridized to target DNA immobilized on PhotoGene™ nylon membranes after 4-h prehybridization. Probes were detected using the Gibco BRL PhotoGene Nucleic Acid Detection System.

**TABLE 1. Components of the PCR Nonradioactive Labeling System.**

| Component | Amount |
|---|---|
| 10X PCR reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] | 500 µl |
| $MgCl_2$ (50 mM) | 200 µl |
| dATP (10 mM) | 50 µl |
| dCTP (1 mM) | 200 µl |
| dGTP (10 mM) | 50 µl |
| dTTP (10 mM) | 50 µl |
| biotin-14-dCTP (1 mM) | 375 µl |
| LacFWD primer (5 µM) | 125 µl |
| LacREV primer (5 µM) | 125 µl |
| *Taq* DNA polymerase (5 U/µl) | 25 µl |
| sterile water | 1.25 ml |
| control GAPDH template (1 ng/µl) | 50 µl |
| silicone oil | 4 ml |

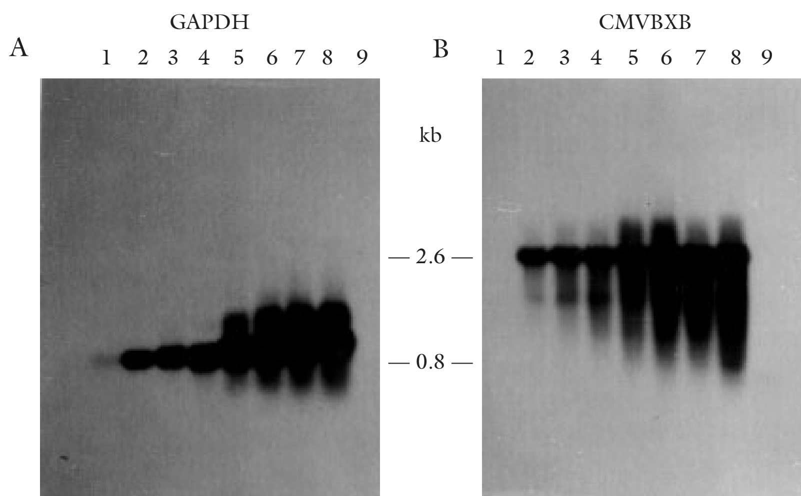


**FIGURE 1. Detection of biotinylated amplification products**. Amplification of the pGAPDH (Panel A) and pCMVBXB (Panel B) inserts was performed with various proportions of biotin-14-dCTP followed by gel transfer to PhotoGene™ nylon membrane and detection with the PhotoGene System. The blot was exposed to film (XRP; Kodak). The proportions of biotin-14-dCTP used were 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100% (lanes 1–9, respectively).

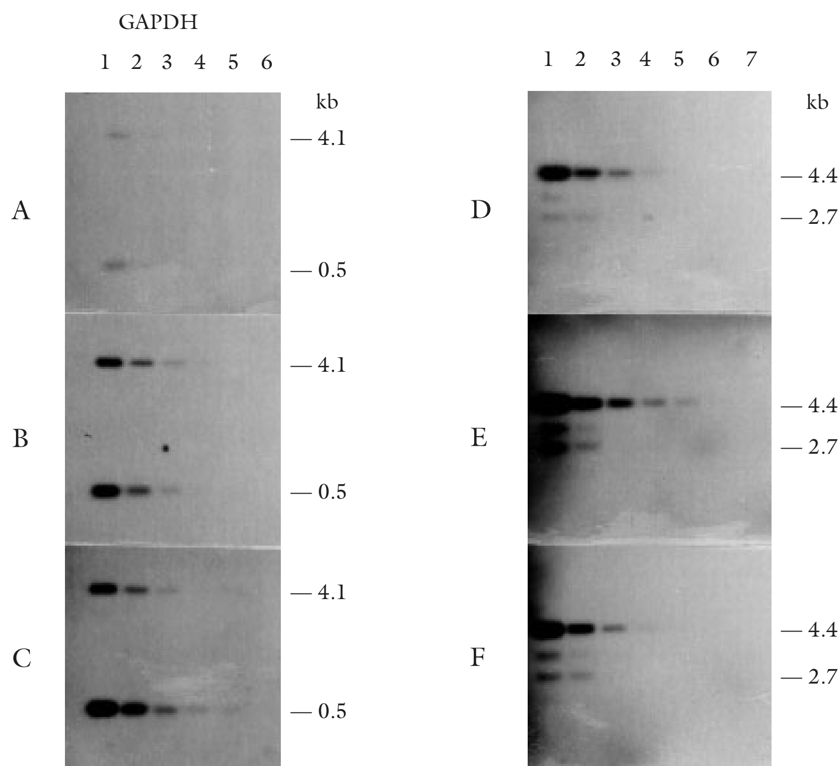


**FIGURE 2. Analysis of pGAPDH and pβ-globin biotinylated amplification products as probes**. pGAPDH probes were generated from reactions where biotin-14-dCTP constituted 25, 50, and 75% of the total dCTP pool (A–C, respectively). For the β-globin insert, biotin-14-dCTP comprised 50, 62.5, and 75% of the added dCTP (D–F, respectively). The probes were purified, hybridized to pGAPDH or pβ-globin plasmid DNA digested with restriction endonucleases, and detected with the PhotoGene System. The amounts of plasmid loaded were 100, 30, 10, 3, 1, 0.3, and 0.1 pg (lanes 1–7, respectively). The vector DNA is 4.1 kb for GAPDH and 2.7 kb for pβ-globin.

## Results and Discussion

Amplification of pGAPDH (800 bp) and pCMVBXB (2.6 kb) was performed with varying concentrations of biotin-14-dCTP. The biotinylated probe was examined after electrophoresis and transfer to a membrane (figure 1). The chemiluminescent signal increased with increasing concentration of biotin-14-dCTP (12.5% to 87.5% of the total dCTP pool). Absence of product with 100% biotin-14-dCTP indicated increased difficulty with incorporation of the modified nucleotide by *Taq* DNA polymerase and/or ability of the enzyme to synthesize DNA on biotinylated parent strands. Decreased electrophoretic mobility of the major band was seen as the proportion of biotin-14-dCTP in the reaction increased, indicating incorporation of the larger biotinylated nucleotide. Each addition of biotin-14-dCMP is equivalent to the addition of 1¾ nucleotides. Measurement of this shift with respect to nonbiotinylated controls and molecular weight standards indicated that when biotin-14-dCTP constituted 50% and 75% of the dCTP pool, products were obtained with ~30% and 60%, respectively, of incorporated biotin-14-dCMP in place of dCMP.

As the proportion of biotin-14-dCTP increased, some smaller amplification products were observed on the blot. However, they were not detected on the ethidium bromide-stained agarose gel (data not shown). These smaller products may result from the inefficiency of *Taq* DNA polymerase to incorporate modified dNTPs, resulting in chain-terminated material. Increasing the concentration of enzyme or doubling the elongation times during thermocycling did not alter this result (data not shown). Nevertheless, these small products represent useful probe since they were generated from the target sequence.

The ability of the biotinylated amplification products to function as probes on Southern blots was tested. The target plasmid DNAs were digested with restriction endonucleases and transferred to membranes following agarose gel electrophoresis. Several bands were seen (figure 2). The band of strongest intensity represented the insert (0.5 kb for GAPDH and 4.4 kb for β-globin), which has complete homology with the biotinylated probe. The weaker band represented the linearized vector,

**TABLE 2. Yields of amplification product with different sizes of plasmid inserts and amounts of starting template.**

| Amount of Template (ng) | Plasmid Insert Size (kb) ≤ 1.4 | 2.6 | 4.4 |
|---|---|---|---|
| 0.1 | 1 µg | 0.89 µg | 0.18 µg |
| 1 | 1.3 µg | 1.2 µg | 0.85 µg |

Supercoiled plasmids were used as templates. Biotin-14-dCTP concentrations were 75% for the 1.4- and 2.6-kb products, and 62.5% for the 4.4-kb product of the total dCTP pool. Quantitation was performed by including a trace label of [$\alpha^{32}$P]dATP in the reaction.

which has limited homology because short vector sequences between the insert and *LacZα* primer-binding sites were copied in the probe. The third band in the β-globin experiment was undigested plasmid. The GAPDH probe, synthesized from the reaction where biotin-14-dCTP consisted of 75% of the dCTP pool, gave the best signal. Increasing the biotin-14-dCTP proportion from 75% to 87.5% led to the synthesis of less product and did not increase the chemiluminescent signal (data not shown). We also made similar conclusions regarding the 2.6-kb pCMVBXB probes (data not shown). However, the most sensitive β-globin probe was obtained from reactions where the proportion of biotin-14-dCTP was 62.5% of the total dCTP pool. Product yields obtained with various insert sizes were determined (table 2). For PCR fragments >2.6 kb, it may be useful to use 1 ng of starting template.

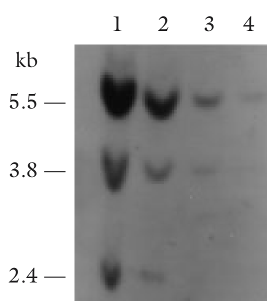


**FIGURE 3. Detection of β-globin gene sequences in genomic DNA using a biotinylated probe**. Human genomic DNA isolated from K-562 cells was *Eco*R I-digested, electrophoresed on an agarose gel, and transferred to PhotoGene nylon membrane. DNA amounts were 10, 5, 2.5, and 1.25 µg (lanes 1–4, respectively). Bound probe was detected using the PhotoGene System.

The β-globin probe synthesized with 62.5% biotin-14-dCTP in the reaction was used to detect a single-copy gene on a genomic Southern blot (figure 3). Hybridization of biotinylated probe resulted in detection of 5.5-kb and 3.8-kb *Eco*R I fragments of the β-globin gene. The 2.4-kb band resulted from cross-hybridization with the β-globin gene.

We described the development of a method for synthesis of biotinylated probes using PCR. This has advantages over probe synthesis by random primer-labeling and nick translation. Subnanogram quantities (0.1 to 1 ng) of template are used to generate probe. Other methods require ≥25 ng of starting template. Also, target sequences contained within pUC and pUC-derived plasmids can be amplified in the presence of biotin-14-dCTP, with only a small amount of vector sequence from the multiple cloning segment in the probe. To obtain comparable insert-specific probes with random primer-labeling, a time-consuming procedure involving restriction endonuclease digestion and DNA purification is often performed. The PCR Nonradioactive Labeling System is a one-step procedure that obviates these purification steps. Biotinylated probes generated by this method are detected by chemiluminescent detection.

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# DNA Mass Ladder™: Estimation of PCR Products in Gels

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Researchers have estimated amounts of DNA by the appearance of bands (thickness and intensity) in agarose or acrylamide gels. The accuracy and convenience of this process have been improved with the GIBCO BRL DNA MASS LADDER (Cat. No. 10068). This Ladder contains an equimolar mixture of six DNA fragments ranging from 100 to 2,000 bp. The mass of the bands covers a 20-fold range (table 1). The sizes are convenient for the quantitation of PCR products.

Several parameters related to agarose gel electrophoresis affect estimation of DNA quantities. For example, the timing of ethidium bromide staining of the DNA (figure 1) is important. The gel stained after electrophoresis shows more uniform band intensity and thickness than the gel where the DNA was electrophoresed in the presence of ethidium bromide. This uniformity is desirable for determining size of DNA fragments, but it is counterproductive to the estimation of DNA quantity. We suggest agarose gel electrophoresis be done with ethidium bromide in both the gel and the electrophoresis buffer for estimation of DNA mass.

Sample loading volume also affects band appearance (figure 2). The difference in band thickness was greatest where the initial loading volume was the smallest. This result is reasonable, since the local concentration of DNA entering the gel surface is highest in this sample. Since maximal differences in band thickness allow maximum accuracy in comparing sample bands and the bands of the DNA MASS LADDER, it is desirable that the loading volume be minimized (*i.e.*, concentration of the DNA in the loading buffer be maximized) for DNA quantitation. It follows that both the sample and the DNA MASS LADDER should be loaded in the same volume.

Several notes and observations to optimize the use of the DNA MASS LADDER follow:

- Accurate pipetting is critical to accurate estimation of DNA mass. Manufacturers of pipetting instruments suggest that for maximum accuracy, more than the volume to be delivered be drawn up into the pipet tip. For example, to deliver 4 µl, push the plunger of the pipette past the first stop, draw the liquid into the tip, then push to the first stop to dispense, leaving the excess liquid in the tip. The advantage is that the correct volume leaves the tip without the necessity of expelling absolutely all the liquid. Thus liquid left on the inside walls of the tip does not result in less delivered DNA. Also, the DNA should be at room temperature for maximum pipetting accuracy.

**TABLE 1. Amount of DNA in each band.**

| Fragment size (bp) | Volume of DNA Mass Ladder 4 µl | 8 µl |
|---|---|---|
| 2,000 | 200 ng | 400 ng |
| 1,200 | 120 ng | 240 ng |
| 800 | 80 ng | 160 ng |
| 400 | 40 ng | 80 ng |
| 200 | 20 ng | 40 ng |
| 100 | 10 ng | 20 ng |

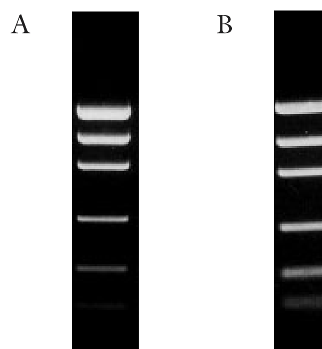


**FIGURE 1. The effect of ethidium bromide staining on the appearance of DNA MASS LADDER.** Electrophoresis of 4 µl (470 ng) of the DNA MASS LADDER on 2% agarose gels in Tris-acetate buffer under identical conditions (102 V, HORIZON® 58 apparatus, 30 min; the gels were poured from the same flask of agarose and electrophoresed simultaneously on the same power supply). One gel contained 1 µg/ml ethidium bromide in both buffer and gel and was photographed immediately (panel A). The other gel was stained with 1 µg/ml ethidium bromide in water for 12 min and then destained 10 min in water following electrophoresis (panel B).

- The closer in size the sample band and the DNA MASS LADDER band with which it is being compared, the more accurate the mass estimation will be.
- The six bands of the DNA MASS LADDER are present in equimolar amounts. Therefore, quantitation after end labeling is not appropriate.
- The DNA MASS LADDER has also been used with agarose and polyacrylamide gels in TBE buffer.
- We have analyzed the DNA MASS LADDER (2% agarose) with an image analysis instrument and associated software. The signal of the upper DNA bands increased less than linearly with increased mass, while quantitation of the smallest bands depended greatly on the mode of background subtraction and boundary definition.

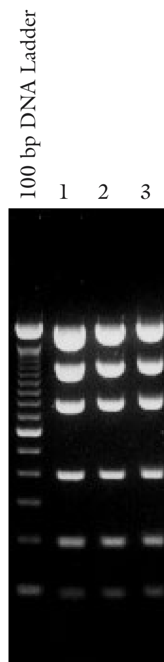


**FIGURE 2. The effect of loading volume on the appearance of the DNA MASS LADDER.** The 2% agarose gel in Tris-acetate buffer was formed with the HORIZON 58 14-tooth, 1.5-mm-thick comb. Identical masses (470 ng) of DNA MASS LADDER were applied, in the same loading buffer, but in final volumes of 5, 10, or 15 µl (lanes 1, 2, and 3). Both the gel and the buffer contained 1 µg/ml ethidium bromide.

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# PURIFICATION OF BIOTINYLATED DNA: A COMPARISON OF TWO CHROMATOGRAPHY BUFFERS

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Column chromatography with SEPHADEX® G-50 is a common method for isolation of DNA probes from unincorporated nucleotides. We investigated the effectiveness of this method for purification of biotinylated DNA. Interaction of the biotinylated DNA with plastic and/or column resin surfaces might pose a problem during purification. Initially, the recovery of purified biotinylated DNA from column chromatography with TE was 40% to 50% of the amounts applied to the column. The yields were improved with 1X SSC buffer containing 0.1% SDS. This buffer also increased resolution of biotinylated probe from unincorporated nucleotides.

A column was constructed using a 6-inch Pasteur pipet that was plugged with siliconized glass wool and filled to a 5-cm column height with SEPHADEX G-50. The probe preparation was divided and one-half (containing 195,000 cpm of biotinylated probe) was fractionated with the column using TE buffer. The column was cleaned with TE; equilibrated with 1X SSC, 0.1% SDS buffer; and then the other sample half was fractionated with 1X SSC, 0.1% SDS.

Elution profiles contained 2 peaks (figure 1). The first peak coeluted with dextran blue and contained the biotinylated 4.4-kb probe that was TCA-precipitated, whereas the second peak consisted of free nucleotide. The elution profile with 1X SSC, 0.1% SDS was more optimal for probe separation from free nucleotide as compared to TE buffer. Further, more probe was obtained in fractions 4–9 of the 1X SSC, 0.1% SDS purification than in fractions 5–7 of the TE purification. For example, 91,800 cpm (~47%) and 146,870 cpm (~75%) of probe were recovered from the column using TE and 1X SSC, 0.1% SDS buffers, respectively.

In summary, the use of 1X SSC, 0.1% SDS column buffer is an improvement over TE. SDS may help to limit interaction of the biotinylated DNA with resin, resulting in an increased biotinylated probe recovery.

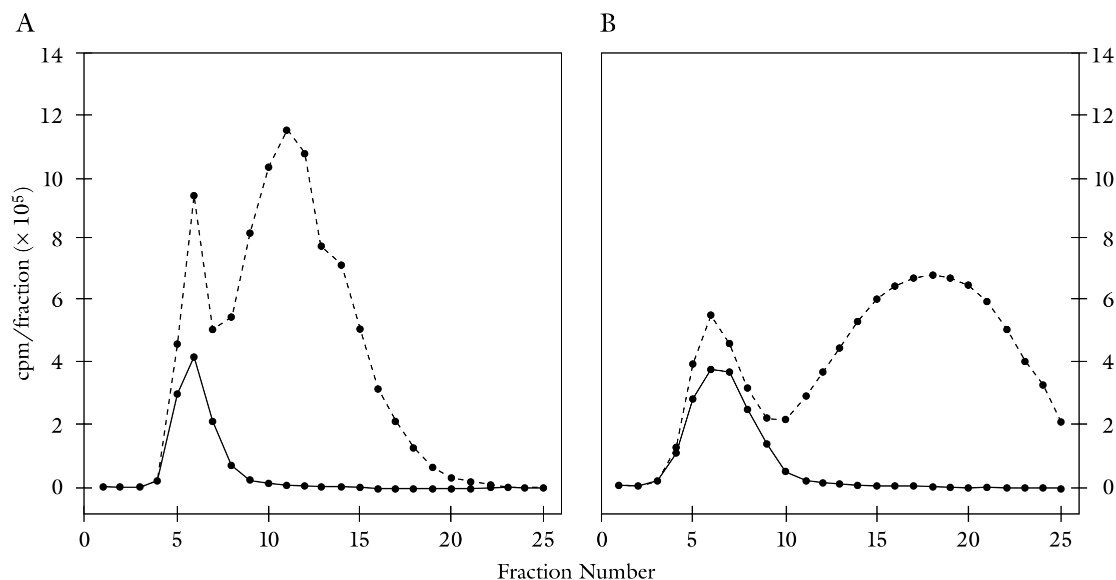


**FIGURE 1. Elution profiles of biotinylated DNA**. The biotinylated 4.4-kb β-globin probe was synthesized using the GIBCO BRL PCR Nonradioactive Labeling System. A trace label of [$\alpha^{32}$P]dATP was added to easily monitor the column elution profile. The probe preparation contained free biotinylated nucleotide and unincorporated [$\alpha^{32}$P]dATP before column chromatography. 5-drop fractions were collected and analyzed for radioactivity (●---------●), followed by TCA precipitation to determine probe elution and yield (●———●). Chromatography buffers were TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] (panel A) or 1X SSC [0.015 M sodium citrate (pH 7.0), 0.15 M sodium chloride], 0.1% SDS (panel B).

# Efficient PCR Amplification: Application to Limited Template and Large DNA Fragments

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While amplification of small DNA fragments (<2 kb) from abundant template (200 ng) by polymerase chain reaction (PCR) is a routine procedure, amplification of larger DNA fragments and amplification from limited amounts of template have been more challenging. Factors affecting efficiency of PCR amplification for large DNA fragments remain ill-defined. Of the parameters affecting amplification, much attention has been focused on the length of time allowed for primer extension. While this is an important factor in amplification, it is not enough to ensure efficient amplification of large DNA fragments. In this paper, we report amplification of DNA fragments ranging from 0.8 to 10.5 kb in length using PCR.

## Methods

*Amplification of brain-derived neurotrophic factor (BDNF) gene.* All components used for the PCR amplifications were from GIBCO BRL PCR Reagent System (Cat. No. 10198). 1 to 200 ng of GIBCO BRL TYPING GRADE™ K562 DNA was used as template. Each 100-µl reaction contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM sense (AUG GAG AUC UCU GGA TCC ATG ACC ATC CTT TTC CTT) and antisense (ACG CGU ACU AGU GGA TCC CTA TCT TCC CCT TTT AAT) primers, and 2.5 U *Taq* DNA polymerase. The reactions were overlaid with 2 drops of silicone oil and cycled in a Perkin-Elmer DNA Thermal Cycler. An initial denaturation of the template at 94°C for 3 min was followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 90 s. A final incubation was performed at 72°C for 10 min. Amplification products were analyzed on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide, followed by fluorescence detection using short-wave UV light. The GIBCO BRL DNA Mass Ladder (Cat. No. 10068) was used for quantitation by comparison of band intensities.

*Amplification of large DNA fragments.* Several fragments cloned into the multiple cloning site of pDELTA 1 (2) were amplified, in a reaction volume of 25 µl each. The amount of template used was 4 to 8 ng. The conditions were not optimized for individual fragment size. The reactions were performed in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 200 µM of each dNTP; 0.4 µM of each of the GIBCO BRL M13/pUC Forward and Reverse Amplification Primers (Cat. No. 18430), and 5 U of *Taq* DNA polymerase. An initial denaturation of the templates at 95°C for 5 min was followed by 20 cycles of 95°C for 45 s, 55°C for 30 s, and 72°C for 3 min. A final extension at 72°C for 10 min was included. The amplified products were visualized on 1.2% agarose gels.

## Results and Discussion

The single-copy BDNF gene was amplified from genomic DNA purified from K562 cells.

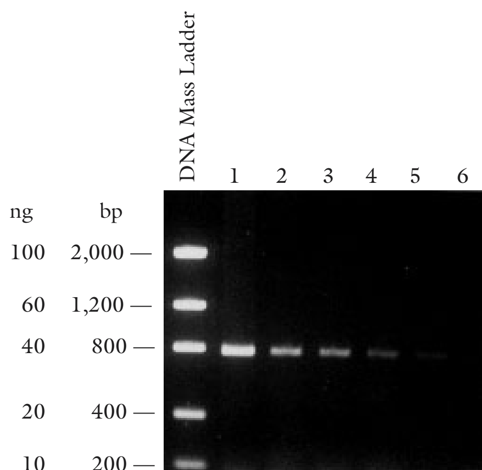


**FIGURE 1. Amplification of BDNF sequences from total human genomic DNA.** The entire BDNF gene, representing a 764-bp fragment, was amplified according to *Methods.* Template amounts were 200, 20, 10, 5, 1, and 0 ng in lanes 1–6, respectively.

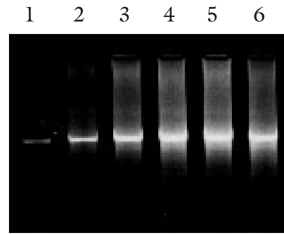


**FIGURE 2. Effect of enzyme concentration on amplification**. *Taq* DNA polymerase used was 1.25, 2.5, 5, 10, 15, and 20 U in lanes 1–6, respectively for amplification of a 10.5-kb fragment.

The expected amplification product is 764 bp and contains the complete coding sequence for this gene (1). The BDNF gene was easily amplified from as little as 1 ng of genomic DNA (figure 1). This amount of template represents ~300 copies of the BDNF coding sequence. The quantitation of the amplified product showed $4 \times 10^8$-fold amplification of the original template.

While there are no standard conditions for PCR, use of 2.5 U *Taq* DNA polymerase in a 100-µl reaction has been applicable for most purposes, as demonstrated above for amplification from 300 copies of BDNF template. Despite this efficiency, amplification of DNA fragments longer than 3 to 4 kb has been difficult and unreliable. There are reports of successful amplification of large DNA fragments after altering some of the reaction conditions (3). The limit in the length of amplification

**FIGURE 3. Amplification of large DNA fragments by PCR**. Clones containing various inserts were amplified using PCR primers that are complementary to the *LacZ* region of the vector (pDELTA1) and flanking the multiple cloning site. DNA template used was 4, 5, and 8 ng in lanes 1–3, respectively. The sizes of the products are 6.4, 8.0, and 10.5 kb in lanes 1–3, respectively.

fragment is not improved by simply increasing the extension time. Therefore, we investigated altering other parameters including enzyme concentration, reaction volume, template concentration, and number of cycles, for amplification of larger DNA fragments.

A number of plasmids with inserts ranging from 3 to 16 kb were used as amplification templates, allowing the use of the same set of primers. To study the effect of the amount of *Taq* DNA polymerase, the reactions were performed in 25 µl using the plasmid containing a 10.5-kb insert (figure 2). Efficient amplification of the insert was seen using >5 U of *Taq* DNA polymerase. Although product was observed with <5 U, the optimal level was between 5 and 10 U of enzyme.

The effect of volume on amplification efficiency was investigated with the 10.5-kb insert. Volumes between 25 and 100 µl resulted in similar amounts of amplification product as long as the concentration of DNA template (8 ng in 25 µl, 0.32 ng/µl) remained the same (data not shown). With 8 ng of template, no product was seen when the volume was increased to 50 µl (data not shown). Note that the template used in these experiments was 18.5 kb and the amount of DNA may need to be adjusted for other templates based on their size.

We investigated the number of cycles and their effect on product yield. An increase from 20 to 35 cycles increased the yield as expected (data not shown). However, it also resulted in the appearance of smaller nonspecific amplification products. Our attempts to improve the specificity by the "hot start" method and by increasing the annealing temperature were not successful.

Using the optimized protocol of 5 U of enzyme in 25-µl volume with 20 cycles, amplification of a range of template sizes was studied. Plasmids with inserts ranging from 6 to 10.5 kb were efficiently amplified (figure 3). These fragments contained different sequences. A plasmid with a 16-kb insert did not amplify. We have not tested any DNA fragments between 11 and 16 kb to investigate where the size limit for amplification lies under these reaction conditions.

Previous reports characterizing *Taq* DNA polymerase have reported the native enzyme to

be more processive than other truncated derivatives (4). Enzymes with higher processivity would possibly be more suitable for primer extension of large templates. In this study, a higher concentration of native *Taq* DNA polymerase was used to amplify large fragments, which is consistent with this notion. In our previous attempts to amplify large DNA fragments using a standard concentration of *Taq* DNA polymerase, the sequence of the DNA affected the amplification. Two fragments of the same size with different sequences did not amplify to the same extent (unpublished data). With the use of the higher amount of enzyme and several plasmids, we did not observe any obvious sequence dependence. While this needs further investigation, we suspect that higher concentration of enzyme may facilitate extension of nascent DNA in difficult-to-read spots.

In summary, we have presented protocols for amplification of small amounts of DNA template and large DNA fragments by PCR. Enzyme concentration was found to be critical for amplification of large DNA fragments.


## Acknowledgements

We wish to thank Alice Young for providing the plasmids used in this study.


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**What are the recommended conditions for a standard PCR reaction?**

There are several parameters that need to be adjusted for optimal PCR, such as $MgCl_2$, dNTP, primers, and template concentrations, as well as annealing temperature. The following conditions are a starting point. In a 100-µl reaction volume add:

template DNA, $10^5$ to $10^6$ target molecules (~1 ug human genomic DNA, 10 ng yeast DNA, 1 ng *E. coli* DNA, or 1% of an M13 plaque),

0.1 to 1 µM each primer,

50 to 200 µM each dNTP,

1 to 4 U of *Taq* DNA polymerase

20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25–50 mM KCl.

Overlay with 75 µl of silicone oil.

Perform 25–35 cycles, 94°C for 30–60 s, 55°C for 30–60 s, 72°C for 30–90 s.

**Should I use BSA in my PCR?**

Some sources of BSA have been shown to inhibit PCR. This phenomenon can be due to denaturation of BSA at high temperatures or to the protein purification and deacetylation. Reports in the literature about the use of BSA in PCR have been contradictory. Gelatin (100 µg/ml) is recommended as a stabilizing agent over BSA.

**How do I design my PCR primers?**

PCR primers are typically between 18 and 25 nucleotides having 40% to 60% GC. Complementary sequences at the 3'end of the primer pairs must be avoided to prevent primer-dimer formation. Avoid more than 3 C's or G's at the 3'end. For highly degenerate primers, it is preferable that the most unambiguous sequence be at the 3'end. At least the last 3 bases adjacent to the 3'end need to be correctly base paired to initiate PCR.

**How can I remove the oil after PCR?**

There are several methods to remove the oil.

- It can be removed by extraction with chloroform/isoamyl alcohol.
- The reaction can be transferred to –20°C, where the aqueous layer freezes but the oil does not.
- The reaction can be transferred to parafilm, where the aqueous PCR product beads at the top of the oil layer and is easily pipetted to a new tube.

**What is "hot start" PCR?**

Hot start is a modification of PCR that decreases the tendency to get nonspecific PCR products. One of the reagents, usually *Taq* DNA polymerase, is left out of the initial denaturation. Then, the tube is held at 80°C and the enzyme is added.

**What is the recommended concentration of $MgCl_2$ for PCR?**

The $MgCl_2$ has to be optimized for each template and primer pair. The final concentration varies between 1 and 2.5 mM. The presence of EDTA can inhibit amplification by chelating the magnesium ions. Excess nucleotides also bind magnesium and may necessitate an increase in magnesium.

# RAPID CHARACTERIZATION OF pSV-SPORT1 cDNA LIBRARIES BY PCR

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After the construction and titering of cDNA libraries, it is essential to determine the percentage of clones containing inserts and those resulting from vector religation events or incomplete vector digestion during preparation. We have used the polymerase chain reaction (PCR) to examine plasmid libraries constructed with the eukaryotic expression vector pSV-SPORT1 for the number of clones containing inserts and the average cDNA size.

Human follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC) cDNA libraries were constructed in pSV-SPORT1 using the SUPERSCRIPT™ Plasmid System. The initial library complexities were $1.81 \times 10^6$ and $1.17 \times 10^6$ clones, respectively. Amplification in semi-solid media was performed in agarose (1). One-half of the amplified library was aliquoted and frozen in 2X LB broth containing 25% glycerol. The remaining half of the cells were washed in fresh LB broth and used for purification of plasmid DNA by alkaline lysis (2) and polyethylene glycol precipitation (3). Plasmid DNA (2 µg) from both libraries was digested with *Mlu* I and analyzed by agarose gel electrophoresis (figure 1). *Mlu* I sites are within the 3' primer-adapter and the 5' adapter used in construction of the SUPERSCRIPT cDNA libraries. A range of cDNA fragment sizes from <600 bp up to 4.4 kb was evident.

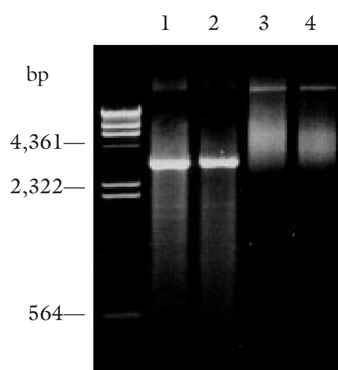


**FIGURE 1. Release of cDNA inserts by *Mlu* I.** Human cDNA libraries constructed from FTC (lanes 1 and 3) and PTC (lanes 2 and 4) were amplified in semi-solid agar, harvested, and total library plasmid DNA purified by the alkaline lysis procedure. Lanes 1 and 2 are plasmid DNAs digested with *Mlu* I. Lanes 3 and 4 are undigested plasmid DNAs. The pSV-SPORT1 vector is 3,160 bp.

Also, individual colonies were randomly selected, picked with a sterile pipet tip, and placed in 0.5-ml PCR-microcentrifuge tubes containing 20 µl of sterile water for analysis. The tubes were agitated continuously at room temperature for 30 min on a high-speed rotary vibrating platform to dislodge the bacteria off the pipet tips. The tips were removed, 3 drops of silicone oil were overlaid on the suspension, and the tubes were heated at 99°C for 15 min. The samples were quick chilled on ice and 100 µl of sterile water was added. After centrifugation for 5 min at $13{,}000 \times g$, the supernatants (25 µl) were added directly to 25 µl of a 2X PCR master mix containing 0.2 µM T7 primer, 0.2 µM SP6 primer, 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 400 µM dNTPs, and 0.1 U/µl GIBCO BRL *Taq* DNA Polymerase.

The reactions were mixed, overlaid with 3 drops of silicone oil, and denatured for 5 min at 95°C followed by 30 cycles of 95°C for 45 s, 45°C for 45 s, and 70°C for 2 min in a Perkin-Elmer DNA Thermal Cycler Model 480. A final incubation at 70°C for 5 min was performed prior to cooling at 4°C.

Analysis of 20 colonies of the PTC library is shown in figure 2. Amplification of the pSV-SPORT1 vector with the T7 and SP6 sequencing primers yielded a 150-bp product derived from the multiple cloning site. Of the 20 colonies tested, 18 gave PCR products >150 bp, indicating the presence of a cDNA insert. The sizes of inserts ranged from 450 bp to 3,800 bp. One clone gave rise to a product very light in intensity (clone 7), while most gener-

ated abundant PCR products. Two reactions (clones 5 and 17) did not yield any amplification product. Amplification of these clones with altered cycling parameters was effective at generating a 150-bp and 450-bp PCR product from clone 5 and clone 17, respectively (data not shown).

Furthermore, we have found that amplification is more consistent across multiple samples (data not shown) when using greater amounts of enzyme (5 U *Taq* DNA polymerase per 50 µl reaction) than that used for most routine PCR reactions (1 to 2.5 U/100 µl).

PCR offers a simple alternative for rapidly characterizing cDNA libraries for insert size and percentage. The analysis of the library reported here has shown an average insert size of 1,380 bp and a ratio of 95% insert-containing clones. The primers and conditions described have proven reliable for amplification of most inserts from cDNA libraries constructed in the pSV-SPORT1 plasmid vector.

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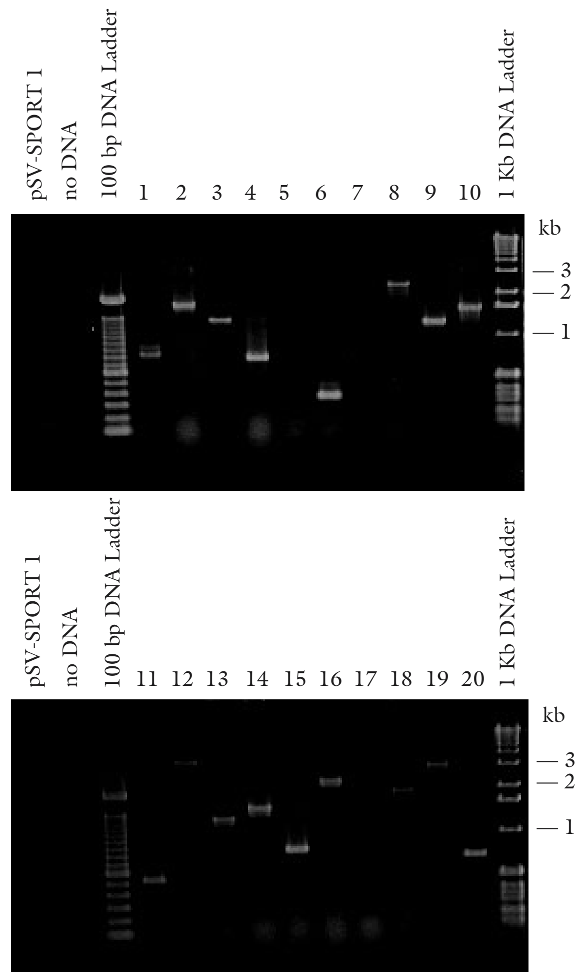


**FIGURE 2. Identification of cDNA inserts by PCR**. Twenty bacterial colonies from the PTC library were picked and analyzed as described in the text. After amplification, sample buffer was added to the reactions and one-sixth of the reaction was electrophoresed on 1% agarose gels in TBE buffer.

# PCR-SSCP Screening of M13 Plaques


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## Abstract

**A rapid, simple, and efficient procedure for screening large numbers of M13 plaques after mutagenesis procedures is described. The protocol used PCR-SSCP (single-stranded conformation polymorphism) to identify potential mutant DNAs, followed by cycle sequencing of double-stranded DNA to identify the specific base changes. Both analytical procedures, along with production of a phage stock, were performed with phage from individual plaques obtained in the initial transformation step.**


Site-directed mutagenesis with synthetic oligonucleotides has become the standard method of introducing specific point mutations (1). The same approach can be used for saturation mutagenesis of a small region using a degenerate oligonucleotide (2). Degenerate oligonucleotides are prepared so that on average there is one misin-

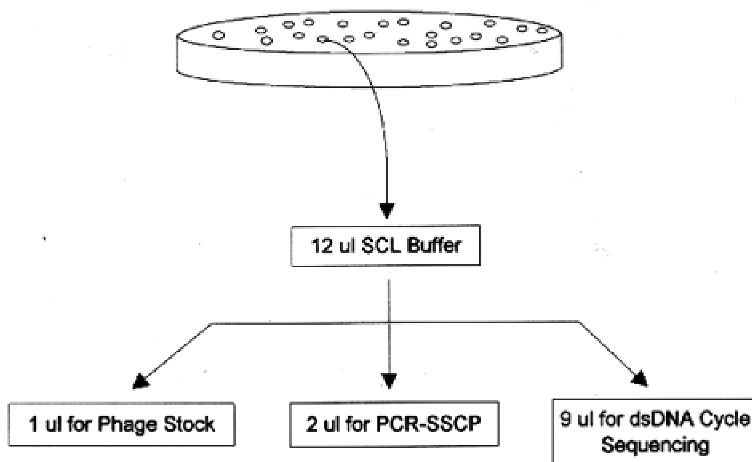


**FIGURE 1. Summary of the plaque screening procedure.** Single plaques were picked into SCL buffer for processing.

corporation per molecule, randomly distributed throughout the chain (2). Under these conditions, the Poisson distribution indicates that ~37% of the molecules will have no base changes, 37% will have a single base change, and the remaining 26% will have two or more changes. When a homogeneous mutagenic oligonucleotide is used for second strand synthesis, the mutation frequency can approach 80% in some systems (3). This makes direct screening by DNA sequencing feasible. However, with the heterogeneous population of the degenerate oligonucleotide, we found that the mutation frequency was more typically 20%. While even this lower frequency would be tolerable if only a single mutation was required, when trying to isolate a series of point mutations across every position in the primer, this low frequency necessitated too much sequencing of nonmutated clones. This report describes the use of PCR-SSCP to screen the initial plaques for potential mutants. Only the plaques identified as positive by PCR-SSCP need to be sequenced.

## Methods

*Mutagenesis.* The template for the mutagenesis reaction was M13BOR. This phage has the bovine papillomavirus (BPV) origin of replication (nucleotides 7892 to 52) cloned into M13mp18. Single-stranded M13BOR DNA was prepared by standard methods (4). The mutagenic oligonucleotide was a degenerate 30-mer with the wild-type sequence TGT GAT TAT TGT TAA CAA CAA TTA TTC ACT. To produce the degenerate 30-mer, 4% of each of the four individual phosphoramidite solutions used for synthesis was replaced with an equimolar mixture of all four phosphoramidites. Phosphoramidites doped at this concentration should yield a 30-mer with an average of 1 substitution per molecule (2). The degenerate 30-mer was phosphorylated with T4 polynucleotide kinase (4).

Mutagenesis was performed with the T7-GEN® *In Vitro* Mutagenesis Kit (United States Biochemicals). Phosphorylated degenerate 30-mer (0.2 pmole) was hybridized to 1.0 µg of single-stranded M13BOR DNA for 5 min at 70°C, followed by slow-cooling to room temperature over ~1 h. Subsequent reactions and transformation were performed as recommended by the manufacturer.

For analysis, individual plaques were isolated by stabbing through the agarose with a sterile Pasteur pipet. The top agar plug was lifted with a sterile toothpick and deposited in 12 µl of SCL [Single Colony Lysis buffer; 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 µg/ml GIBCO BRL Proteinase K]. Samples were vortexed 30 s, then centrifuged 5 to 10 s in a microcentrifuge at room temperature. 1 µl of SCL sample was removed into 200 µl of LB broth to serve as the phage stock. The remainder was incubated for 15 min at 55°C, followed by 15 min at 80°C. Samples were microcentrifuged for 30 s at room temperature, and the supernates were stored at 4°C for use in PCR-SSCP and DNA sequencing.

*PCR-SSCP.* 2 µl of the SCL sample were PCR amplified in a 10-µl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.25 mM each of the four dNTPs, 0.5 µM each of primer 1 (TGT GGA ATT GTG AGC GGA TAAC) and primer 2 (TTC CCA GTC ACG ACG TTG TAA A), 3.3 nM [$\alpha^{32}$P]dGTP, and 0.5 units of *Taq* DNA polymerase. Samples were overlaid with 1 drop of silicone oil and amplified for 30 cycles using conditions previously described (5). The primers corresponded to M13 sequences flanking the BPV insert, and the resultant amplified product was 256 bp. After amplification, 5 µl of formamide sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) were

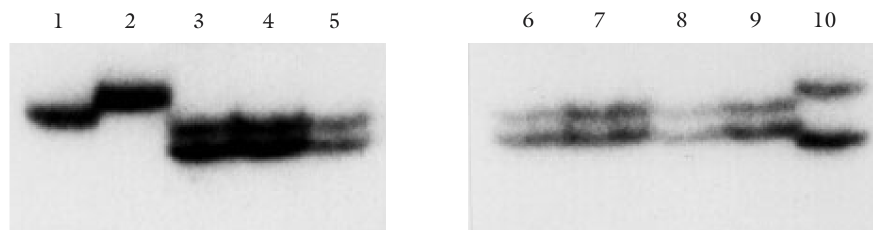


**FIGURE 2. PCR-SSCP analysis of plaque samples.** Shown are PCR-amplified and denatured DNAs from 10 plaques. DNA samples were electrophoresed on nondenaturing acrylamide gels. Samples 1-5 and 6-10 were from two separate amplification reactions. Single-stranded DNAs from samples 1, 2, and 10 have altered electrophoretic mobility compared to the wild type.

added to each sample. Samples were heated for 5 min in a boiling water bath, and 4 µl of each denatured sample were loaded directly onto 0.4 × 160-mm 6% TBE polyacrylamide gels (4) containing 5% glycerol. Sharkstooth combs were used for well formation during sample loading. Gels were submersed in 1X TBE buffer maintained at 20°C by connection to a circulating water bath. Samples were electrophoresed at 1,000 V until the xylene cyanol reached the bottom of the gel. Gels were dried and exposed with intensifier screens.

*Cycle sequencing*. DNA sequencing was performed directly on 9 µl of SCL sample using the GIBCO BRL dsDNA Cycle Sequencing System as described by the manufacturer. Sequencing reactions were analyzed on standard 6% acrylamide sequencing gels.

## Results and Discussion

Saturation mutagenesis of the BPV origin region was performed with a degenerate oligonucleotide. Initially, 20 plaques were picked at random and analyzed by DNA sequencing. Only 4 of the 20 (20%) had mutations, the remainder having the wild-type sequence. To avoid sequencing the predominant number of wild-type phage, a simplified screening and processing procedure was developed (figure 1). A phage suspension was prepared from each plaque from the initial transformation step and was used to prepare a phage stock, screen for possible mutations by PCR-SSCP, and directly sequence potential mutants. This eliminated the need for replaquing or for preparation of single-stranded DNA. Consequently, hundreds of plaques could be screened in several days.

A total of 169 additional plaques were screened for possible mutations by PCR-SSCP. The presence of even a single point mutation in the PCR-amplified region will cause aberrant migration of one or both of the single strands on a nondenaturing gel (6). Of 169 total plaques examined, 28 showed strand migration patterns different from the wild-type strands (figure 2). Sequencing of these 28 samples showed that 27 possessed point mutations in the region of the mutagenic 30-mer (figure 3). Of the 27 clones with mutations, 19 had single point mutations and the rest had double mutations. The 19 single point mutations

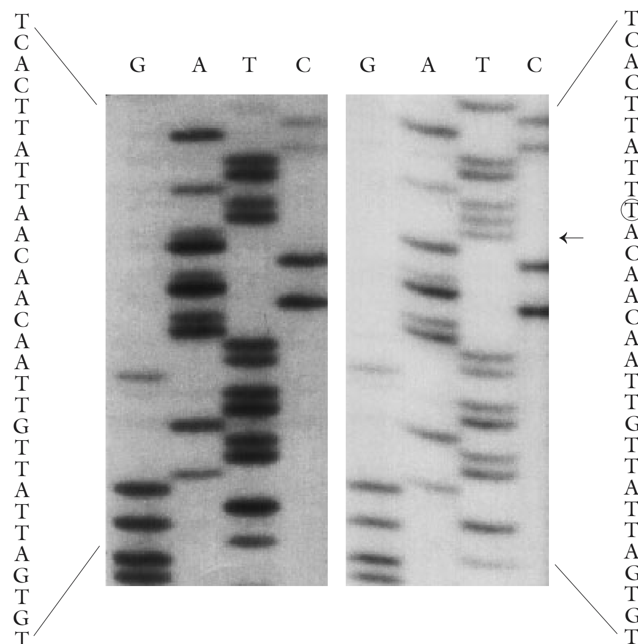


**FIGURE 3. Cycle sequencing analysis of plaque samples.** Plaque samples were subjected to DNA sequencing as described in *Methods*. Shown is a portion of the autoradiographs from two samples that contain sequence from the 30-mer region. The left panel shows a wild-type sequence and the right panel shows sequence from a sample that was identified by PCR-SSCP as containing a mutation. The suspected mutant was found to possess a single A to T base change in the 30-mer region (marked with an arrow on the autoradiograph and circled on the sequence).

occurred at 15 of the possible positions in the 30-mer sequence, with 4 of the 15 positions each being identified in two independent clones. The negative sample may have acquired a mutation elsewhere in the 256-bp amplified fragment as the sequence of the entire fragment was not determined.

SSCP identified mutations in 16% of the plaques examined (27/169). This frequency was similar to the 20% mutation rate determined by direct sequencing of 20 plaques, suggesting that the number of false negatives was low. To further explore the possibility of false negatives, 37 of the amplified DNAs that appeared wild-type by SSCP were digested with *Hpa* I. *Hpa* I has a 6-base recognition sequence that was entirely within the boundaries of the region spanned by the mutagenic 30-mer. Of the 37 DNAs digested, 2 failed to cut with *Hpa* I, and DNA sequencing confirmed that each had a mutation in the *Hpa* I recognition sequence.

The 5% (2/37) false negative rate for *Hpa* I site mutations was somewhat surprising as the *Hpa* I recognition site constitutes only 20% (6 of 30 bp) of the mutagenized region. This suggests that the overall rate of false negatives for the entire 30-bp region would be much higher. However, the concordance between the overall positive rate for SSCP (16%) and direct sequencing (20%) argues against an overall high rate of false negatives. A possible explanation for this discrepancy is that the *Hpa* I site is at the center of an 18-bp inverted repeat region. The extreme symmetry may facilitate formation of a stable stem-loop structure in this region of the 256-base fragment after denaturation of the DNA duplex, and it is likely that the conformation of the stem-loop would be only slightly affected by single point mutations. Consequently, mutations in this symmetry region may have little effect on the overall folding of the single-stranded 256-base fragments. Since SSCP depends on base changes causing an alteration in fragment mobility due to significant changes in the fragment folding pattern, mutations in regions of dyad symmetry may be difficult to detect. As a result, the 5% false negative rate at the *Hpa* I site may be an overestimate of the rate for the remainder of the sequence.

It has also been observed that detection of some mutations depends on electrophoretic conditions (6). Variation in temperature during electrophoresis, acrylamide concentration, ionic strength, and glycerol concentration have all been shown to alter single strand migration (7). These parameters have not been systematically varied to determine if other mutations could be detected.

In summary, we present a simple procedure for screening M13 plaques for the presence of mutations in cloned sequences. Single plaques are sufficient to prepare a phage stock, perform the SSCP analysis, and sequence potential mutants identified by SSCP. While our *Hpa* I results indicate that some mutations may be missed by this screening procedure, the ease of screening large numbers of plaques offsets the potential failure to detect all possible mutations. This protocol should be useful for screening the products of any mutagenesis procedure with a low mutation frequency and no phenotypic selection. It could easily be adapted to other phage and to bacterial colonies as well as M13 phage plaques.

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