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Protocol

Polymerase Chain Reaction (PCR) Amplification of GC-Rich Templates

Michael R. Green and Joseph Sambrook

The efficiency of polymerase chain reaction (PCR) amplification is influenced by the nucleotide composition and sequence of the template DNA. Problematic templates include those with long homopolymeric runs, inverted repeats, or GC-rich tracts—such as those containing >60% G + C residues—that are found in the regulatory regions of many mammalian genes. Localized regions of templates rich in GC residues tend to fold into complex secondary structures that might not melt during the annealing phase of the PCR cycle. Also, the primers used to amplify GC-rich regions often have a high capacity to form self- and cross-dimers and a strong tendency to fold into stem–loop structures that can impede the progress of the DNA polymerase along the template molecule. Predictably, amplification of full-length template DNA is inefficient, and the products of the reaction contain a high proportion of shorter molecules that result from blockage of the DNA polymerase. Altering the design of the primers and using a combination of hot start and touchdown PCR can sometimes improve the efficiency of amplification. More often, a multipronged approach is required, such as the use of enhancers in the amplification reaction, adjustment of the cycling protocol, and, if necessary, designing new sets of primers. This protocol uses a mixture of four additives—betaine, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA)—for use with *Taq* DNA polymerase.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Additive solution (5×) <R>

Bystander DNA

Bystander DNA does not contain target sequences. It should resemble the template DNA in all other respects: complexity, size, and concentration.

Chloroform (optional; see Step 6)

dNTP mix (all four dNTPs, 20 mM each; pH 8.0)

Ethidium bromide (optional; see Step 5)

GC-rich amplification buffer (10×) <R>

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The concentration of $MgCl_2$ in this buffer is 30 mM; however, the concentration of Mg^{2+} should be optimized in a series of pilot reactions containing different concentrations of Mg^{2+} (0.5–5.0 mM).

Gel, polyacrylamide or agarose (see Step 5)
Mineral oil (optional; see Step 3)
Paraffin wax (optional; see Step 3)
Primers:
- Forward primer (20 µM, in H_2O)
- Reverse primer (20 µM, in H_2O)

Use the following formula to calculate the molecular mass of the oligonucleotides:

$$M_r = (\mathrm{C} \times 289) + (\mathrm{A} \times 313) + (\mathrm{T} \times 304) + (\mathrm{G} \times 329),$$

where C *is the number of C residues in the oligonucleotide,* A *is the number of A residues,* T *is the number of T residues, and* G *is the number of G residues. The molecular mass of a 20-mer will be ~6000 Da; 100 pmol of the oligonucleotide will be equivalent to ~0.6 µg.*

SYBR Gold (optional; see Step 5)
Taq DNA polymerase (e.g., Agilent, Thermo Fisher, etc.)
Template DNA (100–500 ng/mL in 10 mM Tris-Cl at pH 7.6)

High concentrations of template DNA are reported to inhibit amplification of GC-rich sequences.

To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only.

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Equipment

Barrier tips (for automatic micropipetting device)
Gel electrophoresis equipment (for polyacrylamide or agarose gels)
Microcentrifuge tubes, 0.5 mL, thin-walled (optional; see Step 1)
Microtiter plates (optional; see Step 1)
Positive-displacement pipette
Thermal cycler

A number of programmable thermal cyclers marketed by different commercial companies (e.g., Mastercycler [Eppendorf], PTC-100 [MJ Research]) are licensed by PerkinElmer for use in PCR. The choice among these instruments depends on the investigator's inclination, the available budget, and the range of uses to which the machine will be put. Before purchasing a thermal cycler, we recommend soliciting as many opinions as possible to discover the advantages and disadvantages of different machines.

Bake all glassware for 6 h at 150°C, and autoclave all plasticware.

METHOD

1. In a sterile 0.5-mL microcentrifuge tube, amplification tube, or the well of a sterile microtiter plate, mix in the following order:

GC-rich amplification buffer (10×)	5 µL
dNTP mix (20 mM each, pH 8.0)	1 µL
Forward primer (20 µM)	2.5 µL
Reverse primer (20 µM)	2.5 µL
Thermostable DNA polymerase (1–5 units/µL)	1–2 units
Template DNA	5–10 µL
Additive solution (5×)	5 µL
H_2O	to a final volume of 50 µL

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2. Prepare positive and negative controls, as detailed below:

   Positive controls are required to monitor the efficiency of the PCR, whereas negative controls are required to detect contamination with DNAs that contain the target sequence.

	Bystander DNA	Template DNA[a]	Target DNA[b]	Specific primers[c]
Positive controls				
1	+	−	+	+
2	−	−	+	+
Negative controls				
3	−	−	−	+
4	+	−	−	+

[a]Template DNA is the DNA under test.

[b]Target DNA contains the target sequence. It can be a recombinant DNA clone, a purified DNA fragment, or a sample of genomic DNA. It should be added to the positive control at concentrations equivalent to those expected in the template DNA. It is often necessary to set up a series of positive controls containing different amounts of target DNA spanning the amount predicted in the template DNA. An appropriate dilution of the target sequence should be prepared ahead of time in an area of the laboratory different from that used for the preparation of other PCR reagents. This precaution reduces the risk of contaminating equipment and plasticware in the area of the laboratory set aside for PCRs.

[c]Specific primers are oligonucleotide primers specific for the segment of target DNA.

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3. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (~50 µL) of light mineral oil. Alternatively, if using a hot start protocol (see Protocol: **Hot Start Polymerase Chain Reaction (PCR)** [Green and Sambrook 2018]), place a bead of wax into the tube. Place the tubes or the microtiter plate in the thermal cycler.
4. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed below:

Cycle number	Denaturation	Annealing	Polymerization
1–30	30 sec at 94°C	30 sec at 55°C	1 min at 72°C
Last cycle	1 min at 94°C	30 sec at 55°C	1 min at 72°C

   Normally, polymerization is performed for 1 min for every 1000 bp of length of the target DNA. Times and temperatures might need to be adapted to suit the specific thermal cycler used and/or reaction volumes.

   Most thermal cyclers have an end routine in which the amplified samples are incubated at 4°C until they are removed from the machine. Samples can be left overnight at this temperature but should be stored thereafter at −20°C.

5. Analyze a sample (5–10 µL) from the reaction mixtures and each of the control reactions by electrophoresis through an agarose or polyacrylamide gel. Be sure to include DNA markers of an appropriate size. Stain the gel with ethidium bromide or SYBR Gold to visualize the DNA.
6. If mineral oil was used to overlay the reaction (Step 3), remove the oil from the sample by extraction with 150 µL of chloroform.

   The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.

   For many purposes, for example, purification of the amplified DNA using a Centricon microconcentrator or cloning amplification products, it is desirable to remove the oil from the sample before proceeding.

   Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.

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DISCUSSION

When designing primers for amplification of GC-rich sequences, use an oligonucleotide design program to check the Gibbs free-energy values (ΔG) for (1) the duplexes between the primers and their binding sites on the targets and (2) the secondary structures predicted for each oligonucleotide. Choose pairs of primers with the highest percent match score and lowest entropy (i.e., a minimum ΔG of about –4 kcal/mol) (Hubé et al. 2005). If these oligonucleotides are inefficient in hot start PCRs, use enhancers in the amplification reaction (see below). If all else fails, consider reducing the entropy of the oligonucleotides by introducing null mutations into the central regions of GC-rich oligonucleotides (see, e.g., Sahdev et al. 2007).

Many additives have been reported to enhance PCRs containing GC-rich templates and primers (see Table 1). Their effects are unpredictable, and using them—either singly or in combination—does not guarantee improvement in the efficiency of amplification of DNA substrates with substantial secondary or tertiary structure. The chances of success are increased if several enhancers are used simultaneously (see, e.g., Musso et al. 2006; Ralser et al. 2006; Zhang et al. 2009). Alternatively, kits containing combinations of additives are available from commercial manufacturers. Most of the kits are believed to contain betaine, in addition to other unspecified additives.

If the combination of touchdown PCR, hot start PCR, and cocktails of additives fails to improve the amplification of a recalcitrant GC-rich template, altering the cycling conditions can solve the problem. For example, Frey et al. (2008) describe the use of a combination of slow ramping rates (2.5°C/sec) and slow cooling rates (1.5°C/sec) to approach the annealing temperature. An essential component of "slowdown" PCR is the inclusion in the reaction mixture of 7-deaza-2′-deoxyguanosine.

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TABLE 1. Enhancers commonly used to improve the efficiency of amplification of GC-rich templates

Enhancer	Mode of action	Reference
Betaine (*N*,*N*,*N*-trimethylglycine) (0.5–1 M)	Reduces the formation of secondary structures by lowering the T_m of GC-rich regions	Rees et al. 1993; Henke et al. 1997
7-Deaza-2′-deoxyguanosine	Eliminates Hoogsteen bond formation but does not impair Watson–Crick base pairing.	McConlogue et al. 1988
DMSO and low-molecular-weight sulfones (1%–10%)	Bind to the major and minor grooves of template DNA and destabilize the double helix	Winship 1989; Pomp and Medrano 1991; Varadaraj and Skinner 1994; Chakrabarti and Schutt 2001
Formamide (1%–5%)	Interferes with hydrogen-bond formation between the two strands of DNA	Sarkar et al. 1990
Polyethylene glycol (5%–15%)	A crowding agent; can also destabilize regions of DNA with high T_m	
Ethylene glycol and 1,2-propanediol	Decrease melting temperature of DNAs by an unknown mechanism apparently different from that of betaine	Zhang et al. 2009
Glycerol (5%–20%), bovine serum albumin (0.1 mg/mL), or gelatin (0.1%–1.0%)	General enzyme-stabilizing agents	Giambernardi et al. 1998
Nonionic detergents (e.g., Triton X-100 [0.1%–0.5%]; Nonidet P-40 [0.1%–0.5%])	Displace traces of ionic detergents used in preparation of templates	Gelfand and White 1990

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RECIPES

Additive Solution (5×)

Reagent	Quantity (for 10 mL)	Final concentration
Betaine (5 M)	5.4 mL	2.7 M
Dithiothreitol (1 M)	67 μL	6.7 mM
Dimethyl sulfoxide	670 μL	6.7% (v/v)
Bovine serum albumin (2 mg/mL)	275 μL	55 μg/mL

Store at 4°C for no longer than a week.

GC-Rich Amplification Buffer (10×)

Reagent	Quantity (for 10 mL)	Final concentration
Ammonium sulfate (1 M)	1.66 mL	166 mM
$MgCl_2$ (1 M)	300 μL	30 mM
Tris-HCl (1 M, pH 8.5)	6.6 mL	0.66 M
Tween 20	10 μL	0.1% (v/v)

Store at 4°C.

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