Downloaded from genome.cshlp.org on April 26, 2023 - Published by Cold Spring Harbor Laboratory Press

Optimized PCR Using *Vent* Polymerase

Kemp B. Cease, Caroline A. Potcova, Cortland J. Lohff, and Mary E. Zeigler

Division of Hematology–Oncology, Department of Internal Medicine, University of Michigan Medical Center, and Veterans Administration Medical Center, Ann Arbor, Michigan 48109

PCR has become an essential tool of the molecular biologist, and substantial literature exists characterizing the performance of *Taq* polymerase in this reaction.[1–4] Use of a thermostable polymerase that, unlike *Taq*, possesses 3′ → 5′ exonuclease activity is desirable in situations where higher fidelity and/or blunt-end products are required.[5–8] *Vent* polymerase is one such enzyme, but information on optimal use of this enzyme in PCR is limited. Ling et al. identified conditions that optimize the fidelity of PCR using *Vent* polymerase.[5] We commonly need high-fidelity blunt PCR products for use in DNA constructions. Our initial experience with *Vent* polymerase convinced us that, within the previously defined conditions that optimize fidelity, careful optimization for PCR product quantity in addition to quality was essential. Therefore, we systematically examined a number of potentially important PCR conditions in a stepwise manner. This analysis led to a highly optimized procedure described below.

MATERIALS AND METHODS

Reactions were set up in 100-μl volumes in 0.5-ml polypropylene tubes (Eppendorf). Primers flanking the multiple cloning site of a pBluescript II KS-derived plasmid were used. These primers, termed S-Near and A-Near, have sequences as follows: S-Near, 5′-CAGGAAACAGCTATGACCATG-3′; A-Near, 5′-GTTTTCCCAGTCACGACGTTG-3′. The plasmid pLFx6/BMX2BS was used as a standard amplification template. Amplification on this template with these primers yields an 861-bp PCR product that includes 131 bp of pBluescript II KS, 56 bp of insert "leader" sequence, six direct repeats of an 81-bp sequence, and 70 bp of insert "trailer" sequence, followed by 118 bp from pBluescript II KS. The leader and trailer sequences were the result of reengineering the pBluescript II KS multiple cloning site at its unique *Sma*I site, and the repeats were derived from the amino-terminal region of HIV-1 (BH10) gp41.[9] The construction and utility of pLFx6/BMX2BS will be described in detail elsewhere (K.B. Cease, C.L. Lohff, S.H. Kim, and M.E. Zeigler, in prep.). The above segment was chosen for these studies as PCR had yielded both higher- and lower-molecular-weight spurious bands in addition to product of the predicted molecular weight. In addition, a variety of different primer pairs and templates were examined to corroborate findings with pLFx6/BMX2BS. Primers typically had a length of 22 bp and a predicted melting temperature (T_m) of 66°C based on G+C content. Study reagents included *Vent* polymerase (*Vent*$_R$ New England BioLabs), *Escherichia coli* gp32 (Pharmacia), single-strand binding protein (SSB) from *Staphylococcus aureus* (U.S. Biochemical), deoxyribonucleotides (dNTPs, Boehringer Mannheim), glycerol (Fisher), tartrazine (Sigma), dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), and 1-methyl-2-pyrrolidinone (NMP) (all from Aldrich). SOC and Luria–Bertani (LB) media were prepared according to standard procedures.[10]

Initial conditions for PCR were based on those recommended by the manufacturer for primer extension and were as follows: 10 mM KCl, 10 mM $(NH4)_2SO_4$, 20 mM Tris-HCl (pH 8.8 at 25°C), 2 mM $MgSO_4$, dNTPs at 200 μM each, 100 μg/ml of BSA, 0.1% Triton X-100, 25 ng of DNA template, primers at 0.2 μM, and 1 unit of *Vent* polymerase in a final volume of 100 μl. Reactions were performed in an MJ Research PTC-60 thermal cycler using programs discussed below. PCR products were electrophoresed on 1.6% agarose gels, stained with ethidium bromide, visualized by UV transillumination, and photographed using standard methods.[10] Analysis of the reaction products consisted of assessment of (1) signal (band intensity at the predicted molecular weight) and (2) signal-to-noise ratio (intensity of predicted band vs. intensity of any other bands present), as judged by four independent examiners. Optimal conditions were identified by varying individual parameters over a range of conditions as indicated in Table 1, with other conditions held constant. Parameters were reexamined as necessary to assure optimal performance with all other parameters optimized.

RESULTS AND DISCUSSION

Initial PCR optimization consisted of varying the annealing temperatures. As expected, a temperature 5°C below the lower of the predicted melting temperatures for the primers was generally found to be optimal. We frequently perform PCR using a bacterial colony or glycerol

3:298–300©1994 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/94 $5.00

Downloaded from genome.cshlp.org on April 26, 2023 - Published by Cold Spring Harbor Laboratory Press

TABLE 1 Results of PCR Optimization using *Vent* Polymerase

Variable	Conditions studied	Optimum	Units
Annealing temperature	$T_m - 25, -15, -10 -5, T_m$	$T_m - 5$	°C
Annealing time	30, 60, 120	30	sec
Cycle number	14, 16, 18, 20, 22, 24, 25, 30, 35, 40, 45, 50, 55, 60	24	cycles
Broth (SOC or LB)	0, 5, 10, 15, 20, 25, 30, 35	15–30	%
Template			
CsCI DNA (no medium)	0.05, 0.5, 5, 50, 500	≥5	ng
Template with medium	0.05, 0.5, 5, 50, 500	≥0.5	ng
CsCI DNA			
bacteria stock, boiled	5, 10	5	µl
Primer (each)	0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4	≥1.0	µM
dNTP	0.125, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5	≥0.5	mM
$MgSO_4$	2, 3, 4, 5, 6, 7	2–4	mM
Vent polymerase	0.25, 0.5, 1, 2, 4	≥1	units
DMSO	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	5	%
NMP	2, 4, 6, 8, 10	2	%
DMF	2, 4, 6, 8, 10	0	%
BSA	10	0	µg
SSB	0.5, 1.0, 1.5	0	µg/100 µl
gp32	0.5, 1.0, 1.5	0	µg/100 µl
Glycerol	5	0	%
Tartrazine	25, 50	0	µg/100 µl
Top oil	with vs. without	without	
Hot start	with vs. without	without	

TABLE 2 50/50 Protocol for Vent Polymerase PCR

Reagents

Template

colony pick, 5 µl culture, or 0.5 ng of DNA in 20 µl of broth (LB or SOC) per tube 20 µl

P Mix (primers)

Reagent	Stock conc. (µM)	Volume/ tube
Water	—	20
Primer S	20	5
Primer A	20	5
Total		30

R Mix (reagents)

Reagent	Stock conc.	Volume/ tube (µl)
Water	—	30.5
Buffer, *Vent*	10×	10
DMSO	100%	5
dNTP pool	25 mM each	2
BSA (optional)	10 mg/ml	1
$MgSO_4$	100 mM	1
Polymerase, *Vent*	2 U/µl	0.5
Total		50

Procedure

1. Add a colony pick or other template sample to an aliquot of LB in each reaction tube to obtain a total volume of 20 µl per tube. If doing colony picks, archive each colony by touching the toothpick to a new agar master plate for incubation and subsequent recovery of clones of interest.
2. Make the necessary amount of P mix and add 30 µl to each reaction tube to obtain 50 µl of complete TP mix per tube.
3. Boil tubes containing TP mix for 5 min.
4. Snap-chill each tube containing boiled TP mix by immediately placing on ice slush.
5. Add 50 µl of R mix to each tube.
6. Place tubes in the thermal cycler and begin cycling.
7. Determine the predicted melting temperature of the hybridizing segment of your primers by summation using 4°C for each G or C and 2°C for each A or T. The primer with the lower melting temperature will determine the annealing temperature. Run the following thermal cycling program: (1) Denature initial 95°C×3 min; (2) denature 95°C×1 min; (3) anneal T_m − 5°C×30 sec; (4) extend 72°C×1 min; (5) cycle 24× to step 2; (6) extend final 72°C×5 min; (7) hold 4°C indefinitely.

stock as the source of template.[11] Such experiments serendipitously revealed that often the PCR products were actually qualitatively and quantitatively superior to reactions using highly purified DNA template. To our surprise, we found this to be attributable to culture medium introduced with the bacteria. Either SOC or LB broth worked equally well in a range from 5% to 35% with a broad optimum between 15% and 30%. Inclusion of medium permitted use of 10-fold less purified template DNA. Theoretically, a component introduced with the broth could enhance the reaction directly or could act indirectly by reducing an inhibitory influence. We elected to use this empiric observation without further elucidation of its basis. Although inclusion of SOC would be expected to increase the magnesium concentrations to supraoptimal levels (e.g., 6 mM for a reaction containing 30% SOC), adverse effects were not observed, perhaps owing to magnesium binding by proteins introduced proportionately with the broth. The optimal primer concentration was found to be 1 mM, although acceptable signal was seen at lower concentrations. A dNTP concentration of 0.5 mM for each dNTP was required for optimal reactions. At SOC or LB concentrations of 5% or less, magnesium concentration was found to be a critical variable with concentrations in excess of 4 mM showing substantially decreased signal and signal-to-noise ratios. An optimal level of 3 mM was chosen to ensure that the reaction was within the range described by Ling et al. for optimal fidelity.[5] BSA was found to be optional when SOC or LB medium was present in the reaction. Experiments with several polar aprotic solvents revealed DMSO to be superior to DMF and comparable to NMP, with optimal concentrations of 5% for DMSO and 2% for NMP. *E. coli* gp32[12] and SSB from *Staphylococcus aureus*[13] have been reported to be useful in PCR reactions under other conditions but did not contribute to reactions with either the monomeric or the multimer-containing templates. We found that glycerol can be tolerated at 5% as can tartrazine at 50 µg/100 µl, in agreement with the findings of others using *Taq* polymerase, although neither improved the reaction.[14] Between 20 and 25 cycles generally yielded optimal signal and signal-to-noise ratios. Creation of a vapor barrier (e.g., with mineral oil) and dena-

Downloaded from genome.cshlp.org on April 26, 2023 - Published by Cold Spring Harbor Laboratory Press

turation of template prior to addition of other reagents (hot start strategy) were both found to be unnecessary. To define the final procedure, critical parameters were reexamined to assure their optimization with all other variables optimized. These studies have resulted in the optimized protocol presented in Table 2 that we refer to as the 50/50 protocol. All conditions specified fall within the ranges found by Ling et al. for minimizing polymerase error rate.[5] In our experience, the procedures described work well with any template ranging from bacterial colonies or stock samples to highly purified DNA.

ACKNOWLEDGMENTS

This work was supported in part by grant AI26818 to K.B.C. and grants P30 CA46592, P60 AR20557, and M01 RR00042. We thank Dr. Jon Oscherwitz for critical reading of the manuscript.

REFERENCES

1. Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350–1354.
2. Mullis, K.B. and F.A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Methods Enzymol.* **155:** 335–350.
3. Kleppe, K., E. Ohtsuka, R. Kleppe, I. Molineux, and H.G. Khorana. 1971. Studies on polynucleotides. XCVI. Repair replications of short synthetic DNAs as catalyzed by DNA polymerases. *J. Mol. Biol.* **56:** 341–361.
4. Keohavong, P. and W.G. Thilly. 1989. Fidelity of DNA polymerases in DNA amplification. *Proc. Natl. Acad. Sci.* **86:** 9253–9257.
5. Ling, L.L., P. Keohavong, C. Dias, and W.G. Thilly. 1991. Optimization of the polymerase chain reaction with regard to fidelity: Modified T7, *Taq*, and *Vent* DNA polymerases. *PCR Methods Applic.* **1:** 63–69.
6. Lohff, C.J. and K.B. Cease. 1992. PCR using a thermostable polymerase with 3′ to 5′ exonuclease activity generates blunt products suitable for direct cloning. *Nucleic Acids Res.* **20:** 144.
7. Cease, K.B. and C.J. Lohff. 1993. A vector for facile PCR product cloning and modification generating any desired 4-base 5′ overhang: pRPM. *BioTechniques* **14:** 250–255.
8. Damak, S. and D.W. Bullock. 1993. A simple two-step method for efficient blunt-end ligation of DNA fragments. *BioTechniques* **15:** 448–452.
9. Ratner, L., W. Haseltine, R. Patarca, K.J. Livak, B. Starcich, S.F. Josephs, E.R. Doran, J.A. Rafalski, E.A. Whitehorn, K. Baumeister, L. Ivanoff, M.L. Pearson, J.A. Lautenberger, T.S. Papas, J. Ghrayeb, N.T. Chang, R.C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313:**(6000) 277–284.
10. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
11. Zon, L.I., D.M. Dorfman, and S.H. Orkin. 1989. The polymerase chain reaction colony miniprep. *BioTechniques* **7:** 696–698.
12. Schwarz, K., T. Hansen-Hagge, and C. Bartram. 1990. Improved yields of long PCR products using gene 32 protein. *Nucleic Acids Res.* **18:** 1079–1080.
13. Chou, Q. 1992. Minimizing deletion mutagenesis artifact during *Taq* DNA polymerase PCR by *E. coli* SSB. *Nucleic Acids Res.* **20:** 4371.
14. Hoppe, B.L., B.M. Conti-Tronconi, and R.M. Horton. 1992. Gel-loading dyes compatible with PCR. *BioTechniques* **12:** 679–680.

Received December 20, 1993; accepted in revised form February 14, 1994.

Downloaded from genome.cshlp.org on April 26, 2023 - Published by Cold Spring Harbor Laboratory Press

GENOME RESEARCH

Optimized PCR using Vent polymerase.

K B Cease, C A Potcova, C J Lohff, et al.

Genome Res. 1994 3: 298-300

References This article cites 13 articles, 2 of which can be accessed free at:
http://genome.cshlp.org/content/3/5/298.full.html#ref-list-1

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

Affordable, Accurate Sequencing.

gencove

To subscribe to *Genome Research* go to:
https://genome.cshlp.org/subscriptions

Copyright © Cold Spring Harbor Laboratory Press