2009

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Published in final edited form as:
J Microbiol Methods. 2009 December ; 79(3): 295–300. doi:10.1016/j.mimet.2009.09.015.
Published by Elsevier B.V.

Gene synthesis by integrated polymerase chain assembly and PCR amplification using a high-speed thermocycler

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Abstract

Polymerase chain assembly (PCA) is a technique used to synthesize genes ranging from a few hundred base pairs to many kilobase pairs in length. In traditional PCA, equimolar concentrations of single stranded DNA oligonucleotides are repeatedly hybridized and extended by a polymerase enzyme into longer dsDNA constructs, with relatively few full-length sequences being assembled. Thus, traditional PCA is followed by a second primer-mediated PCR reaction to amplify the desired full-length sequence to useful, detectable quantities. Integration of assembly and primer-mediated amplification steps into a single reaction using a high-speed thermocycler is shown to produce similar results. For the integrated technique, the effects of oligo concentration, primer concentration, and number of oligonucleotides are explored. The technique is successfully demonstrated for the synthesis of two genes encoding EPCR-1 (653 bp) and pUC19 β-lactamase (929 bp) in under 20 min. However, rapid integrated PCA–PCR was found to be problematic when attempted with the TM-1 gene (1509 bp). Partial oligonucleotide sets of TM-1 could be assembled and amplified simultaneously, indicating that the technique may be limited to a maximum number of oligonucleotides due to competitive annealing and competition for primers.

Keywords

Gene synthesis; PCA; Polymerase chain assembly

1. Introduction

The capability to create synthetic genes *de novo* is important within many biotechnology fields. A plethora of synthesis options are available which rely on basic molecular biology techniques, and one is referred to Czar et al. (2009) for a good review. Polymerase chain assembly (PCA) followed by PCR amplification (Stemmer et al., 1995) has been frequently used to synthesize genes from a few hundred to many kilobases (Mamedov et al., 2007; Mehta et al., 1997; Smith et al., 2003). PCA also is a means to create synthon precursors that can be utilized to create even larger constructs (Kodumal et al., 2004). PCA is a variation of the PCR process in which many oligonucleotides (“oligos”) are repeatedly hybridized, extended by a polymerase, and denatured. The desired gene sequence is segmented into short phosphoramidite synthesized oligo sequences of ~15 to 100 bp in length. The oligo design is typically aided by an oligo design software program (Rouillard et al., 2004). Using PCR thermocycling, the oligos are hybridized to one another and undergo polymerase chain extension to construct longer dsDNA. After repeated thermal cycling, the desired full-length sequence is assembled. In traditional equimolar PCA, relatively little full-length dsDNA is

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completely assembled during the PCA process. Thus, PCA is usually followed by a second primer-mediated PCR amplification of the full-length template to produce useful (and detectable) quantities of the synthetic gene. In contrast, integrated PCA and PCR amplification consists of a single reaction using both assembly oligos and amplification primers. Fig. 1 gives an overview of both traditional 2-step PCA synthesis method and the integrated 1-step approach.

The ability to conduct PCA and primer-mediated amplification simultaneously is highly attractive due to reduced labor, reagents, and instrument time. A more subtle advantage of the integrated technique is that a reduction of synthetic errors attributable to thermal damage can be logically assumed due to fewer thermal cycles (Pienaar et al., 2006). A one-step synthesis approach has been noted by others for its appeal. Chen and Choi (1994) described PCR based *de novo* gene synthesis of a 779 bp bacteriorhodopsin gene. Chen and Choi (1994) employed purified 70- to 100-mers with ~20 bp overlap between neighboring oligos, along with short outer oligos (primers). Barnes and Frawley (2003) used approximately picomole amounts of each 40-mer per 100μl reaction volume with outer primers present at 200 nM each. However, their recommended annealing times were 20 min (resulting in 7–10 hour runtimes). Tian et al. (2004) added outer primers (400 nM) with a 2μl oligo mixture to perform integrated PCA–PCR. Gao et al. (2004) presented a unique thermodynamically balanced inside-out (TBIO) synthesis scheme to conduct PCA with gradient oligo concentrations from 40 nM (innermost oligos) to 200 nM (outermost oligos).

Wu et al. (2006) explored key parameters in a conventional PCR thermocycler for their one-step simplified gene synthesis approach. Wu et al. (2006) tested polymerase selection, oligo concentration, and ratio of outer primers for three different genes (209, 777, and 936 bp). Similarly, we have focused our efforts on exploring several key variables that are important to the development of a rapid technique—the number of thermal cycles, the concentration of assembly oligos, the concentration of outer primers, and the maximum number of oligos that can be used. In instances where the rapid protocol failed, the Barnes and Frawley (2003) protocol was attempted using a conventional block cycler.

To investigate the integrated PCA–PCR technique, three genes were selected as representative candidates: a 653 bp gene containing the endothelial protein C receptor (EPCR) sequence, a 929 bp gene sequence encoding the pUC19 β-lactamase gene, and a 1509 bp gene sequence encoding the thrombomodulin-1 (TM-1) gene. All three of these genes have been previously shown to work well under the traditional two-step approach (Mamedov et al., 2007; TerMaat et al., in press).

2. Materials and methods

2.1. Gene sequences and oligonucleotide design

Oligonucleotides spanning the templates coding the EPCR gene (653 bp), pUC19 β-lactamase gene (929 bp), and TM-1 gene (1509 bp) were designed from cDNA sequences (GenBank accession nos. L35545, L09137, and BC035602, respectively). The Gene2Oligo computer program of Rouillard et al. (2004) was used to design the assembly oligos. Oligos and primers were obtained from Integrated DNA Technologies (Coralville, IA) at 50 μM in nuclease-free water.

2.2. PCR conditions

2.2.1. Standard thermocycling and reaction composition—Thermocycling protocols were performed in a rapid compressed air PCRJet® thermocycler (Quintanar and Nelson, 2002) using glass capillaries. All reaction volumes were 25 μl with the following

reagents fixed: 200 μM of each dNTP, 5 mM $MgSO_4$, 400 μg/ml non-acetylated BSA, and 0.5 unit KOD hot-start polymerase from Novagen (Madison, WI) in 1× manufacturer's buffer. All thermocycling protocols had fixed conditions of a 30 second hot start at 94 °C, denaturation of 94 °C for 2 s, extension of 72 °C for 10 s, and a final extension at 72 °C for 15 s. The number of cycles and annealing conditions were varied.

2.2.2. Traditional 2-step synthesis—*Traditional PCA*: 20 or 30 cycles with annealing conditions of 56 °C for 10 s. Each reaction contained 0.1 μM of each oligonucleotide. *Follow-up PCR*: 20 or 35 cycles with annealing conditions of 56 °C for 3 s, and each reaction contained 1 μl PCA product template and 0.7 μM of each primer.

2.2.3. Integrated PCA–PCR experiments—For the integrated PCA–PCR experiments, a variety of thermocycling conditions and reaction compositions were used. The standard conditions for the integrated PCA–PCR experiments were 40 cycles with annealing conditions of 56 °C for 10 s. Each standard reaction contained 10 nM of each oligonucleotide and 0.7 μM of each primer. For oligo concentration experiments, 1 nM to 100 nM of each oligo was employed, with an additional 1 nM sample with 30 second annealing time. For primer concentration experiments, 0 μM to 1.5 μM of each primer was employed. To investigate the number of integrated PCA–PCR cycles, 20 to 50 cycles were used. For integrated PCA–PCR experiments with the TM-1 oligo set, the number of oligos present in each reaction was varied from 46 to 76 oligos. The thermocycling conditions were 70 cycles with annealing at 62 °C for 15 s. Each reaction contained 5 nM of each oligonucleotide along with an additional 700 nM each outer oligo.

2.2.4. Integrated PCA–PCR with long annealing times—The protocol described by Barnes and Frawley (2003) was also attempted with the β-lactamase and TM-1 oligo sets. Klentaq1 Polymerase and Rockstart buffer from DNA Polymerase Technologies (St. Louis, MO) were employed in the reaction mixture along with 1.3 M betaine (Sigma Aldrich) and 20, 10, or 5 nM of each oligo. A conventional block thermocycler was used to execute the 20 minute annealing time protocol.

2.3. Gel electrophoresis

All reaction products were electrophoresed on 1% agarose gels stained with ethidium bromide using 6 μl of each product.

3. Results

3.1. Number of thermal cycles

Fig. 2 shows integrated PCA–PCR of EPCR (Fig. 2A) and β-lactamase (Fig. 2B) using 20 to 50 cycles. The left lanes of each gel show the equimolar PCA and follow-up PCR, respectively, for the traditional two-step approach with 30 cycles of PCA and 35 cycles of PCR. The right lanes of Fig. 2B show the equimolar PCA and follow-up PCR for β-lactamase with 20 cycles each.

3.2. Assembly oligo concentration

The integrated technique using 1 nM to 100 nM each oligo was tested for both EPCR (Fig. 3A) and β-lactamase (Fig. 3B). The right lane of each gel represents a 1 nM of each oligo sample with the annealing time increased to 30 s. For both genes, 100 nM did not yield any distinguishable product from the background. For 25 nM reactions, EPCR showed a band at the correct molecular weight while β-lactamase did not. Both genes had strong product amplification at 10 nM and 5 nM. The 1 nM EPCR sample with 30 second annealing had an

extremely faint band, while 10 second annealing product did not. The reverse situation was observed for 1 nM β-lactamase samples.

3.3. Primer concentration

The effect of primer concentrations (0 to 1.5 μM) is shown in Fig. 4 (EPCR: Fig. 4A, β-lactamase: Fig. 4B). Wu et al. (2006) specified a primer to assembly oligo ratio, but it is our impression that absolute primer amounts may have more weight to integrated PCA–PCR techniques than a primer ratio. Note that primers can be replaced by simply spiking the outer assembly oligos (with consideration given to optimal annealing temperatures).

3.4. Maximum number of oligos

When rapid integrated PCA–PCR was attempted on TM-1 (76 oligos, 1509 bp), no desired amplification product was visible on the gel (results not shown). Numerous adjustments to TM-1 oligo concentrations, primer concentrations, and increased number of cycles did not alter the outcome (results not shown). We hypothesized that integrated PCA–PCR may be practically limited to a maximum number of oligos. Thus, the number of oligos used within the reaction was decreased to investigate the point at which the integrated technique failed.

Fig. 5A shows the resulting products using the TM-1 oligo set for 46 to 76 oligos. The 76 oligo reactions are the full-length 1509 bp gene, while fewer oligo reactions result in shorter products (partial gene sequence). All oligos were set at a concentration of 5 nM. An additional 700 nM each outer oligo employed in lieu of primers. The optimal annealing conditions were found to be 62 °C for 15 s since longer oligos were used. The number of cycles was increased to 70 to maximize yield. As can be seen from the figure, the amount of amplified product decreases as the number of oligos increases and integrated PCA–PCR fails to produce a distinct product at about 60 to 66 oligos. The decreased band intensity for 56 oligos is due to a compromise in annealing temperatures (not optimal) for the oligo set. Fig. 5B shows the traditional equimolar PCA and follow-up PCR products using the full TM-1 oligo set. The desired band at ~1509 bp is visible after completion of the two-step approach.

4. Discussion

We have developed an integrated PCA–PCR technique using short annealing times (~10 s), a high-speed thermocycler, and fast KOD polymerase. Fig. 2 shows that the amplification of the desired product increased up to a maximum along with the number of thermal cycles. With the use of a rapid thermocycler, the 40 cycle protocol was completed in 19.2 min, and was used for further reactions. However, one can see that a high yield of product can be obtained in 30 cycles (14.5 min).

Wu et al. (2006) found that KOD polymerase performed better in their simplified gene synthesis than *Taq* or *Pfu* enzymes. Additionally, the fast extension rate of KOD enables minimal extension times that are well-suited for use with rapid thermocycling. Integrated PCA–PCR using Vent (exo-) polymerase (New England Biolabs) resulted in successful amplification of the 653 bp EPCR gene with the elongation time increased to 30 s (results not shown). While polymerase selection is a critical element in light of Wu et al. (2006), the rapid technique may accommodate other polymerases if adequate elongation time is given. Similarly, the rapid thermocycling was performed in the PCRJet® thermocycler (Quintanar and Nelson, 2002). While not tested, other fast thermocyclers with comparable heating and cooling rates may also be amenable to the rapid integrated PCA–PCR technique.

The concentration of oligonucleotides is paramount to simultaneous assembly (PCA) and amplification (PCR) in a single reaction. As can be seen in Fig. 3, the oligo concentration of

100 nM typically employed in the traditional two-step PCA and follow-up PCR is unsuitable for use in the integrated approach. While 25 nM of each oligo did yield product in the case of the 653 bp EPCR gene, it did not in the case of the longer 929 bp β-lactamase sequence. Only when the oligo concentrations were about 5 to 10 nM did the integrated technique show high yield of the desired product in a single step. This is in good agreement with the findings of Barnes and Frawley (2003) with their long annealing time integrated protocol. Interestingly, Wu et al. (2006) was able to obtain the desired product at higher concentrations up to 100 nM, but only for short genes with few oligos. At 5 nM, Wu et al. (2006) had only the 209 bp gene construct visible product. In exploring oligo concentrations, both the conventional and rapid techniques became less forgiving as the number of oligos present increased. We recommend 5 nM to 10 nM concentration of each oligo as close to optimal for rapid integrated PCA–PCR.

Further reduction of oligo concentrations to 1 nM resulted in little to no product. This indicates that the optimum of around 5 to 10 nM drops off sharply. In essence, proper assembly is intermittent at 1 nM or lower concentrations and is not advisable. There is no theoretical minimum on the necessary oligo concentrations for PCA per se, as it is conceivable that only one strand of each oligo would be needed. However, the oligos become so dilute within the reaction mixture that the probability of correct hybridization at each cycle becomes miniscule. This is exacerbated by the fact that, upon later cycles, these sparse intermediate products must hybridize. Thus, the propagation of assembly is contingent upon intermediate product concentrations that are considerably less than 1 nM. As intermediate assembly products are at extremely low (or non-existent) concentrations, even the longest of annealing times may be insufficient to promote continued assembly.

The maximum amount of full-length product attainable clearly depends on the amounts of the outer primers as shown in Fig. 4. With no primers present, no distinct band at the expected size was visible for either gene while primer concentrations of 0.1 μM did produce weak amplification. 0.4 μM to 0.7 μM of each primer provided good amplification of the product. High primer concentrations of 1 μM or more also produced good results. Just as in regular PCR, high primer concentrations can cause increased probability of misannealing and thereby inhibit the desired amplification. Thus, we recommend concentrations of primers or outer oligos of about 0.4 to 0.7 μM each.

Logically, both PCA and PCR amplification become more difficult as the number of oligos and gene length increase. Given the failure of the integrated technique on TM-1 but the success of the 2-step approach, it is fair to say that the 2-step approach is more robust. In general, one would expect such a result, as two separate steps facilitate the use of different oligo concentrations. In equimolar PCA, higher oligo concentrations (25 nM to 100 nM) allow for efficient assembly even for longer gene sequences with a large number of oligos. Follow-up PCR on an aliquot of the PCA product is performed with a low concentration of background oligos (~5 nM) due to the dilution of the aliquot. This dichotomy of oligo concentrations is not possible with the integrated approach.

To allow for efficient amplification, the background oligos should be present in minimal concentrations. All background oligos are complementary to the full-length PCR amplification templates. Thus, these oligos may inhibit polymerase extension by annealing to the template downstream of the primer. Experiments using Vent polymerase (New England Biolabs) with strand displacement activity did not result in successful amplification of TM-1 (results not shown). Additionally, inner oligos that have been extended to completion on their 3′ end will compete for primers and be linearly amplified. As the number of oligos in the reaction mixture increase, so does the primer competition and upstream annealing. For example, 26 possible forward oligos are present for β-lactamase. In

contrast, 38 forward oligos are present in the full TM-1 set. As an additional complication, the formation of DNA duplex pairs and gel analysis inferences will depend on the relative oligo pool at the last thermal cycle. A distinct band may not be visible even though a significant amount of the full-length top strand and bottom strands may be present. It is expected that using 100-mers (as described by Chen and Choi, 1994) instead of 40-mers would likely allow for longer genes to be assembled and amplified with an integrated technique. However, PAGE purification of assembly oligos would be needed to limit unwanted deletion errors in the synthesized product.

The use of integrated PCA–PCR to assemble longer genes is possible given the findings of Barnes and Frawley (2003). To see if the TM-1 gene could be assembled with longer annealing times, we increased the annealing times from 10 s up to 14 min in the compressed air thermocycler. In no case was the integrated PCA–PCR technique successful for the TM-1 gene. Negative results (not shown) were also found for another 76 oligo set that was available in our lab. Follow-up PCR was performed and demonstrated that the oligos had assembled full-length templates, but never reached an efficient amplification phase. The exact integrated PCA–PCR protocol described by Barnes and Frawley (2003) was then attempted with the β-lactamase and TM-1 oligo sets using a conventional block thermocycler. A discernable band was visible after 25 cycles for β-lactamase with 5 nM each oligo. However, none of our experiments with the TM-1 oligo set was successful even with the same reaction conditions and more cycles (results not shown). While we certainly cannot rule out the possibility of success as we did not use PAGE-purified oligos or KlentaqLA polymerase mix, the TM-1 gene was not simultaneously assembled and amplified in any of our reactions.

To further validate the reliability of the technique, four other oligo sets were successfully synthesized using rapid integrated PCA–PCR (results not shown). The standard reaction and thermocycling conditions of the technique were used without further optimization, with the oligos at 10 nM each and primers at 700 nM each. The yield and background for all four products were comparable to the presented results for the 929 bp β-lactamase product in Fig. 2B, lane 6. The oligo sets consisted of 50 or 52 oligos and yielded products ranging from 936 to 1000 bp in length. The GC content for the four different gene sequences ranged from 48.2% to 61.8%.

5. Conclusion

Integration of PCA assembly and primer-mediated PCR amplification into a single reaction using rapid thermocycling has been demonstrated. Unlike the traditional 2-step synthesis approach, the concentration of initial oligos must be lowered with an optimum about 5 nM to 10 nM each oligo. Standard PCR reaction and primer conditions may be used. The rapid integrated PCA–PCR technique was successful for two genes, a 653 bp EPCR gene (35 oligos) and a 929 bp β-lactamase gene (52 oligos). Annealing times of 10 s were found to be sufficient for the technique. Both genes were assembled and amplified in a single reaction, requiring less than 20 min total (for 40 cycles) of rapid thermocycling to obtain the full-length genes in high yield.

As the number of oligos present within the reaction increases, one must reconcile the dichotomy of optimal oligo concentrations. Lower concentrations favor PCR amplification, but hinder efficient oligo assembly due to extremely dilute intermediate products. Higher concentrations promote assembly but inhibit PCR amplification of constructed full-length templates by upstream annealing of complementary oligos and competition for primers. For the 1509 bp TM-1 gene synthesis with 76 oligos, we were unable to obtain a distinct gel electrophoresis product. The long annealing protocol of Barnes and Frawley (2003) was

equally poor for TM-1 but successful for β-lactamase synthesis. Using partial oligo sets for TM-1, it was determined that about 60 oligos is the maximum number that can be routinely used for rapid integrated PCA–PCR. This corresponds to about 1200 bp if ~40-mers are used. While there is likely some dependence on the specific TM-1 gene sequence, some insights can be drawn from the number of oligo results. Above 60 oligos, results may be unsatisfactory or optimization too laborious and the two-step approach preferable.

Acknowledgments

This work was supported by funds from the National Institutes of Health (R33 RR022860).

References

Barnes, WM.; Frawley, ER. Stream-lined gene assembly PCR. In: Dieffenbach, CW.; Dveksler, GS., editors. PCR Primer: a Laboratory Manual. Cold Spring Harbor Laboratory Press; New York: 2003. p. 475-481.

Chen GQ, Choi I. Total gene synthesis: novel single-step and convergent strategies applied to the construction of a 779 base pair bacteriorhodopsin gene. J. Am. Chem. Soc. 1994; 116:8799–8800.

Czar MJ, Anderson JC, Bader JS, Peccoud J. Gene synthesis demystified. Trends Biotechnol. 2009; 27:63–72. [PubMed: 19111926]

Gao X, Yo P, Keith A, Ragan TJ, Harris TK. Thermodynamically Balanced Inside-Out (TBIO) PCR-based gene synthesis: a novel method of primer design for high-fidelity assembly of longer gene sequences. Nucleic Acids Res. 2004; 31:e143–e164. [PubMed: 14602936]

Kodumal SJ, Patel KG, Reid R, Menzella HG, Welch M, Santi DV. Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. Proc. Natl. Acad. Sci. U. S. A. 2004; 101:15573–15578. [PubMed: 15496466]

Mamedov TG, Padhye NV, Viljoen H, Subramanian A. Rational *de novo* gene synthesis by rapid polymerase chain assembly (PCA) and expression of endothelial protein-C and thrombin receptor genes. J. Biotechnol. 2007; 131:379–387. [PubMed: 17875333]

Mehta DV, DiGate RJ, Banville DL, Guiles RD. Optimized gene synthesis, high level expression, isotopic enrichment, and refolding of human interleukin-5. Protein Expr. Purif. 1997; 11:86–94. [PubMed: 9325143]

Pienaar E, Theron M, Nelson M, Viljoen HJ. A quantitative model of error accumulation during PCR amplification. Comput. Biol. Chem. 2006; 30:102–111. [PubMed: 16412692]

Quintanar, A.; Nelson, RM. A process and apparatus for high-speed amplification of DNA. U.S. Patent No. 6,472,186. 2002.

Rouillard JM, Lee W, Truan G, Gao X, Zhou X, Gulari E. Gene2Oligo: oligonucleotide design for in vitro gene synthesis. Nucleic Acids Res. 2004; 32:W176–180. [PubMed: 15215375]

Smith HO, Hutchison CA, Pfannkoch C, Venter JC. Generating a synthetic genome by whole genome assembly: ΦX174 bacteriophage from synthetic oligonucleotides. Proc. Natl. Acad. Sci. U. S. A. 2003; 100:15440–15445. [PubMed: 14657399]

Stemmer WPC, Crameri A, Ha KD, Brennan TM, Heyneker HL. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene. 1995; 164:49–53. [PubMed: 7590320]

TerMaat JR, Mamedov TG, Pienaar E, Whitney SE, Subramanian A. Automated two-column purification of Iminobiotin and BrdU labeled PCR products for rapid cloning: application to genes synthesized by Polymerase Chain Assembly. J. Chromatogr. Sci. in press. Accepted to.

Tian J, Gong H, Sheng N, Zhou X, Gulari E, Gao X, Church G. Accurate multiplex gene synthesis from programmable DNA microchips. Nature. 2004; 432:1050–1054. [PubMed: 15616567]

Wu G, Wolf JB, Ibrahim AF, Vadasz S, Gunasinghe M, Freeland SJ. Simplified gene synthesis: a one step approach to PCR-based gene construction. J. Biotechnol. 2006; 124:496–503. [PubMed: 16516321]

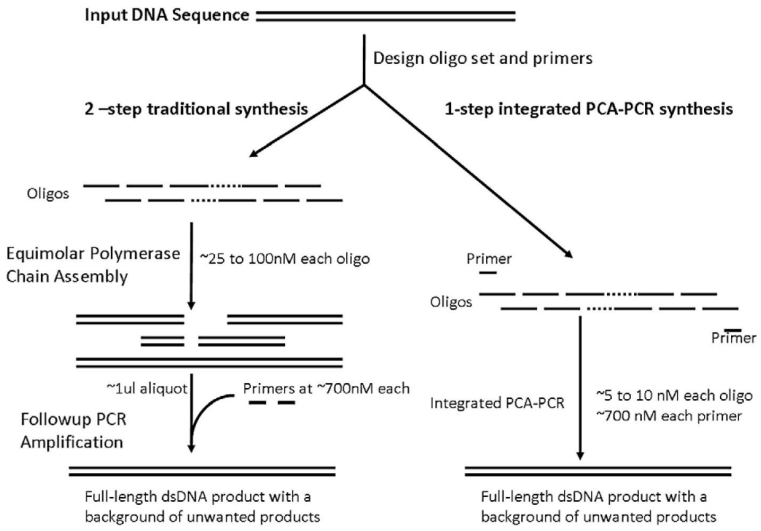

Fig. 1.

Schematic of the two synthesis methodologies. The traditional 2-step approach uses equimolar PCA synthesis followed by a 2nd PCR amplification reaction. The integrated PCA–PCR approach employs both assembly oligos and amplification primers in a single reaction.

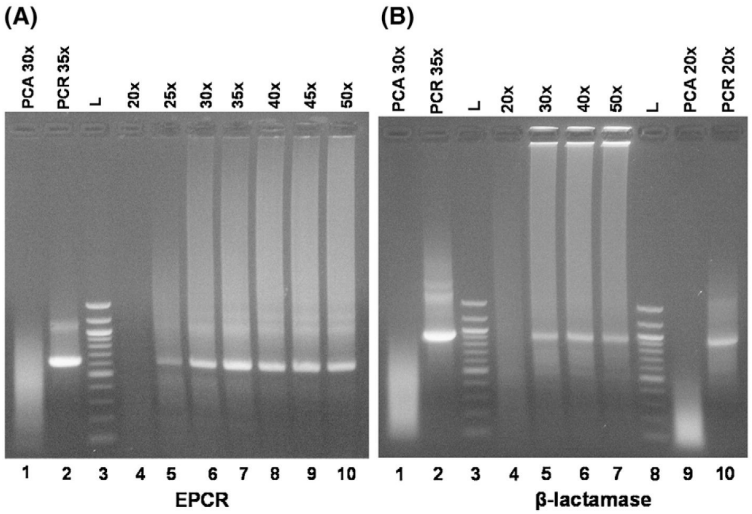

Fig. 2.

Traditional 2 step PCA/PCR and integrated PCA–PCR vs. # of cycles. (A) EPCR gene. Lane 1: equimolar PCA with 30 cycles; lane 2: follow-up PCR with 35 cycles; lanes 4 thru 10: integrated PCA–PCR vs. # of cycles. (B) β-Lactamase gene. Lane 1: equimolar PCA with 30 cycles; lane 2: follow-up PCR with 35 cycles; lanes 4 thru 7: integrated PCA–PCR vs. # of cycles; lane 9: equimolar PCA with 20 cycles; lane 10: follow-up PCR with 20 cycles. Lanes (L): 100 bp DNA ladder (NEbiolabs).

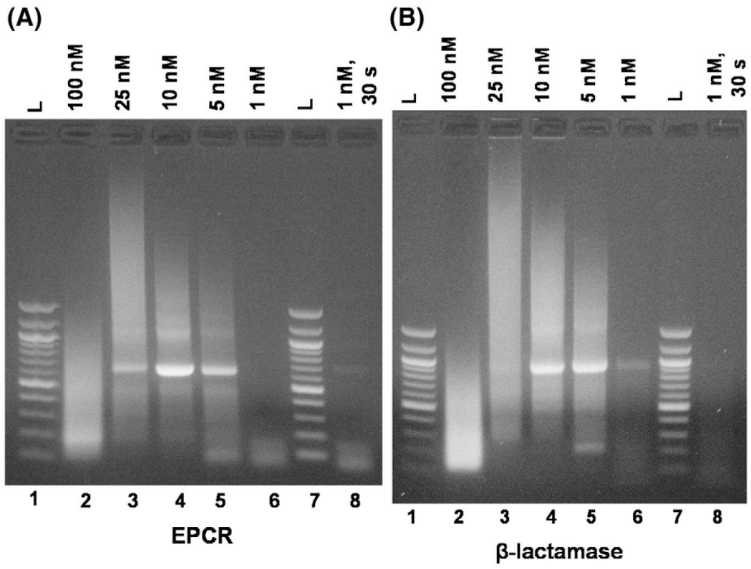

Fig. 3.

Effect of oligo concentration on integrated PCA–PCR. (A) EPCR gene. (B) β-Lactamase gene. Lanes 2 thru 6: 100, 25, 10, 5, and 1nM of each oligo, respectively. Lane 8: 1 nM of each oligo with 30 second annealing time. Lanes (L): 100 bp DNA ladder (NEbiolabs).

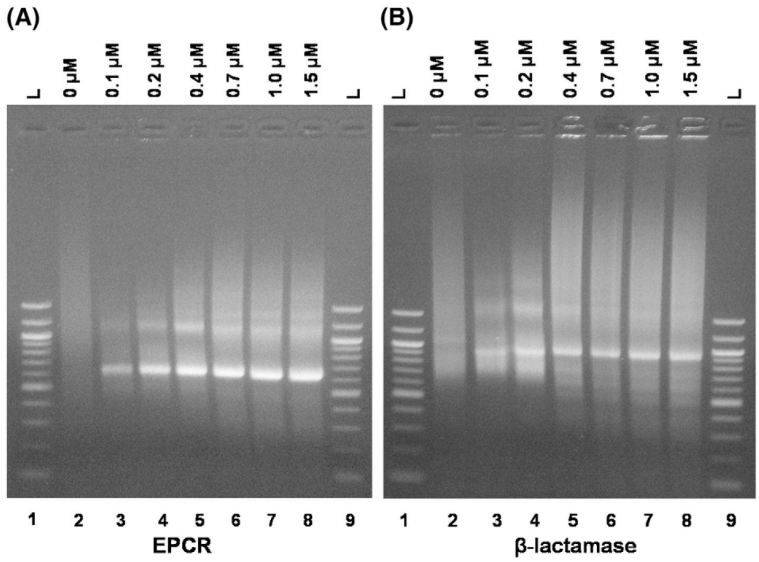

Fig. 4.

Effect of primer concentration on integrated PCA–PCR. (A) EPCR gene. (B) β-Lactamase gene. Lanes 2 thru 6: (0 (none), 0.1, 0.2, 0.4, 0.7, 1.0, and 1.5) μM of each primer, respectively. Lanes (L): 100 bp DNA ladder (NEbiolabs).

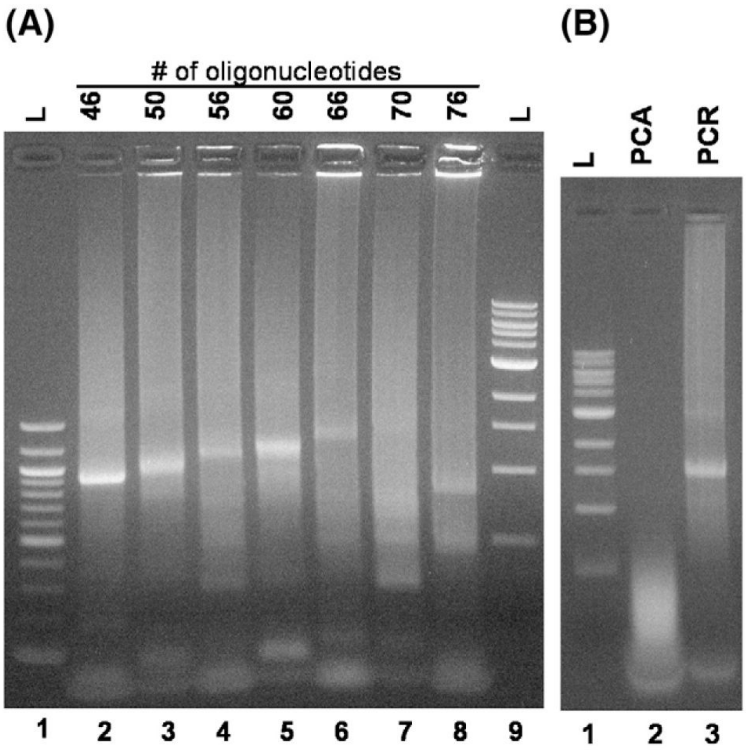

Fig. 5.

Traditional 2 step PCA/PCR and integrated PCA–PCR vs. # of oligos using the TM-1 oligo set. (A) Integrated PCA–PCR using different # of oligos from the TM-1 oligo set. Lanes 2 thru 8: 46, 50, 56, 60, 66, 70, and 76 total oligos, respectively. (B) Equimolar PCA (lane 2) with follow-up PCR (lane 3) of the full TM-1 gene 76 oligo set. Lanes (L): 100 bp DNA ladder or 1 kb DNA ladder (NEbiolabs).