

Research article

Signal and noise in bridging PCR

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Abstract

Background: In a variant of the standard PCR reaction termed bridging, or jumping, PCR the primer-bound sequences are originally on separate template molecules. Bridging can occur if, and only if, the templates contain a region of sequence similarity. A 3' end of synthesis in one round of synthesis that terminates in this region of similarity can prime on the other. In principle, Bridging PCR (BPCR) can detect a subpopulation of one template that terminates synthesis in the region of sequence shared by the other template. This study considers the sensitivity and noise of BPCR as a quantitative assay for backbone interruptions. Bridging synthesis is also important to some methods for computing with DNA.

Results: In this study, BPCR was tested over a 328 base pair segment of the *E. coli lac* operon and a signal to noise ratio (S/N) of approximately 10 was obtained under normal PCR conditions with Taq polymerase. With special precautions in the case of Taq or by using the Stoffel fragment the S/N was improved to 100, i.e. 1 part of cut input DNA yielded the same output as 100 parts of intact input DNA.

Conclusions: In the *E. coli lac* operator region studied here, depending on details of protocol, between 3 and 30% per kilobase of final PCR product resulted from bridging. Other systems are expected to differ in the proportion of product that is bridged consequent to PCR protocol and the sequence analyzed. In many cases physical bridging during PCR will have no informational consequence because the bridged templates are of identical sequence, but in a number of special cases bridging creates, or, destroys, information.

Background

Bridging, or jumping, PCR (BPCR) can convert DNA backbone interruptions anywhere in the region of shared sequence between primer binding sites into a single and unique amplified band [1]. The logic of BPCR is illustrated in Figure 1. In principle BPCR can detect backbone in-

terruptions anywhere within a chosen sequence region. This manuscript reports a study in which BPCR is used to detect a minority of interrupted molecules in the *lac* operon of *Escherichia coli* and describes a system to measure noise in BPCR.

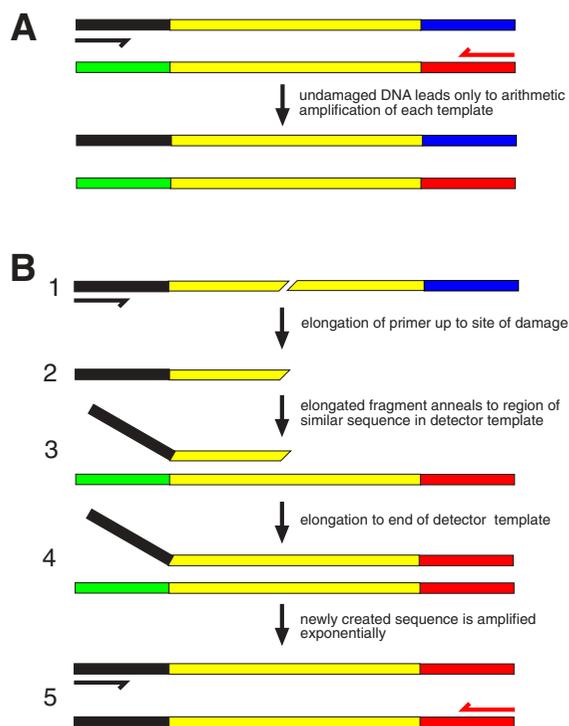


Figure 1
The principle of Bridging PCR (BPCR) A. Only arithmetic amplification occurs when there are two templates and two primers but only one primer is complementary to each template. B. Consider the case in which two templates share a central region of similar sequence (shown in yellow), flanked by regions of different sequence (black, blue, green, red). 1) If one template is interrupted, then its primer can extend only up to the point of interruption. 2) Synthesis up to the break creates a new 3' end at the site of the interruption. 3) On the next round of PCR the new 3' end created by the interruption anneals to the second template is in the region of shared (yellow) sequence and, 4) primes synthesis to the end of the second template. 5) Extension to the end of the second template creates a recombinant molecule with both primer binding sites. This new molecule is exponentially amplified in subsequent rounds of normal PCR.

Results

Signal and noise in bridging PCR

Bridging PCR (BPCR) is a combination of two processes, a recombination between two template sequences and an amplification of the recombinant template. Two parental sequences share a homologous region (a region in which the sequence is identical) and diverge in the nonhomologous flanking sequences. One side of the nonhomologous flanking sequence of each of the two "parental" molecules contains a different PCR primer-binding site. Neither pa-

rental template has both primer-binding sites. Therefore a "chain reaction" cannot occur and only linear amplification is expected.

However, during the course of cycling, recombination can occur between the homologous regions via a copy choice mechanism (illustrated in Figure 1). A 3' end of one template sequence, if it is within the homologous region, can anneal to the other template. During the next extension cycle the invading strand will grow from its 3' end, and newly synthesized DNA will cross the region of sequence similarity and continue into sequences unique to the second template, including the second template's primer binding site. The result is a recombinant sequence, identical to the product of *in vivo* homologous recombination, with one flank from the original strand and the other flank from the second template. Because the two primer sites are now *in cis*, the recombinant sequence can be amplified exponentially.

If the 3' end of either template is not within the region of similar sequence, then no 3' end will hybridize to the other template and synthesis does not lead to recombination. The creation of recombinant sequences during PCR reflects the presence of templates whose 3' ends are within the region of sequence similarity. BPCR ought to be utilizable to assay the proportion of input templates with 3' ends in the region of sequence similarity. However, the reaction itself can produce 3' ends which then lead to recombinant templates. For maximum sensitivity and S/N ratio, the PCR reaction itself should not produce ends at any sites that did not exist in the input DNA. PCR itself generates new 3'ends [2,3]. In BPCR new ends yield recombinant products, even if the initial template was completely intact.

BPCR between plasmid pBS and Phagescript

Figure 2 shows the map and sequence of templates and primer sites used in BPCR between a portion of plasmid pBS and Phagescript, a derivative of the ssDNA M13 phage. This pair of substrates meets the requirement for BPCR substrates because the region of shared sequence, the *lac* operator and the *lac* operon, are flanked by sequences that are different between the two vectors. The *lac* operon is one of the most frequently used genes for the study of *in vivo* mutagenesis [4-7].

Determination of signal to noise (S/N)

Two samples with known quantities of templates were prepared: one sample contained the pBS (-) sequence template with a cut in the homologous region created by *PvuII* digestion (cut template) and the intact ssDNA Phagescript DNA (reference template). In the other sample both the pBS (-) (intact template) and the Phagescript DNA (reference template) were intact. The molar ratio of

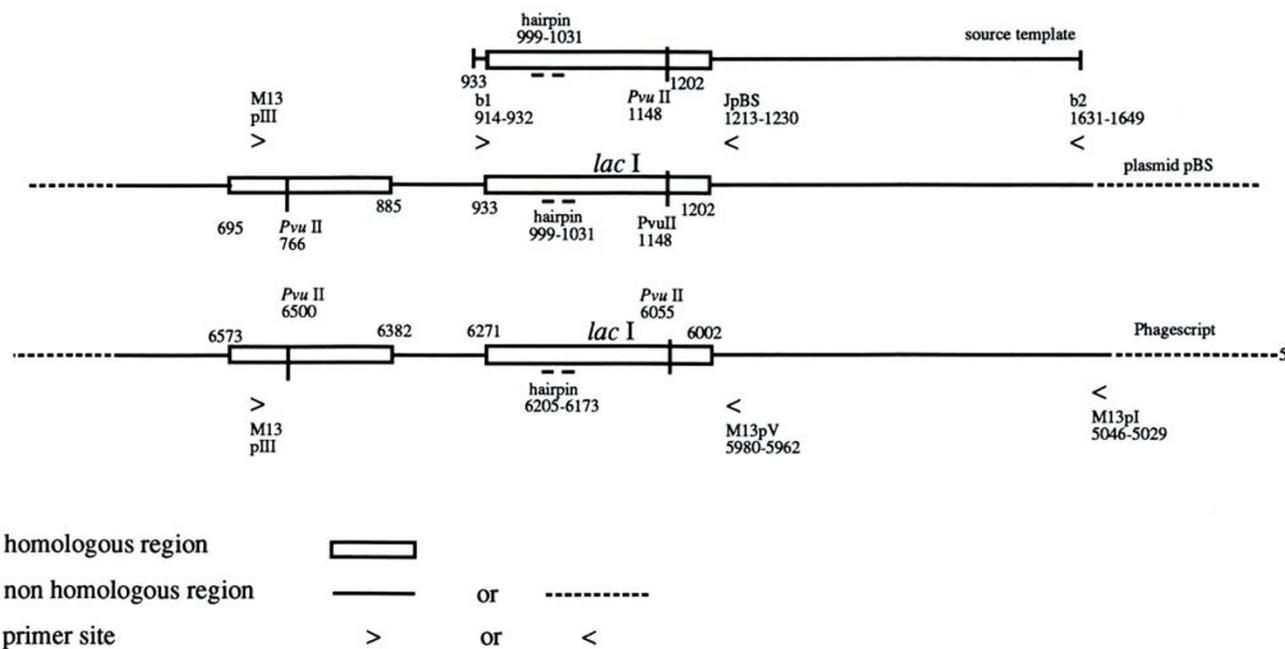


Figure 2
Maps and primer locations Maps of the templates and of the primer locations for bridging PCR between plasmid pBS(-) and phagescript particles. The base numbers (the numbers of on the maps) are from the published sequences by the supplier (Stratagene). The DNA of pBS(-) is double stranded. The sequences for the primers whose positions are shown in Figure 2 are given in Table 1.

reference template (ssDNA) to either intact template (dsDNA) or to cut template (dsDNA, considering only one of the two fragments) was about 10.

The concentration of pBS (-) sequence template, either cut or intact, was adjusted to be about 0.1 nM and to be equal in the two samples. The equality was confirmed by a 20 thermal cycle conventional PCR with primers b1 and M13 revI. The PCR outputs from serial dilutions (10 fold dilution between each step) were similar between the two samples (data not shown).

Each of the two samples was serially diluted into BPCR reactions, the dilution involving only the cut and intact template, with everything else unchanged. The highest concentration of template was about 10¹⁴ molecules/μl. Typically, dilution steps were 10 fold each and covered a million fold range. The PCRs were run in parallel (same concentrations, same volume and running on the same thermal block at the same time). After the BPCR, output of recombinant DNA was assayed by electrophoresis. For each dilution one can obtain the concentration of output [R] as a function of [Ts], the concentration of template (cut or intact depending on the sample). For each sample

one defines [Ts]_{off} as a cut-off concentration below which [R] is no longer saturated. The signal to noise is the ratio between [Ts]_{off} in the two samples:

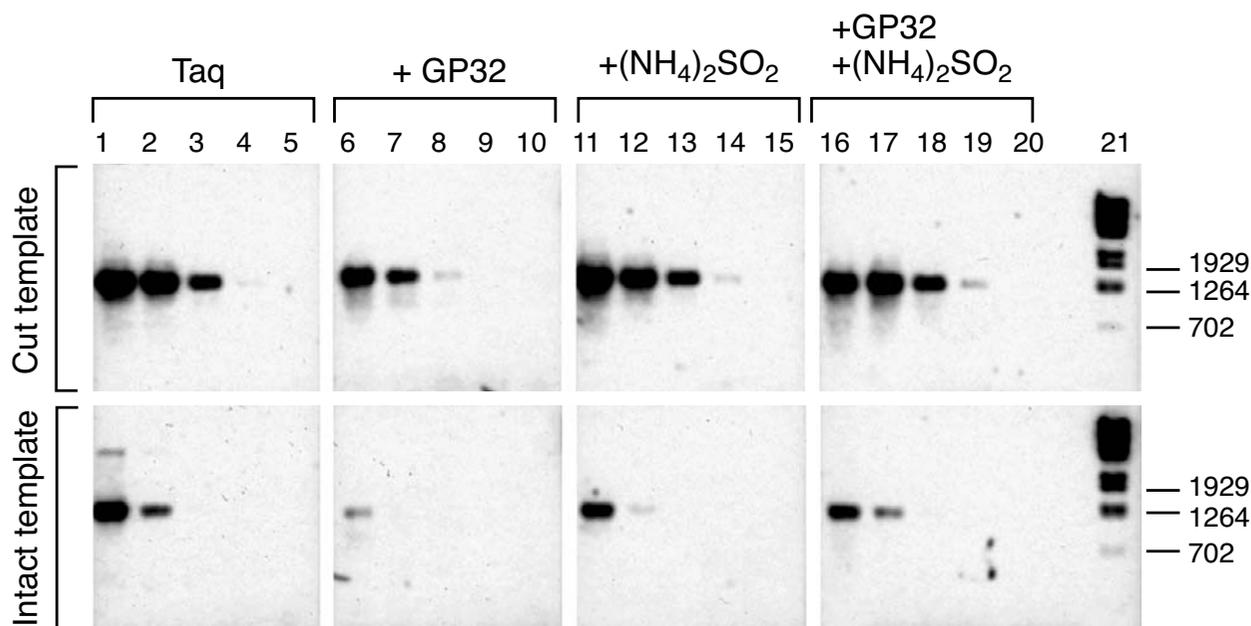
$$S/N = \frac{[Ts]_{off, intact}}{[Ts]_{off, cut}}$$

For example, if the cut-off concentration of the cut sample is 100 times less than the intact cut-off concentration then the S/N is 100.

Pre-annealing and pre-extension

A pre-annealing and pre-extension regime improved the S/N. In this regime, templates in the reaction tube with 1 × PCR buffer and dNTP's were heated to 90°C for 10 minutes then gradually cooled (by approximately 1°C/minute) to 72°C.

Pre-extension was carried out by holding the temperature at 72°C and adding polymerase to the pre-annealing tube. The temperature was held at 72°C for three more minutes before it was dropped to room temperature. The rationale

**Figure 3**

Taq polymerase alone yields a S/N of 10 but can be improved Bridging PCR with Taq polymerase and the improvement of the S/N by addition of gp32 and (NH₄)₂SO₄. The cut or intact templates was prepared by PCR with Taq polymerase and primers b1 and Jp on double-stranded plasmid pBS(-). BPCR was carried out with Taq polymerase and primers b1 and M13 pl. (See Figure 2 for location of primers and Table 1 for their sequence.) The other template was Phagescript phage particles. The 1 × PCR buffer for lanes 1–10 was 50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM MgCl₂. The 1 × PCR buffer with (NH₄)₂SO₄, (lanes 11 to 20) was 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 2.0 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100 and 0.1 mg/ml bovine serum albumin. The ethidium bromide fluorescence image of the 1.0% agarose electrophoresis gel was photographed. The photograph was digitized and processed with Scion Image Adobe Photoshop software on a Macintosh computer. The image intensity was inverted.

Each lane has two rows which are labeled as cut, i.e. *PvuII* digested (upper row), or intact (lower row). The BPCR product bands are about 1261 bp, as expected from the template sequences (see figure 2). Lanes 1 to 5 show BPCR with Taq polymerase and no other additions. Lanes 6 to 10 show the enhancement of the S/N of BPCR by the addition of 0.01% gp32. Lanes 11 to 15, show the enhancement of BPCR by the addition of 10 mM (NH₄)₂SO₄. Lanes 16 to 20, show BPCR with gp32 and (NH₄)₂SO₄. The addition of both gp32 and (NH₄)₂SO₄ did not improve the S/N. Each lane has two samples separately loaded into the two separate loading wells, one on each row. In the figure, the upper row is of source template cut with *PvuII*, and the lower DNA sample is of the same source template intact (without *PvuII* cut). The source template concentrations in the two samples were equal. Lanes 2, 3, 4 and 5 are serial template dilutions of lane 1. Lanes 7, 8, 9 and 10 are serial template dilutions of lane 6. Lanes 12, 13, 14 and 15 are serial template dilutions of lane 11. Lanes 17, 18, 19 and 20 are serial template dilutions of lane 16. Lane 21 contains DNA size markers made from Lambda DNA, *BstE II* digested, 125 ng. The source template concentrations of lanes 1, 6, 11 and 17 are adjusted to equal to 1 × 10⁻¹⁷ mol/μl PCR solution.

for pre-extension [8] was that cut template molecules that are annealed to reference molecules should be extended by the polymerase to become full length. There are no primers present in the pre-extension cycle and therefore this cycle does not generate the class of noise due to incomplete extension from normal primers.

Immediately following pre-extension, i.e. without storage at 4°C, the samples were serially diluted and PCR amplified with the BPCR primers.

BPCR with Taq polymerase yielded S/N of approximately 10

Templates were prepared by 25 cycles of conventional PCR with Taq polymerase [9] using primer b1 and Jp on template plasmid pBS(-). Phagescript phage particles, rather than purified DNA prepared from the particles by phenol extraction, were used as the reference template [10]. The BPCR primer pair was b1 and M13 pl (see figure 2 for primer locations) which would give a 1261 bp recombination product. The S/N of BPCR with Taq

polymerase was approximately 10 (see figure 3, lanes 1–5). This result is consistent with the report that Taq polymerase's nuclease activity [2,11] cuts in the *lac* operator [3]. The *lac* operator cut site is within the homologous region in this system. BPCR was conducted for 30 cycles with Taq polymerase.

Several variations of BPCR with Taq polymerase were carried out (data not shown), including: 1) cut and intact templates were prepared with primers b1 and b2, 2) purified ssDNA instead of phage particles was used as a reference template and 3) cut and intact templates were prepared with the Stoffel fragment of Taq polymerase. The S/N was about 10 in these variations. Increase in the number of PCR cycles and/or a longer extension time (5 minutes) were also tested and found to be not acceptable because non-specific output, in the form of multiple gel bands or smearing gel lanes, frequently appeared. Combining Taq polymerase with a small quantity of Pfu polymerase [12] had little effect on the S/N.

Additives that improved Taq polymerase S/N to approximately 100

Some additives were found to enhance the S/N ratio. Among the additives tested, gp32 protein [13] or $(\text{NH}_4)_2\text{SO}_4$ improved the S/N most efficiently and the S/N reached 100 (See figure 3. Compare cut vs intact rows in lanes 6–10 and 11–15). Combining gp32 and $(\text{NH}_4)_2\text{SO}_4$ did not further improve S/N (See figure 3. Compare cut vs intact rows in lanes 16–20.).

The Stoffel fragment of Taq polymerase gave a S/N of approximately 100

The Stoffel fragment of Taq polymerase is a truncated portion of the enzyme which retains polymerase activity but lacks the 5'→3' exonuclease activity of Taq polymerase [14]. The Stoffel fragment does not cut at the *lac* operator as does Taq polymerase [3]. It might therefore be a good choice of polymerase for jumping PCR. Consistent with the expectation, results of using the Stoffel fragment, without additives such as gp32, $(\text{NH}_4)_2\text{SO}_4$ or detergent, gave a S/N of 100 (See figure 4, compare intact lanes 3, 4, 5, 6 to cut lanes 7,8,9,10). Addition of any of the above additives did not further improve the S/N. The source templates used were also prepared by standard PCR with the Stoffel fragment and the primer pair b1 and Jp on pBS (see figure 2).

BPCR primers b1 and M13 pV, produced the expected 328 bp recombinant sequence. In this size range the Stoffel fragment of Taq polymerase yielded a better S/N than Taq polymerase. However, the Stoffel fragment had low output in BPCR between primers b1 and M13 pI. This is consistent with the manufacturer's description that the Stoffel

fragment is not optimal for the amplification of sequences longer than 1 kb.

Vent polymerase lacking nuclease activity [15] is also expected to give a low noise level. However, it yielded insufficient output (data not shown).

Discussion

Bridging, or jumping, PCR (BPCR) was discovered during the analysis of ancient DNA [1]. In reconstruction experiments new alleles of lysozyme genes were generated *in vitro*, during a normal PCR reaction, and, since damage to DNA promotes bridging, it was suggested that BPCR could be developed as an assay for DNA damage. Since its fortuitous discovery, BPCR has been used to make pools as part of algorithms for the generation and selection of new alleles [16,17], as well for the assembly of genes [18,19] and replicons [19] from oligonucleotides. BPCR has the potential to be developed into a method for the detection of nucleotide backbone interruptions or other factors that impede 3' extension by polymerase.

Certain algorithms for "Computing with DNA" [20] utilize bridging synthesis [21,22]. Noise in the context of computing with DNA could follow bridging between unintended sequences via the 3' end of a partially-extended primer. Bridging errors in the computation could limit the size of problem that can be solved experimentally using algorithms that involve bridging synthesis. Forensic DNA fingerprinting can be carried out via PCR and analysis of tandem repeats (e.g. [23]). Forensic samples may contain damaged DNA [24] and it seems likely that they are thereby prone to BPCR between repeated sequences [8] leading to artifacts.

Sources of noise in BPCR

PCR itself creates DNA damage and aberrant molecules. Taq polymerase harbors nuclease activities [2,3]. Sites of stem-loop structure are particularly vulnerable to both degradation and to polymerase stalling [25–27] both of which generate noise in BPCR. Gp32, an additive that increased S/N for Taq, is a single stranded binding protein that reduces the stem-loop structures [13] associated with both cleavage and stalling by polymerases [25][2,3,11,28]. Templates are damaged by thermal cycling [29] even in the absence of enzyme.

The buffer for Taq polymerase including $(\text{NH}_4)_2\text{SO}_2$ and for the Stoffel fragment had a low KCl concentration (10 mM). Since the stem-loop structure of the template is less likely to form at low KCl, this low concentration could also contribute to the increase of S/N. Because the Taq and Stoffel polymerase both reached the same optimum S/N of 100, it seems most likely that template damage via

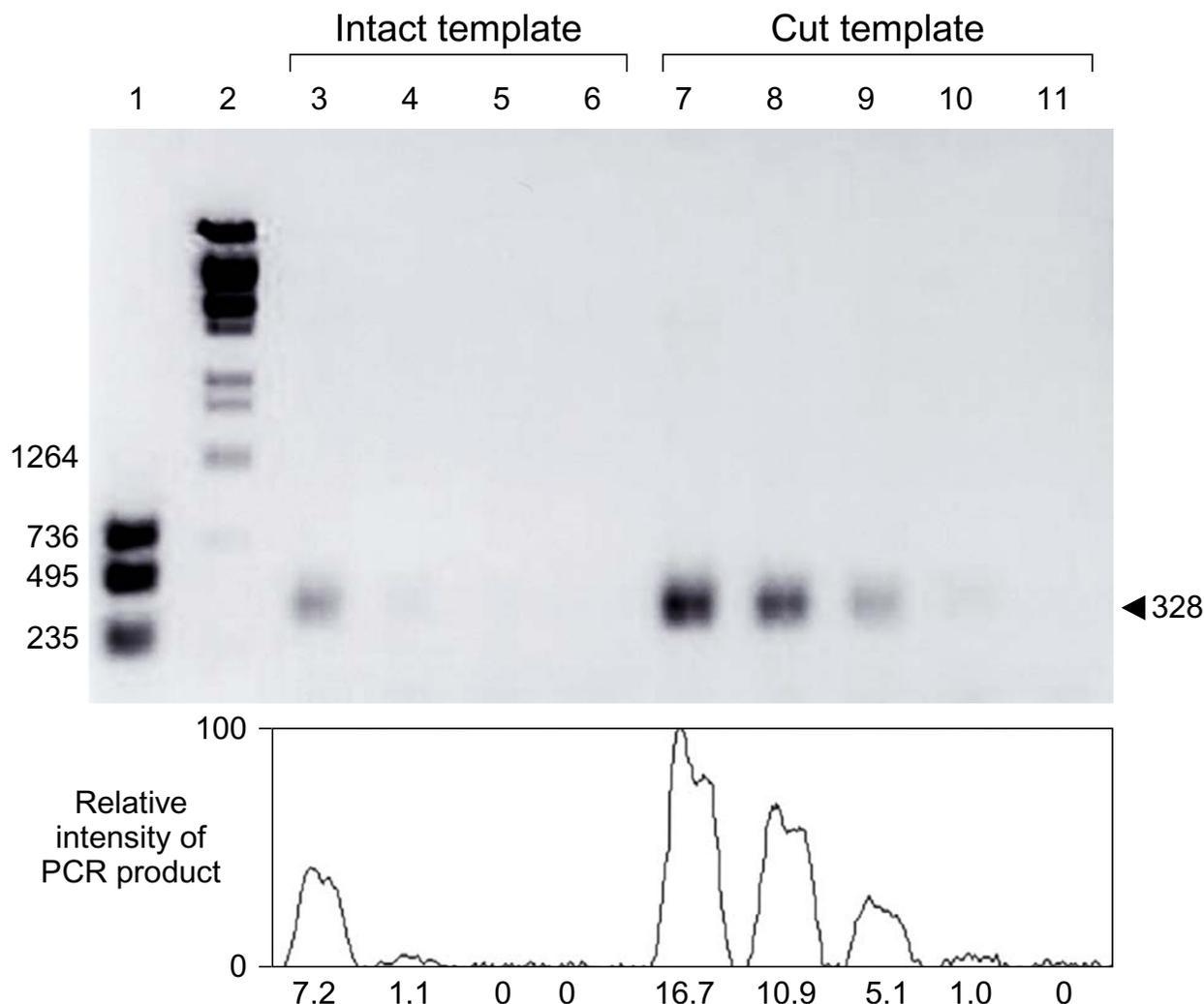


Figure 4
Stoffel fragment of Taq yields a S/N of 100 The Stoffel fragment of Taq polymerase was used in BPCR. The gel was 1% agarose containing ethidium bromide. The fluorescent image of the gel was acquired and processed with a Macintosh computer, a video camera and the software Scion Image 1.59 (from NIH). The gel image intensity was inverted, and the background was subtracted (horizontal ID). The band intensity of the 328 bp PCR product was recorded. The intensity profile curve of the 328 bp band is aligned and plotted below the gel lanes. The relative area under each peak is given below the peak. Area values are equalized to Lane 10, whose area value is set as 1.0. Lane 1, DNA size markers: a mixture of three separate PCR products: 235 bp (with primers b1 and M13 revl and template pBS(-)), 495 bp (primers M13 pIII and M13 revl and template Phagescript) and 736 bp (primers b1 and b2 and template pBS (-)). Lanes 2, size markers of Lambda DNA, BstE II-digested, 75 ng/lane. Lanes 3, 4, 5 and 6, PCR with intact source template. Each lane had 5 µl PCR product. Lanes 7, 8, 9, 10 and 11, PCR with source template cut with PvuII. Each lane had 5 µl PCR product. The concentrations of source template of lanes 3 and 7 are adjusted to be equal, 1×10^{-16} mol/µl PCR solution. Lanes 4, 5 and 6 are serial template dilutions of lane 3. Lanes 8, 9, 10 and 11 are serial template dilutions of lane 7.
 BPCR was carried out with the Stoffel fragment of Taq polymerase and primers b1 and M13 pV. (See Figure 2 for location of primers and Table 1 for their sequence.) The cut or intact template was made by PCR with the Stoffel fragment, primers b1 and Jp and a plasmid preparation of pBS(-). The other template (reference template) was ssDNA from Phagescript particles. The 1 × PCR buffer contains 10 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C) and 2.5 mM MgCl₂.

heating-cooling cycles as found by Gustafson et al. [29] is limiting S/N in this system.

Applications and limitations of bridging PCR as an assay for DNA damage

It is a basic aspect of materials science in any system to study discontinuities. This is especially the case in materials that store information. Several methods are available to assay discontinuities in DNA, and for many applications, one of the several current methods may suffice [30][31–36]. DNA backbone interruptions are key intermediates in genetic metabolism [37][38][39][40][41,42][43]. Chemotherapy [44], ionizing radiation [45] and dietary mutagens [46] also cause distributed DNA strand interruptions [47]. Chemical modifications [48–50][51] need not break the DNA backbone to impede the progress of DNA polymerase. Other modifications can be converted into backbone interruptions. For example, it has been proposed that alkali-sensitive ribonucleotides in DNA are a source of mutations [10].

It is likely that the presently attained S/N of 100 for BPCR is barely adequate for *in vivo* studies. Further improvement of the S/N by another factor of 10 or 100 would give greater assurance. Even with the achieved S/N of 100, we are faced with a dilemma because of the lack of other techniques that can detect a defect in less than 1% of the input DNA over a long region, 328 bp in this study. Background breaks at any site in the region contribute to noise, therefore the background breakage at any given site is apparently less than one in 30,000.

Conclusions

Break-copy recombination [52][53] almost inevitably occurs during PCR; it has been called variously: bridging PCR, jumping PCR, recombinogenic PCR, PCR sewing, gene shuffling, and sexual PCR. Sometimes this process is a 'bug' in the system leading to artifacts; sometimes it is a 'feature' leading to diverse libraries of products. Bridging PCR is to a certain extent- ca 3%/kb- found in this work, unavoidable in PCR. With a proper configuration of markers, BPCR can be used as a sensitive method for detecting discontinuities in DNA. In another context, fidelity of bridging synthesis is important for computing with DNA. For assay and computing applications it is necessary to consider noise in BPCR. In this work, BPCR was studied over a 328 base segment of the *lac* operon, and the noise level was measured as approximately 10% when no special precautions were taken with Taq polymerase. With buffer modifications or the addition of T4 gp32 the S/N of Taq was improved to 1%. The Stoffel fragment yielded a S/N of 1% without modifications of the standard protocol but was not further improved by buffer modifications or gp32. In this system with normal PCR using Taq polymerase,

the level of bridging that occurred was ca 30% per kilobase of amplified sequence. It must be cautioned that our results have been obtained in only one system with respect to the test sequence and the primers. Differences of the sequence and of reaction conditions are expected to alter the proportion of bridging that occurs in any particular system. With respect to sequence, it is anticipated that regions of potential secondary structure or other sites that are targets for the nuclease activity of Taq may become hotspots for "crossing over" during PCR.

Materials and Methods

The sources of materials used in this study: Phagescript and pBS(-) plasmid were from Stratagene; Taq polymerase and the Stoffel fragment of Taq were from Perkin Elmer Cetus; gp32 was from Pharmacia; *BstE* II-digested Lambda DNA was from New England Biolabs. *Pvu*II digestion was carried out according to the manufacturer's directions (New England Biolabs), and completeness was assayed by gel electrophoresis. Single stranded phage DNA was prepared from phage particle suspension with phenol extraction [54]. Phenol was tris-equilibrated at pH 8.0 (Sigma). The Bridging PCR reaction solution contained 0.5 mM dNTP's, 70 nM of each primer, 2.5 units/100 µl of polymerase in 1× buffer. (NH₄)₂SO₄ at 10 mM was used in some reactions (see Figure 3) as was Triton X-100 at 0.1%. When used (see Figure 3), gp32 was 0.01% (wt/vol).

The PCR thermal cycles were as follows: An initial 3 minutes at 94°C followed by 30 cycles, each cycle consisting of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C.

PCR was "hot start", i.e., the polymerase mixed in 10% of the final reaction volume in 1 × buffer was added to the rest of the mix during the initial denaturing step when the sample temperature was already at 94°C. Hot start and cycling was carried out on a Perkin Elmer Gene Amp 9600. PCR products were visualized by ethidium bromide staining and fluorescence after electrophoresis through 1.0% agarose gel.

Table 1: Primer sequences

Primer name	5'- sequence -3'
Jp	GCGAGTCAGTGAGCGAGG
b1	GTCGACCTGCAGGCATGCA
b2	CCCACACACGTGCTTGG
M13 pI	GTCTGTGCCACGTATTCT
M13 pV	GCTGTTGCCCGTCTCGCTG
M13revI	CTGGCAGCAGGTTTCCCGA
P11I	GCAACTGTTGGGAAGGGCGATCG

Primer sequences used in this study are given in Table 1 and primer locations are shown in Figure 2.

Authors' contributions

Author 1, SL, carried out the experiments and drafted part of the manuscript. Author 2, DST, conceived of the study, with author 1 created the experimental design, and wrote parts of the manuscript. Author 3, AJL, made intellectual contributions.

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