A plasmid-based lacZa gene assay for DNA polymerase fidelity measurement

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ABSTRACT
A significantly improved DNA polymerase fidelity assay, based on a gapped plasmid containing the lacZa reporter gene in a single-stranded region, is described. Nicking at two sites flanking lacZa, and removing the excised strand by thermocycling in the presence of complementary competitor DNA, is used to generate the gap. Simple methods are presented for preparing the single-stranded competitor. The gapped plasmid can be purified, in high amounts and in a very pure state, using benzoylated-naphthoylated DEAE-cellulose, resulting in a low background mutation frequency (~1 × 10⁻⁸). Two key parameters, the number of detectable sites and the expression frequency, necessary for measuring polymerase error rates have been determined. DNA polymerase fidelity is measured by gap filling in vitro, followed by transformation into Escherichia coli and scoring of blue/white colonies and converting the ratio to error rate. Several DNA polymerases have been used to fully validate this straightforward and highly sensitive system.

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DNA replication is a complex process that requires a multiprotein assembly (the replisome) to bring about the efficient, rapid, and accurate copying of the genome [1,2]. The replisome minimizes the appearance of detrimental mutations, thereby maintaining genomic stability, and the base substitution error rate of the Escherichia coli replication machinery in vivo is approximately 1 × 10⁻⁷ to 10⁻⁸ [3]. DNA polymerases make a major contribution to accuracy, and some replicative polymerases result in only one error per 10⁹ [4,5]. DNA polymerases are essential components of many biotechnological applications, especially the polymerase chain reaction (PCR), where accuracy is important [6–8]. Therefore, it is important both to understand the mechanisms that contribute to DNA polymerase fidelity and to develop assays sensitive enough to detect the infrequent errors that many polymerases make.

Several approaches to measure fidelity have been described; however, many are complicated or do not detect the whole spectra of mutations that may occur [9–11]. One of the most successful methods uses replication of a gap in the bacteriophage M13mp2 lacZa gene, encoding an inactive fragment of β-galactosidase, the α-peptide. When accurately copied, and subsequently introduced into E. coli that bears a complementing copy of the remaining β-galactosidase gene, functional β-galactosidase is reconstituted, resulting in the hydrolysis of X-gal and blue bacterial plaques [12]. Inaccurate polymerase activity may result in a defective α-peptide, eventually resulting in reduced or abolished β-galactosidase activity, indicated by light blue or colorless plaques. The error rate is calculated from the blue/colorless plaque ratio, and further information can be obtained from DNA sequencing. This method allows the detection of all 12 possible base substitutions as well as insertions and deletions in varying sequence contexts, is very well characterized, and has been used extensively by the Kunkel group for many investigations of DNA polymerases [4,5,12,13].

Previously, we described a plasmid-based method for measuring polymerase fidelity using pSJ1, a derivative of pUC18 [14]. In pSJ1, a segment encoding lacZa is flanked by two single-strand nicking endonuclease sites, allowing one of the DNA duplex strands to be specifically cut on both sides of the lacZa gene. Removal of the nicked DNA fragment results in a gapped plasmid containing the lacZa gene in the single-stranded region. The accuracy of a polymerase can be determined by copying the gene in vitro and then introducing the plasmid into E. coli, an approach similar in concept to that described above for the bacteriophage system. The plasmid-based system benefits from the simple preparation of pSJ1 in reasonable quantities and straightforward use in fidelity evaluation [14]. Because plasmids are compatible with many cell types, gapped variants can be used to study DNA replication and repair in vivo [15]. Nevertheless, the pSJ1 method is currently underdeveloped as compared with the much longer established bacteriophage approach. In particular, two key parameters, the number of detectable mutations (alterations in lacZa that

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1 Abbreviations used: PCR, polymerase chain reaction; dNTP, deoxyribonucleotide triphosphate; BND–cellulose, benzoylated–naphthoylated DEAE–cellulose; Pfu-Pol B, P. furiosus family B DNA polymerase; Taq-Pol, T. aquaticus DNA polymerase.

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result in an inactive gene and a white phenotype) and the expression frequency (the degree to which the polymerase-synthesized strand is expressed in *E. coli*), have yet to be determined. Knowledge of these features is required for converting the observed ratio of blue/white colonies/plaques to a polymerase error rate, measured as mistakes per base incorporated [12,13]. In this study, both the detectable mutations and the expression frequency have been measured, making the plasmid system fully equivalent to the bacteriophage. Furthermore, considerable improvements in the preparation and purification of the gapped plasmids are outlined, resulting in larger amounts of purer product and lowered background mutation rates. Together, these refinements substantially increase the scope and power of plasmid methods for polymerase fidelity measurements.

**Materials and methods**

**Materials**

Deoxyribonucleotide triphosphates (dNTPs), lambda (λ)-exonuclease, ExoIII, DNA ligase, and the restriction endonucleases EcoRI, DpnI, NdeI, and Sall were supplied by Fermentas (York, UK). Taq-Pol, for use in routine PCR applications, was also purchased from Fermentas. The nicking enzymes Nt.Bpu10I and Nt.BbvCI and the restriction enzyme BbvCI were purchased from New England Bio-labs (Ipswich, MA, USA). Plasmid-safe DNase was supplied by Cambio (Cambridge, UK). PCR cleanup, nucleotide removal, and miniprep and maxiprep kits were obtained from Qiagen (Crawley, UK). Benzoylated–naphthylated DEAE–cellulose (BND–cellulose) was obtained from Invitrogen (Paisley, UK), and Velocity PCR cleanup kit according to the supplier's protocol. With the product eluted in 35 μl of H2O preheated to 50 °C. 15 μl of this product was supplemented with 7 U of DpnI in the supplier's recommended buffer and incubated at 37 °C for 3 h to degrade the parental plasmid. Then 1 μl of this reaction was used to transform 60 μl of competent *E. coli* Top 10 cells, and recovered plasmids were completely sequenced to confirm the integrity of the mutagenesis.

**Identifying detectable sites in pSJ2 and pSJ3**

The lacZa DNA sequences in both pSJ2 and pSJ3 are very similar to that found in M13mp2. Therefore, nearly all of the detectable sites (i.e., base changes that result in an inactive α-peptide) are known. The status of the small number of lacZa bases in pSJ2 and pSJ3, not found in the bacteriophage, was determined using site-directed mutagenesis. The deoxynuclcoside at each of these uncharacterized positions was separately changed to all other three bases (for the promoter region) and to all bases that resulted in a new amino acid (for the protein coding region). PCR-based site-directed mutagenesis [18] was used, as described above, to make these alterations.

**Preparation of single-stranded lacZa competitor DNA**

Single-stranded DNA was generated using a PCR/λ-exonuclease method [14]. Two primers, one containing a 5′-phosphate and the second containing three phosphorothioates at the phosphodiesterases nearest the 5′ end (see Fig. S1 in Supplementary material), were used to amplify the lacZa gene in pSJ2 and pSJ3. The PCR mixture (100 μl) consisted of: plasmid (300 ng), primers (1.5 μM of each), dNTPs (200 μM of each), MgCl2 (1.5 mM), and Taq-Pol (5 U as defined by the supplier) in the supplier's recommended buffer. PCR consisted of 30 cycles: 35 s at 95 °C (1 min at 98 °C for the first cycle), 35 s at 55 °C, and 30 s (90 s on last cycle) at 70 °C. The amplified lacZa DNA was purified with a PCR cleanup kit. The 5′-phosphorylated strand was then specifically degraded by treating 2 μg of amplified product with 5 U of λ-exonuclease in 50 μl of the supplier’s recommended buffer for 30 min at 37 °C. The resulting single-stranded DNA was purified using a nucleotide removal kit. Alternatively, chemically synthesized oligodeoxynucleotides were used as competitors (Fig. S1).

**Preparation of gapped DNA**

Plasmid (40 μg) was digested with 40 U of Nt.BbvCI (pSJ2) or Nt.Bpu10I (pSJ3), both enzymes cut twice on the coding strand flanking the lacZa gene. Nicking was performed for 3 h at 37 °C in 1 ml of the supplier’s recommended buffer. This reaction was carried out five times in parallel (total of 200 μg plasmid). Nicked plasmid was gapped in 48 simultaneous reactions, each containing 3.5 μg (~2 pmol) of plasmid using either a 10-fold molar excess of PCR/λ-exonuclease–prepared single-stranded competitor (for pSJ2) or a 50-fold molar excess of chemically synthesized oligodeoxynucleotide competitor (for pSJ3). The aliquots of nicked plasmid and competitor DNA were each incubated in 100 μl of the nicking buffer and subjected to three heat/cool cycles of 95 °C for 1 min, 60 °C for 10 min, and 37 °C for 20 min. The reaction mixtures were then pooled, and gapped plasmid was separated from excess competitor and any displaced double-stranded lacZa DNA using four passes (reducing the volume ~ 10-fold in each pass) through a 100-kDa cutoff Amicon ultrafilter (Millipore). During ultrafiltration, the
gapped plasmid was simultaneously equilibrated to TE buffer (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA [ethylenediaminetetraacetic acid]). The process yielded 160 μg of crude gapped plasmid, which was subsequently purified using 2 g of BND–cellulose prepared as a 50% suspension in TE buffer containing 1 M NaCl. The DNA was gently agitated with 4 ml of BND–cellulose slurry for 10 min at room temperature, and the mixture was loaded into a column. The resin was washed with 60 ml of TE buffer containing 1.0 M NaCl, and the gapped plasmid was eluted with CFS buffer (TE buffer containing 1 M NaCl, 2% [w/v] caffeine, and 50% [v/v] formamide). Then 1-ml fractions were collected and assayed for the presence of gapped plasmid using 1% agarose gel electrophoresis. Appropriate fractions were pooled, and the gapped plasmid was reequilibrated to TE buffer using ultrafiltration as described above. Purified gapped plasmid (80 μg) was stored frozen in 20-μl aliquots at −20 °C.

Expression frequency determination

This investigation was carried out only with pSJ2. Initially, PCR-based site-directed mutagenesis was used to destroy the upstream nicking site, giving a derivative (pSJ2A) with only a single downstream nicking site. A further round of mutagenesis with pSJ2A was used to make a solitary base change that resulted in an in-phase premature stop codon toward the beginning of the lacZ coding sequence, yielding pSJ2B. The two plasmids, pSJ2A and pSJ2B, were then used to produce a heteroduplex with a single-stranded circular DNA and the linear duplex were derived from pSJ1, encoding an active gene. The heteroduplex and the control were used to transform E. coli TOP10 (see below), and the ratio of blue/white colonies was determined, allowing calculation of the expression frequency.

DNA polymerase fidelity assay

The DNA polymerases of pSJ2 and pSJ3 were filled by the DNA polymerase under investigation using 20 μl of 20 mM Tris–HCl (pH 8.0), 10 mM KCl, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 2 μg of bovine serum albumin, and 250 μM of each dNTP. The concentrations of the DNA polymerase and the gapped plasmid were 100 and 1 nM, respectively. Extension reactions were carried out at 70 °C for 30 min and, after this time, were examined using 1% agarose gel electrophoresis. Approximately 9 μl of the reaction mixture was analyzed directly by electrophoresis, and a further 9 μl was tested following digestion with 5 U of EcoRI (30 min at 37 °C). Provided that the electrophoresis/EcoRI analyses indicated complete extension, 1 μl of the remaining original reaction mixture was used for the transformation of competent E. coli Top 10 cells (60 μl). Transformed cells were plated on LB agar and scored for blue/white colonies as described above. To determine the appropriate background mutation rate for polymerase extension assays, the protocol was carried out in the absence of DNA polymerase (in this case, electrophoretic analysis is not required).

Colony counting

To facilitate counting of large numbers of blue and white E. coli colonies, a digital camera was used to take images of the plates, which were then analyzed by ImageQuant colony-counting software.

DNA sequencing

Mutant (white) colonies were grown overnight at 37 °C in LB medium containing 100 μg/ml ampicillin. Plasmid was purified using miniprep kits and sent to be sequenced. (GATC Biotech, Cambridge, UK).

Results

Redesigning the lacZx gene to give pSJ2 and pSJ3

To rigorously evaluate the error rate of a polymerase as mistakes per base incorporated, it is necessary to know the number of detectable mutations in the indicator gene. A particular advantage of the bacteriophage system, based on the lacZx sequence present in M13mp2, is that a large number of experiments have characterized nearly every detectable mutation, that is, exactly which base substitutions and insertions/deletions result in an inactive α-peptide and, hence, a change in plaque color from blue to white [4,5,12,13,21,22]. The plasmid previously used, pSJ1, encodes an active α-peptide, but its gene sequence is derived from pUC18 and, ultimately, M13mp18 [14]. Thus, as shown in Fig. 1A, the M13mp2 and pSJ1 lacZx sequences vary somewhat, making it complicated to apply the wealth of knowledge available for the phage system to this plasmid. As a remedy, two new plasmids have been designed with lacZx sequences that more closely match that found in M13mp2. One of the plasmids, pSJ2, has a lacZx sequence nearly identical to M13mp2, mainly differing in the presence of three and seven extra bases at the 5′ and 3′ ends, respectively (underlined in Fig. 1A), necessary for introducing the N(t/b),BbvCl nicking sites (Fig. 1B). The second plasmid, pSJ3, matches M13mp2 closely over the lacZx sequence that actually encodes protein (the additional upstream sequences present in both M13mp2 and pSJ2 are predominantly the lac promoter), with minor base changes at the 5′ and 3′ termini to accommodate the N(t/b),Bpu10I nicking enzyme used in this case (Fig. 1B). Further very slight sequence deviations are caused by the removal of dam
methyltransferase sites (GATC) from both pSJ2 and pSJ3 (Fig. 1A) to eliminate any complications arising from dam-targeted base mismatch repair [19]. With pSJ2 and pSJ3, the sizes of the gapped regions ultimately used for DNA polymerase fidelity assay are 288 and 163 bases, respectively.

Detectable sites in pSJ2 and pSJ3

The main features of pSJ2 and pSJ3 are illustrated in Fig. 1B. Both plasmids contain a lacZ gene sequence (given in full in Fig. 1A) flanked by two nicking endonuclease sites. The nicking enzymes (N.BbvCl and N.Bpu10I) are available as “t” or “b” forms, which specifically cut the “inner” strand (containing the lacZ coding sequence) or the “outer” strand (containing the lacZ noncoding sequence) of the plasmids shown in Fig. 1B. Thus, the alternative use of “t” and “b” gives two orientations of the gapped plasmid, named “+” (inner coding strand removed) and “−” (outer noncoding strand removed) in earlier studies with pSJ1 [14]. All of the studies reported in this article used the “+” gapped forms, that is, with the inner coding strand removed with the “t” variants of the nicking enzymes. However, a small number of investigations with pSJ2 and pSJ3 have the same “detectability”. The properties of the extra bases present in pSJ2 were determined by site-directed mutagenesis.

| lacZ gene source | Length of lacZ gene analyzed (bases) | Number of detectable base substitutions | Number of detectable insertions and/or deletions | Total number of detectable sites |
|------------------|-------------------------------------|---------------------------------------|-----------------------------------------------|---------------------------------|
| M13mp2           | 729                                 | 324                                   | 204                                           | 548                            |
| pSJ1             | 288                                 | 242                                   | 206                                           | 494                            |
| pSJ3             | 288                                 | 242                                   | 206                                           | 494                            |
| pSJ2             | 288                                 | 242                                   | 206                                           | 494                            |
| pSJ3             | 288                                 | 242                                   | 206                                           | 494                            |

Fig. 1. The main features of pSJ2 and pSJ3, plasmids useful for measuring DNA polymerase fidelity. (A) The lacZ peptide and lac promoter sequences of M13mp2, pSJ1, pSJ2, and pSJ3. Only the bases actually used in fidelity determination are shown; thus, pSJ1 and pSJ3 contain the lac promoter, but because of the nicking site positions, this element is not used in fidelity assays with these two plasmids. The symbol + indicates identical bases in the lacZ peptide (for all four sequences) and in the lac promoter (for M13mp2 and pSJ2). The underlined sequences are the extra bases at the extremities of pSJ2, necessary for introducing the nicking sites. The ATG triplet that encodes the first methionine in the lacZ peptide is shown, as is the dam methylase (GATC) site altered in pSJ2 and pSJ3. The C shown with the symbol + is the base altered in pSJ2 to introduce a stop codon for expression frequency determination. In all cases, the coding sequence of the lacZ gene (as given in full in Table 1) is illustrated. (B) Plasmid maps of pSJ2 and pSJ3 showing the location and orientation of the lacZ sequences and the nicking endonuclease sites, Nt.BbvCl for pSJ2 and Nt/Bpu10I for pSJ3. The EcoRI site used for analytical purposes is also shown. The abbreviations dcs and ucs stand for downstream and upstream cutting sites, respectively.
as expected, provided that the smaller size of the lacZx gene in pSJ3 is taken into account. A full list of the detectable sites is given in the Supplementary material (Fig. S2).

Preparation of gapped pSJ2 and pSJ3

With pSJ1, preparation of the gapped derivative required cutting the plasmid with the appropriate nicking enzyme and heating in the presence of a single-stranded competitor DNA [14]. The competitor was designed to be complementary to the nicked strand and so to sequester it during the heat/cool cycle to give the gapped plasmid. Previously, single-stranded competitor preparation used PCR amplification with two primers: one with a 5'-OH and the other containing a 5'-phosphate. Subsequent degradation of the PCR product with λ-exonuclease, an enzyme that preferentially degrades duplex DNA from ends containing a 5'-phosphate, yields the desired single strand [23]. Unfortunately, λ-exonuclease does not absolutely discriminate between 5'-phosphorylated and 5'-OH ends; therefore, some destruction of the required strand occurs. To further improve selectivity, the 5'-phosphorylated PCR primer has been combined with one containing a 5'-OH group and phosphorothioate modifications at the three internucleotide phosphodiester groups nearest the 5' end (Fig. S1). DNA substituted with phosphorothioates is commonly observed to be nuclease resistant [24], and the new combination of primers is expected to better target λ-exonuclease to the 5'-phosphorylated strand. Indeed, the new combination of primers reliably produced higher concentrations of competitor DNA than those seen when the phosphorothioates were missing. Typically, approximately 17.5 μg of single-stranded product was produced from 100-μl PCR volumes using the triple phosphorothioated primer. In contrast, approximately 7.5 μg of product was produced with a normal primer lacking phosphorothioate protection. A single-stranded competitor produced in this manner, 288 bases in length and fully complementary to the excised lacZ sequence, could be used to produce gapped pSJ2, as shown in Fig. 2A. In this figure, the starting nicked plasmid and desired gapped product are poorly resolved. However, treatment with the restriction endonuclease EcoRI converts the nicked plasmid (which contains a GAATTC EcoRI site in double-stranded DNA) to the linear form. The gapped plasmid, in which GAATTC is in single-stranded DNA, is inert toward EcoRI. The gapped and linear plasmids are well resolved (Fig. 2A), and the relative intensities of their bands are indicative of the gapping yield. In agreement with earlier studies using pSJ1, a 10-fold excess of the competitor was optimal [14].

Although the use of phosphorothioate-containing primers improves the PCR method for producing competitor, it is still difficult to generate large amounts of material, which is important because a 10-fold excess is required. As an alternative, the use of chemically synthesized DNA competitors has been explored. Standard solid-phase synthesis of oligodeoxynucleotides is limited to lengths of approximately 100 bases [25], shorter than the gapped regions produced when using pSJ2 (288 bases) and pSJ3 (163 bases). However, two competitors approximately 80 bases in length, designed to almost completely cover the 163-nucleotide lacZx gene of pSJ3 (Fig. S1), were effective in gapping (Fig. 2B). Again EcoRI treatment was required to distinguish the starting nicked plasmid and the gapped product. These two oligodeoxynucleotides could be used without purification, but a high ratio of competitor to plasmid, 50:1, was needed (with the PCR/λ-exonuclease method, a 10-fold excess suffices). Nevertheless, the higher amounts of material available from chemical synthesis compared with PCR make this approach worthwhile. Unfortunately, the use of chemically synthesized DNA was relatively inefficient with pSJ2. Attempts to gap the 288-nucleotide lacZx region with two oligodeoxynucleotide sets (the first consisting of five strands and the second consisting of four strands, with each DNA strand between 50 and 80 bases long) were largely unsuccessful (Fig. S1). Much of the starting nicked double-stranded plasmid remained in the mixture, and little of the required gapped derivative was produced (see Fig. S3 in Supplementary material). Therefore, the PCR method for competitor preparation needed to be used with this plasmid.

**Purification of gapped pSJ2 and pSJ3**

In earlier work, gapped pSJ1 was purified by gel electrophoresis, a method that limits the amount of product that can easily be prepared [14]. Gapped plasmids contain a stretch of single-stranded DNA, which results in the exposure of relatively hydrophobic bases, normally buried in double-stranded DNA. This enabled their isolation using BND–cellulose, a material that interacts strongly...
with hydrophobic regions and so binds tightly to gapped plasmids [26,27]. Elution of the gapped plasmids from BND–cellulose uses a solution of caffeine. Purification using BND–cellulose is simpler and quicker than gel electrophoresis and produces larger quantities of gapped plasmid. Starting from 200 μg of pSJ2 or pSJ3, 80 μg of gapped plasmid (40% yield) was typically obtained and shown by gel electrophoresis to be extremely pure (Fig. 2C). Treatment of the purified gapped plasmids with EcoRI did not give rise to an additional linear product of faster mobility, as would be expected in cases of contamination with nicked starting plasmid (Fig. 2C). The BND–cellulose purification can be scaled up to handle milligram (mg) quantities of plasmid. As described below, plasmids isolated using BND–cellulose resulted in a lower background mutation rate than that observed using electrophoresis, presumably arising from less damage to the DNA bases. This is a significant advantage for fidelity measurements with highly accurate DNA polymerases.

**Expression frequency of pSJ2 and pSJ3**

When a polymerase makes a mistake during the filling in of a gapped plasmid, either a heteroduplex with a single-base mismatch or a frame shift usually results. However, this will be scored as a polymerase error only if the coding information in the newly synthesized strand is used to direct lacZ peptide synthesis. The gap-filled pSJ2 and pSJ3 plasmids used for *E. coli* transformation are dam-neutral; that is, all of the GATC sites outside the lacZ gene are fully methylated, and the gene itself lacks dam sequences. Therefore, hemi-methylated dam sequences, which would arise from polymerase filling of a lacZ gene containing GATC runs, are lacking. Hemi-methylation of dam sequences is used to guide mismatch repair in *E. coli* [19], and in its absence one would predict that base mismatches would be restored in an undirected manner. Thus, when a mismatch is produced, the polymerase introduced base and the original template base are each replaced 50% of the time, a number that corresponds to the expression frequency. However, polymerase-filled pSJ2 and pSJ3 also contain a nick at the 3’ end of the lacZ gene, which could influence mismatch repair [19], making it important to experimentally determine the expression frequency. To do this, a plasmid identical to that produced after a mistake in gap filling (i.e., with a detectable error in the equivalent of polymerase-generated strand and a 3’ nick) has been generated. Full details are given in the Supplementary material (Fig. S4). In brief, they involve changing a single C to a T (Fig. 1A) and converting a CAA codon (which specifies Gln13 in the lacZ peptide) to a TAA stop codon, giving a truncated inactive α-peptide. The sequences specifying an “active” and “inactive” lacZ peptide can be blended to give a heteroduplex with a G:T mismatch (Fig. S4). In this heteroduplex, the noncoding strand bears the wild-type triplet (TTG, specifying CAA on the coding strand following replication), and the coding strand possesses the stop codon (TAA). The coding strand also has a nick at the end of the lacZ gene; therefore, the coding and noncoding strands are equivalent to newly synthesized and parental sequences in a polymerase extension assay. Depending on how the G:T mismatch is repaired, either an active (blue colonies) or inactive (white colonies) lacZ peptide will be produced, with the ratio of blue/white colonies reporting on which base in the mismatch is changed. When the G:T heteroduplex was used to transform *E. coli*, 44.4% of the colonies observed were white, resulting from a truncation in the lacZ peptide due to the stop codon (Table 2). For completely random repair, a value of 50% would be expected; therefore, the value of 44.4% indicates that repair of base mismatches in pSJ2 (and presumably pSJ3) is essentially random. For all subsequent experiments, 0.444 has been used as the expression frequency. A control experiment used a homoduplex having a wild-type CAA (Gln13) codon but produced in exactly the same manner as the heteroduplex (Fig. S4). Transformation of *E. coli* with this control plasmid resulted in only blue colonies; no white colonies, which would indicate a mutation in the lacZ gene, were observed (Table 2).

**Table 2**

| Nicked plasmid | Total number of colonies | Number of mutant (white) colonies | % Mutant colonies | Expression frequency of lacZ gene |
|----------------|--------------------------|----------------------------------|------------------|----------------------------------|
| Heteroduplex   | 7603                     | 3379                             | 44.4%            | 0.444                            |
| Homoduplex     | 7393                     | 0                                | 0                | Not applicable                   |

* Obtained by summing three independent determinations.

**Table 3**

| Plasmid | Total number of colonies | Number of mutant (white) colonies | Mutation rate (%) | Mutation frequency |
|---------|--------------------------|----------------------------------|------------------|-------------------|
| pSJ2    | 39,119                   | 1                                | 0.0026           | 2.6 x 10^{-5}     |
| pSJ2 (gapped, BND–cellulose purified) | 28,406 | 3                               | 0.011            | 1.1 x 10^{-4}     |
| pSJ2 (gapped, gel purified) | 26,313 | 14                              | 0.033            | 5.3 x 10^{-4}     |
| pSJ3 (gapped, BND–cellulose purified) | 32,519 | 1                               | 0.0031           | 3.1 x 10^{-5}     |
| pSJ1†  | 39,119                   | 1                                | 0.08             | 8.0 x 10^{-4}     |
| M13mp2 (phage) | – | – | 0.04–0.06 | 4.0–6.0 x 10^{-4} |

* Plasmid DNA isolated from the four mutant colonies found with gapped pSJ2 and gapped pSJ3 (purified using BND–cellulose) was sequenced. Three of the mutations in the lacZ gene were C → T/G → A transitions. The fourth was a T → C/A → G transition.

† Each data set with pSJ2 and pSJ3 was the sum of five independent experiments carried out in triplicate.

† Data taken from a previous study[14].

‡ Data taken from previous studies[12,22].
colonies are observed, making it difficult to determine the extremely low background mutation rate accurately. For comparison, the background seen using gapped pSJ2 purified by gel electrophoresis is also given, as are previous values observed for nicked pSJ1 [14] and M13mp2 [12,13]. It is clear that gapped pSJ2 purified using the BND–cellulose method shows a 5-fold reduction in background errors when compared with the gel extraction method. BND–cellulose purified gapped pSJ3 has an even lower background mutation frequency, almost certainly due to the shorter gap in lacZ. The very low backgrounds observed with pSJ2 and pSJ3 represent a significant improvement over earlier lacZ-based systems. Although only four white colonies were observed with pSJ2 and pSJ3, these have been fully sequenced to confirm the presence of a mutation. In three cases, a C → T/G → A transition was observed (Table 3), a mutation also overrepresented in an earlier study using pSJ1 [14]. This transition is most likely caused by the deamination of cytosine to uracil, a thymidine mimic.

Validation of pSJ2 and pSJ3

To check whether the new plasmids are suitable for assessing the fidelity of DNA polymerases, error rates have been determined for a number of well-characterized enzymes. Studies have been carried out with the Klenow fragment of the family A polymerase from T. aquaticus (Taq-Pol) and three variants of the family B polymerase from P. furiosus (Pfu-Pol): wild type, a 3′–5′ proofreading exonuclease-deficient mutant (D215A/E143A) [16], and an even more error-prone variant (D215A/E143A/D473G) [17]. In each case, the polymerase was used to fill gapped pSJ2 and pSJ3 in vitro, and successful extension was confirmed by EcoRI digestion and gel electrophoresis (Fig. 3). The filled plasmids were used for the transformation of E. coli, and the numbers of blue and white colonies were counted, enabling the determination of the mutation frequency (MF) (Table 4). To determine the error rate (ER) the following equation, which makes explicit the reasons for determining the detectable sites (D) and expression frequency (P), was used [12,22]:

\[
ER = \frac{N_i}{N \times MF} \frac{D}{P}
\]

where

- \(N_i\) = number of a particular type of mutation (usually a deletion/insertion or base substitution)
- \(N\) = total number of mutations
- \(MF\) = observed mutation frequency – background mutation frequency
- \(D\) = number of detectable sites for a particular mutation
- \(P\) = probability of expressing the mutant lacZ gene (expression frequency).

With the above equation, the type of mutation (\(N_i\)) can be determined only by DNA sequencing of mutant (white colonies) lacZ genes. In the absence of sequencing, \(N_i/N = 1\), and the equation can determine only total mutations [12,13,22].

The fidelity of wild-type Pfu-Pol has been characterized previously, and typically an error rate of between \(1.3 \times 10^{-6}\) and \(1.6 \times 10^{-6}\) is observed [6,8], similar to the values observed using both pSJ2 and pSJ3 (Table 4). Pfu-Pol lacking the 3′–5′ exonuclease (D215A/E143A) has an increased error rate due to loss of proofreading activity, and an approximately 2- to 4-fold decrease in fidelity is seen with the plasmid systems. An additional mutation (D473G) to a loop in the fingers domain further increases the number of mistakes made on replication [17], and an approximately 3- to 4-fold increase in error rate is observed when compared with Pfu-Pol (D215A/E143A) (equivalent to an ~10-fold increase compared with the wild type). Finally, the fidelity of Taq-Pol, a family A DNA polymerase that lacks proofreading exonuclease activity, was determined. The accuracy of Taq-Pol is strongly dependent

Table 4

| Polymerase | Gapped plasmid | Number of colonies | Number of mutant (white) colonies | Mutation frequency | Error rate |
|------------|----------------|-------------------|-----------------------------------|-------------------|------------|
| Pfu-Pol B  | pSJ2           | 25,700            | 11                                | 3.2 \(\times 10^{-4}\) | 1.6 \(\times 10^{-5}\) |
|            | pSJ3           | 20,116            | 11                                | 5.2 \(\times 10^{-4}\) | 3.5 \(\times 10^{-5}\) |
| Pfu-Pol B (D215A/E143A)* | pSJ2 | 14,601 | 20 | 1.3 \(\times 10^{-3}\) | 6.3 \(\times 10^{-6}\) |
|            | pSJ3 | 24,766 | 25 | 1.0 \(\times 10^{-3}\) | 6.7 \(\times 10^{-6}\) |
| Pfu-Pol B (D215A/E143A/D473G)† | pSJ2 | 78,431 | 296 | 3.7 \(\times 10^{-3}\) | 1.8 \(\times 10^{-4}\) |
|            | pSJ3 | 38,836 | 141 | 3.6 \(\times 10^{-3}\) | 2.4 \(\times 10^{-4}\) |
| Taq-Pol    | pSJ2           | 46,239            | 98                                | 2.0 \(\times 10^{-3}\) | 1.0 \(\times 10^{-4}\) |
|            | pSJ3 | 20,756 | 34 | 1.6 \(\times 10^{-3}\) | 1.1 \(\times 10^{-5}\) |

* All of the gapped plasmids used in these experiments had the coding strand (inner strand in Fig. 1B) removed by treatment with Nt.BbvCl (pSJ2) or Nt.BpuIol (pSJ3).

† Sum of three independent experiments, each consisting of five repeats.

‡ The mutation frequencies given here have had the background mutation frequencies found for gapped pSJ2 and pSJ3 subtracted.

§ Error rates were calculated using the formula given in the text. An expression frequency (P) of 0.444 was used. In the absence of extensive DNA sequencing, an \(N_i/N\) value of 1 was used and the number of detectable sites (D) was the sum of the values for base substitutions plus insertions/deletions, that is, 448 for pSJ2 and 329 for pSJ3 (Table 1).

† The mutation D215A/E143A disables the 3′–5′ proofreading exonuclease activity [16].

† The triple mutation D215A/E143A/D473G has even lower fidelity than the exonuclease-deficient mutant D215A/E143A [17].
on the reaction conditions, and error rates of between $2 \times 10^{-4}$ and $8 \times 10^{-6}$ have been reported [6,8,13,21], in agreement with the value of $1 \times 10^{-5}$ found with pSJ2 and pSJ3. One study [6] showed that Taq-Pol was approximately 6 times less accurate than Pfu-Pol, not too dissimilar to the decrease in fidelity found using pSJ2 and pSJ3 (Table 4). The good general agreement found between earlier investigations and the current studies with pSJ2 and pSJ3 suggests that both plasmids are suitable for determining the error rates of DNA polymerase. The mutant (white) colonies from the pSJ2 assays of Pfu-Pol B wild type and the exonuclease-deficient variant were sequenced to determine further information on the mutation spectra. As was observed previously, a significant proportion of the mutants were C → T/G → A transitions, probably as a result of template strand cytosine deamination as described above for background mutation rate. This form of DNA damage was also observed in the pSJ1 and M13 methods, most likely also as a result of damage during substrate preparation [4,12,14]. However, because the background mutation frequency observed using pSJ2 and pSJ3 is lower, the influence of background mutations is reduced. To obtain rigorous data concerning the mutation spectrum of Pfu-Pol, a far higher number of mutants would obviously need to be sequenced. Nevertheless, this small data set confirms that each time a white mutant colony is observed with pSJ2, it is faithfully reflected in a DNA sequence error.

Discussion

A considerably improved plasmid-based DNA polymerase fidelity assay has been described. The new plasmids, pSJ2 and pSJ3, are elaborations of an earlier version, pSJ1 [14], and contain a lacZα gene flanked by two nicking endonuclease sites. With pSJ2 and pSJ3, both the number of detectable sites and the expression frequency have been determined, factors necessary for determining polymerase error rates and not previously available for pSJ1. The expression frequency of 44.4% found for pSJ2 is near the theoretical maximum of 50% expected for random repair of mismatches, suggesting high plasmid integrity with minimal base damage. The critical step in applying pSJ2 and pSJ3 is gapping. As found with pSJ1, gapping cannot simply be carried out using heat to remove the strand excised in the nicking step. Rather, a complementary single-stranded competitor needs to be added to sequester the excised strand. The assay described in this article improves the preparation of relatively long competitors, using PCR followed by λ-exonuclease digestion, by exploiting the exonuclease resistance of phosphorothioate-protected primers. Although the phosphorothioate improvement consistently resulted in higher yields of competitor, PCR-based methods tend to give relatively small amounts of amplified material. Higher amounts of single-stranded DNA are available by direct chemical synthesis, but here the lengths that can be prepared become limiting. Thus, two competitors were required to successfully gap pSJ3; unfortunately, the use of four or five competitors to gap the longer pSJ2 failed. Gapping remains the most demanding part of the protocol, and research is still required to enable the production of long competitors in high quantity or to develop an alternative protocol. The use of BND–cellulose to isolate gapped plasmids represents a major improvement over gel-based purification techniques. Greater amounts can be prepared in a very straightforward manner and (importantly) the resulting gapped plasmids appear to be of much higher quality with little damage to the bases. This is evidenced by the very low background mutation rates seen for both pSJ2 and pSJ3 prepared using BND–cellulose, reduced approximately 5-fold as compared with gel-purified material. The low backgrounds seen with gapped pSJ2 ($1 \times 10^{-4}$) and pSJ3 ($3 \times 10^{-5}$) are extremely advantageous when studying high-fidelity polymerases. The suitability of the new plasmids was confirmed by determining the error rates of Pfu-Pol and Taq-Pol, which gave values similar to those obtained by a number of other methods. So far, pSJ2 and pSJ3 have been used only to test the fidelity of DNA polymerases in vitro. However, due to the compatibility of plasmids with many bacteria and eukaryotes, the in vivo study of DNA replication and repair should be possible, as has already been described for a plasmid-based mismatch repair activity assay [15].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jab.2012.10.019.

References

[1] A. Johnson, M. O’Donnell, Cellular DNA replicases: components and dynamics at the replication fork, Annu. Rev. Biochem. 74 (2006) 283–315.
[2] P.M.J. Burgers, Polymerase dynamics at the eukaryotic DNA replication fork, J. Biol. Chem. 284 (2009) 4041–4045.
[3] R.M. Schaaper, Base selection, proofreading, and mismatch repair during DNA replication in Escherichia coli, J. Biol. Chem. 268 (1993) 23762–23765.
[4] T.A. Kunkel, P.S. Alexander, The base substitution fidelity of eukaryotic DNA polymerases: mispairing frequencies, site preferences, insertion preferences, and base substitution by dislocation, J. Biol. Chem. 261 (1986) 160–166.
[5] S.D. McCulloch, T.A. Kunkel, The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases, Cell Res. 18 (2008) 148–161.
[6] J. Cline, J.C. Braman, H.H. Hogrefe, PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases, Nucleic Acids Res. 24 (1996) 3546–3551.
[7] A. Pavlov, N.V. Pavlova, S.A. Kozovikin, A.I. Slesarev, Recent developments in the optimization of thermostable DNA polymerases for efficient applications, Trends Biotechnol. 22 (2004) 253–260.
[8] K.S. Lundberg, D.D. Shoemaker, M.W.W. Adams, M. Short, J.A. Sorge, E.J. Mathre, High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosis, Gene 108 (1991) 1–6.
[9] A. Maor-Shoshani, N.B. Reuven, G. Tomer, Z. Livneh, Highly mutagenic replication by DNA polymerase V (UmuC) provides a mechanistic basis for SOS targeted mutagenesis, Proc. Natl. Acad. Sci. USA 97 (2002) 565–570.
[10] K.A. Eckert, A. Mowery, S.E. Hills, Misalignment-mediated DNA polymerase β mutations: comparison of micro-satellite and frame-shift errors using a forward mutation assay, Biochemistry 41 (2002) 10490–10498.
[11] S. Fujii, M. Aoyama, K. Aoki, Y. Sugaya, K. Higuchi, M. Hiraoka, Y. MiKi, N. Saitoh, K. Yoshiyama, K. Ibara, M. Seki, E. Ohtsubo, H. Maki, DNA replication errors produced by the replicative apparatus of Escherichia coli, J. Mol. Biol. 289 (1999) 835–850.
[12] H. Behebnek, T.A. Kunkel, Analyzing fidelity of DNA polymerases, Methods Enzymol. 262 (1995) 217–232.
[13] K.R. Tindall, T.A. Kunkel, Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase, Biochemistry 27 (1998) 6008–6013.
[14] S.K. Jozwiakowski, B.A. Connolly, Plasmid-based lacZα assay for DNA polymerase fidelity: application to archaeal family-B DNA polymerase, Nucleic Acids Res. 37 (2009) e102.
[15] B. Zhou, C. Huang, J. Yang, J. Lu, L. Z.-Sun, Preparation of heteroduplex enhanced green fluorescent protein plasmid for in vivo mismatch repair activity assay, Anal. Biochem. 388 (2009) 167–169.
[16] S.J. Evans, M.J. Fogg, A. Mamone, M. Davis, L.H. Pearl, B.A. Connolly, Improving dideoxynucleotide-triphosphate utilization by the hyper-thermophilic DNA polymerase from the archaeon Pyrococcus furiosus, Nucleic Acids Res. 28 (2000) 1059–1066.
[17] B.D. Biles, B.A. Connolly, Low-fidelity Pyrococcus furiosus DNA polymerase mutants useful in error-prone PCR, Nucleic Acids Res. 32 (2004) e176.
[18] L. Zheng, U. Baumann, J.-L. Reymond, An efficient one-step site-directed and site-saturation mutagenesis protocol, Nucleic Acids Res. 32 (2004) e115.
[19] P. Modrich, Methyl-directed DNA mismatch correction, J. Biol. Chem. 264 (1989) 6597–6600.
[20] J. Chiu, P.E. March, R. Lee, D. Tillett, Site-directed, ligase-independent mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h, Nucleic Acids Res. 32 (2004) e174.

[21] K.A. Eckert, T.A. Kunkel, High fidelity DNA synthesis by the Thermus aquaticus DNA polymerase, Nucleic Acids Res. 18 (1990) 3739–3744.

[22] J.M. Fortune, Y.I. Pavlov, C.M. Welch, E. Johansson, P.M.J. Burgers, T.A. Kunkel, Saccharomyces cerevisiae DNA polymerase delta: High fidelity for base substitutions but lower fidelity for single- and multi-base deletions, J. Biol. Chem. 280 (2005) 29980–29987.

[23] E. Thomas, A. Pingoud, P. Friedhoff, An efficient method for the preparation of long heteroduplex DNA as a substrate for mismatch repair by the Escherichia coli MutHLS system, Biol. Chem. 383 (2002) 1459–1462.

[24] P. Guga, M. Koziolkiewicz, Phosphorothioate nucleotides and oligonucleotides: recent progress in synthesis and applications, Chem. Biodiv 8 (2011) 1642–1681.

[25] R.A. Hughes, A.E. Miklos, A.D. Ellington, Gene synthesis: methods and applications, Methods Enzymol. 498 (2011) 277–309.

[26] H. Wang, J.B. Hays, Simple and rapid preparation of gapped plasmid DNA for incorporation of oligomers containing specific DNA lesions, Mol. Biotechnol. 19 (2011) 133–140.

[27] I. Gillam, S. Millward, D. Blew, M. von Tigerstrom, E. Wemmer, G.M. Terner, The separation of soluble ribonucleic acids on benzoylated diethylaminoethylcellulose, Biochemistry 5 (1967) 3043–3056.