Detection of extremely rare alleles by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification (Bi-PAP-A): measurement of mutation load in mammalian tissues

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Pyrophosphorolysis-activated polymerization (PAP) was developed to detect extremely rare mutations in complex genomes. In theory, PAP can detect a copy of a single base mutation present in $3 \times 10^{11}$ copies of the wild-type allele. In practice, the selectivity of detection is limited by a bypass reaction involving a polymerase extension error from the unblocked oligonucleotide annealed to the opposing strand. Bidirectional PAP allele-specific amplification (Bi-PAP-A) is a novel method that uses two opposing 3′-terminal blocked pyrophosphorolysis-activatable oligonucleotides ($P^*$s) with one nucleotide overlap at their 3′ termini. This eliminates the problematic bypass reaction. The selectivity of Bi-PAP-A was examined using λ phage DNA as a model system. Bi-PAP-A selectively detected two copies of a rare mutated allele in the presence of at least $2 \times 10^9$ copies of the wild-type λ phage DNA. Bi-PAP-A was then applied to direct detection of spontaneous somatic mutations in the mouse genome at a frequency as low as $3 \times 10^{-9}$. A 370-fold variation in the frequency of a specific somatic mutation among different mouse samples was found, suggesting clonal expansion of mutation occurring during early development and a hyper-Poisson variance. Bi-PAP-A is a rapid, general, and automatable method for the detection of rare mutations.

INTRODUCTION

Mutations are substrates of human disease. A method of detecting one mutated allele in $10^6$–$10^9$ wild-type alleles would be advantageous for multiple applications including: detection of minimal residual disease; cancer risk assessment by searching for elevated mutation load due to endogenous DNA repair defects or environmental mutagen exposures (i.e., measuring the frequency and pattern of somatic mutations present in normal tissues); and prenatal diagnosis of paternally derived mutations within fetal cells in the maternal circulation (1).

Multiple genotypic methods for detecting mutations present in less than 10% of cells (i.e., rare alleles) have been developed (2). Three types of methods for the direct analysis of point mutations include: (i) preferential amplification of the rare allele [e.g., PCR amplification of specific alleles (PASA) (3), mutagenically separated PCR (MS-PCR) (4), allele-specific competitive blocker PCR (ACB-PCR) (5), mismatch amplification mutation assay (MAMA) (6), blocker-PCR (7), peptide nucleic acid (PNA) clamping blocker PCR (8), ligation chain reaction (LCR) (9), and Gap-LCR (10)]; (ii) preferential destruction of the wild-type allele [e.g., restriction fragment length polymorphism (RFLP)/PCR (11), PCR/RFLP (12), and mismatch protection from exonuclease cleavage by MutS followed by PCR (MutEx/PCR) (13)]; or (iii) spacial separation of the mutant and wild-type alleles [e.g., single-stranded conformational polymorphism (SSCP) (14), denaturing gradient gel electrophoresis (DGGE) (15), constant denaturant capillary electrophoresis (CDCE) (16), multiple rounds of CDCE (17), competitive mobility shift assay (CMSA) (18), and magnetic mismatch binding beads (M$_2$B$_2$) (Genecheck, Fort Collins, CO, USA)].

PASA [allele-specific PCR (AS-PCR) or amplification refractory mutation system (ARMS)], a widely used method, routinely detects a mutated allele in 40–200 copies of the wild-type allele (3). RFLP/PCR, the most sensitive of the methods, has been reported to detect a mutated allele in $10^6$ wild-type alleles (11); however, it has several drawbacks. RFLP/PCR is highly labor-intensive, the mutation must be within an appropriate restriction endonuclease site, and the cleavage efficiencies of many restriction endonucleases limit the selectivity of the method to a range of 1:10$^3$ to 10$^5$ (2). The above methods...
are not commonly utilized because they do not generally achieve high selectivity, are very laborious, require specific sequence contexts, and/or are not currently robust for routine analysis.

Pyrophosphorolysis-activated polymerization (PAP) is a novel method for nucleic acid amplification (19,20). In PAP, pyrophosphorolysis and polymerization are serially coupled by DNA polymerase using pyrophosphorolysis-activatable oligonucleotides (P*s). P*s are blocked at their 3′ termini with dideoxynucleotide but can be activated by pyrophosphorolysis. When a P* anneals to its complementary template, the 3′-terminal blocker can be removed by pyrophosphorolysis in the presence of pyrophosphate, and the activated oligonucleotide can then be extended by DNA polymerization.

The extreme selectivity of PAP results from the use of P*s to serially couple pyrophosphorolysis (21) and polymerization (Figure 1A) (19). Significant nonspecific amplification (Figure 1A, Type II error) requires mismatch pyrophosphorolysis followed by misincorporation by the DNA polymerase, an event with frequency estimated to be $3.3 \times 10^{-11}$ (19). After demonstrating proof-of-principle (19), the efficiency of PAP was greatly improved using AmpliTaq® FS (Applied Biosystems, Foster City, CA, USA), a genetically engineered DNA polymerase, and it was also demonstrated that PAP was inhibited by mismatches along the length of P* (20). Unfortunately, the actual selectivity of PAP was 1:10$^4$ to 10$^5$ because of misincorporation within the extension product of the unblocked oligonucleotide (Figure 1B). To solve this problem, bidirectional PAP allele-specific amplification (Bi-PAP-A) was developed (Figure 2A). In Bi-PAP-A, PAP is simultaneously performed in two directions with two opposing P*s that overlap by one nucleotide at their 3′ termini.

Bi-PAP-A has been shown to detect a single copy of a template directly from mammalian genomic DNA; achieve an extremely high selectivity for the detection of two copies of a single base substitution in the presence of at least $2 \times 10^9$ copies of the wild-type λ phage DNA; and measure spontaneous somatic mutation in mouse tissues.

**MATERIALS AND METHODS**

**λ Phage DNA Template**

The wild-type λ phage DNA template that contains an inserted wild-

![Figure 1. Pyrophosphorolysis-activated polymerization (PAP) detection of rare mutations in an abundance of wild-type template.](image-url)

(A) Theoretical PAP selectivity. The 3′ terminus of the mutation-specific pyrophosphorolysis-activatable oligonucleotide (P*) matches the mutated A template but mismatches the wild-type T template at nucleotide position 190. Specific amplification occurs efficiently (indicated by thick arrows). When hybridized to the mutated A template, the P* cannot be extended directly from the 3′-terminal dideoxynucleotide; the 3′-terminal ddTMP must be removed by pyrophosphorolysis, and the activated oligonucleotide is then extended. Nonspecific amplification occurs rarely when mismatch pyrophosphorolysis occurs to generate a wild-type product that is not an efficient template for subsequent cycles (type I error is indicated by a thin arrow with an estimated frequency of $10^{-5}$). Once type I error occurs, the product is 10$^{-5}$-fold less for each subsequent cycle and cannot be amplified. The product of type I error can be differentiated from the specifically amplified product due to their sequence difference. When both mismatch pyrophosphorolysis and misincorporation occur (which is extremely rare) to generate a mutated product (type II error) (indicated by thin arrows), it is with an estimated coupled frequency of $3.3 \times 10^{-11}$ (29,30,41). Once the errors occur, the mutated product can be amplified exponentially in subsequent cycles, therefore limiting the selectivity. (B) Unblocked oligonucleotide (U) decreases the selectivity of PAP. In PAP, with a downstream P* and an upstream unblocked oligonucleotide, extension errors from the unblocked upstream can produce the specific mutation of interest, thus reducing the selectivity. Mut, mutant.
type lacI gene of Escherichia coli (22) was purchased from Stratagene (La Jolla, CA, USA). Three mutated λ DNA templates were prepared from λ phage plaques from infected SC5-8 E. coli cells using a procedure that has been previously described (23). They contain an A to T mutation at nucleotide position 190, a T to G mutation at nucleotide 369, and a T to C mutation at nucleotide 369, respectively, of the lacI gene (24). The amount of λ DNA was determined by UV absorbance at 260 nm.

Synthesis of P* by Adding a 3’ Dideoxynucleotide

The 3’-terminal dideoxynucleotide was added to an oligodeoxynucleotide by terminal transferase. The mixture contained 100 mM potassium cacodylate, pH 7.2, 2 mM CoCl₂, 0.2 mM dithiothreitol (DTT), 2 nmol of the oligonucleotide, 2.4 mM 2’-3’-dNTP (the molar ratio of the 3’-OH terminus to ddNTP was 1:30) (Roche Applied Science, Indianapolis, IN, USA), 100 U terminal transferase (Invitrogen, Carlsbad, CA, USA) to a total volume of 25 µL. The reaction was incubated at 37°C for 6 h and then stopped by the addition of EDTA to a 5-mM final concentration. After desalting using a Centri-Spin²⁰™ column (Princeton Separations, Adelphia, NJ, USA), P* was purified by preparative 7 M urea/16% polyacrylamide gel with 30 mM triethanolamine/tricine buffer, pH 7.9, at 25°C (23,25). The amount of P* recovered was determined by UV absorbance at 260 nm.

Because small amounts of unterminated oligonucleotide would result in unexpected PCR amplification, P* was 32P-labeled at the 5’ terminus by T4 polynucleotide kinase and then was electrophoresed through a 7-M urea/20% polyacrylamide gel. Only P* products were visible, even when the gel was overexposed. It is estimated that more than 99.99% of P* contained a dideoxynucleotide at the 3’ terminus.

Figure 2. Bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification (Bi-PAP-A). (A) Schematic of Bi-PAP-A to detection a rare mutation. Example mutation is as in Figure 1. The downstream and upstream pyrophosphorolysis-activatable oligonucleotides (P*s) contain a dideoxy T and a dideoxy A, respectively, at their 3’ termini. They are specific for the T:A allele of the mutated template at nucleotide 190 (on the left) but are mismatched to the A:T allele of the wild-type template at their 3’ termini (on the right). The P*s overlap at their 3’ termini by one nucleotide. If both P*s are 40 nucleotides, the product is 79 nucleotides (40 + 40 - 1). On the left, the products are generated efficiently from both strands of the mutated template. On the right, no substantial amount of product is generated from either strand of the wild-type template because of the mismatch. (B) Bi-PAP-A amplification directly from λ DNA. Each of the wild-type and mutation-specific Bi-PAP-A assays at nucleotide 190 was used to amplify a 79-bp segment of the lacI gene from λ DNAs. For the wild-type assay (lanes 1–3), the two wild-type P*s have 3’-terminal ddA and ddT, respectively. For the mutation-specific assay (lanes 4–6 and 7–9), the two mutated P*s contain ddT and ddA, respectively, at their 3’ termini. In lanes 1, 4, and 7, 2000 copies of the wild-type template were added to each reaction. In lanes 2, 5, and 8, 2000 copies of the mutated template were added to each reaction. In lanes 3, 6, and 9, no template was added. In lanes 7–9, 200 ng human genomic DNA were added as carrier. The product and P* are indicated. Lane M contains 120 ng of φX174-PUC19/HaelIII DNA marker (298, 267, 174, and 80 bp, respectively). The product yields in lanes 1, 5, and 8 were estimated to be 9.3, 9.9, and 14.4 ng per 25 µL of reaction, based on the their intensities and on the intensities of marker bands of 298, 267, and 174 bp. The average efficiency in 35 cycles was estimated to be approximately 0.7 per cycle. WT, wild-type; Mut, mutant; No, no template.
The cycling conditions were 35 cycles of 92°C for 6 s, 68°C for 1 min, and 72°C for 20 s. A denaturing step of 92°C for 1 min was added before the first cycle. Commercially available AmpliTaq FS is formulated with pyrophosphatase, which decreases PAP efficiency; note the short cycling time used to minimize the effect of pyrophosphatase.

Twenty microliters of the 25-µL reaction were electrophoresed through a standard 2.5% agarose gel, and the gel was stained with ethidium bromide for UV photography by a Gel-Doc™ 1000 charge-coupled device (CCD) camera (Bio-Rad Laboratories, Hercules, CA, USA), and the image was analyzed by Multi-Analyst®/PC software (version 1.1) (Bio-Rad Laboratories) (Figure 2B).

To differentiate the mutated product from the wild-type product of the same size, nondenaturing SSCP gel electrophoresis was performed (14). A portion (3.3 µL) of the 25-µL reaction was mixed with 6.6 µL loading buffer (7 M urea and 50% formamide), boiled, and rapidly cooled on ice. The product was electrophoresed through a 8% nondenaturing PAGE-PLUS™ gel (Amresco, Solon, OH, USA) with 30 mM ethanolamine/capsco buffer, pH 9.6, at 4°C (25). The gel was dried and exposed to X-OMAT™ AR film (Eastman Kodak, Rochester, NY, USA) for autoradiography (Figure 3).

**Measurement of Mutation Load**

Liver, heart, adipose tissue, cerebrum, and cerebellum from 10-day-old to 25-month-old mice were snap-frozen and stored under liquid nitrogen until use. DNA was extracted according to the BigBlue® protocol (Stratagene). In brief, the tissues were homogenized and digested with proteinase K. The genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at 4°C. The amount of mouse genomic DNA was determined by UV absorbance at 260 nm.

The mutation-specific Bi-PAP-A assay for 369 T to G (Table 1, assay B) of the lacI gene was performed as described above except that (i) the reaction contained 2 µg of the mouse genomic DNA (approximately 20 kb in size) unless otherwise stated; (ii) mouse DNA in 20 µL 1.25× HEPES buffer, 5% DMSO without MgCl₂ was heated at 100°C for 2 min and quickly cooled on ice before the other components were added; (iii) a denaturing step at 95°C for 1 min was added before the first cycle; and (iv) denaturation was at 95°C for 10 s. Ten microliters of the 25-µL reaction were mixed with 10 µL of the denaturing loading buffer, boiled, and rapidly cooled on ice. The product was electrophoresed through an 8% 7-M urea/polyacrylamide gel with 90
mM TBE (90 mM Tris, pH 8.3, 90 mM boric acid, 7 mM EDTA) buffer at room temperature. The gel was dried and autoradiographed as described above.

RESULTS

Increased Efficiency of Pyrophosphorolysis-Activated Polymerization Amplification

AmpliTaq FS, a genetically engineered DNA polymerase (26), greatly improved the efficiency of PAP (20). Here PAP was further optimized for higher efficiencies, allowing it to amplify directly from one or a few copies of λ phage DNA or genomic DNA template. The optimal reaction components and thermal cycling regime include the following: (i) decreased concentrations of pyrophosphate while keeping the pyrophosphate to dNTP ratio essentially constant; (ii) use of low pH HEPES buffer (pH 6.9 at 25°C); (iii) addition of (NH₄)₂SO₄; (iv) increased amount of AmpliTaq FS; and (v) higher annealing temperature.

Bidirectional Pyrophosphorolysis-Activated Polymerization Allele-Specific Amplification

PAP has a potential selectivity of 1 part in 3.3 × 10¹¹ (Figure 1A). Approaching this potential requires a design that eliminates sources of error. A mutation of the E. coli lacI gene inserted into λ DNA was used as a model system (190 A to T). In PAP, there is one P* and one opposing unblocked oligonucleotide. Extension errors from the unblocked oligonucleotide can produce the rare mutation of interest, thus reducing selectivity (Figure 1B). If the misincorporation rate of AmpliTaq FS is 10⁻⁵ or less per incorporated nucleotide and one of the three possible misincorporations generates the A to T mutation from the unblocked oligonucleotide, the selectivity decreases to 3.3 × 10⁻⁶ due to this bypass reaction. To eliminate this problem, Bi-PAP-A was developed (Figure 2A). In Bi-PAP-A, both the downstream and upstream oligonucleotides are P*s that are specific for the nucleotide of interest at their 3′ termini. The P*s overlap at their 3′ termini by one nucleotide.

Bi-PAP-A amplified efficiently and specifically at nucleotide position 190 using λ DNA containing the lacI gene as template (Figure 2B). The addition of carrier human genomic DNA did not affect the amplification. The 79-bp product of Bi-PAP-A was easily distinguished from unincorporated P*s. P* did not form dimers because P* needs a perfectly matched region at the 3′ terminus for activation. Similar results were observed at nucleotide position 369. Direct sequencing analysis confirmed the correct sequence of the amplified product (data not shown).

Selectivity of Bi-PAP-A

To demonstrate the selectivity of Bi-PAP-A, more than 10¹⁰ copies of DNA template were used for a Bi-PAP-A reaction. The λ DNA containing the lacI gene of E. coli was chosen as the model system because 1 µg of λ DNA contains 2 × 10¹⁰ vector genomes, while 1 µg of human genomic DNA contains only 3.3 × 10⁵ genomes. To avoid potential contamination of the wild-type λ DNA in this laboratory, mutation-specific Bi-PAP-A assays with mutated P*s were chosen to amplify the wild-type λ DNA. The relative frequency of a spontaneous mutation in the lacI gene of the wild-type λ DNA is estimated to be less than 10⁻⁹ by examining λ phage plaques infecting E. coli.

The specificity, sensitivity, and selectivity of Bi-PAP-A were examined using three mutation-specific Bi-PAP-A assays and their corresponding mutated E. coli lacI gene inserted into λ DNAs (Table 1). Four titration experiments were performed for each mutation-specific Bi-PAP-A assay (Figure 3). Three or four bands from each amplified product were visible on each gel. The upper one or two bands were double-stranded DNA (dsDNA) caused by the hybridization of denatured single-stranded segments during electrophoresis, which had resulted from the substantial amounts of amplified product present. Increasing the concentration of the amplified product further increased the intensity of the upper bands (data not shown). Experiment I tested the specificity or the maximum “tolerable” copies of the wild-type template without a detectable mutated product using the mutation-specific Bi-PAP-A assay. The wild-type λ DNA was titrated from 2 × 10¹⁰ copies to 2 × 10⁶ copies by using a 10-fold serial dilution. The specificities were 2 × 10⁹ to 2 × 10¹⁰, 2 × 10⁷ to 2 × 10⁹, and 2 × 10⁵ to 2 × 10⁸, respectively, for the three mutation-specific Bi-PAP-A assays. Experiment II tested the sensitivity of Bi-PAP-A or the minimum copies of the mutated template with a detectable mutated product. We detected as few as

<table>
<thead>
<tr>
<th>Assay</th>
<th>Oligonucleotides</th>
<th>Mutation</th>
<th>Sensitivitya</th>
<th>Selectivityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5′-GATGGCGGAGCTGTAATTACATTTCCAAACCGGTGACAT* and 5′-GGCAACGGCGCAATCGAGCTTGTGCGCCGCAATGTA*</td>
<td>A190T</td>
<td>2</td>
<td>1:10⁶ to 1:10¹⁰</td>
</tr>
<tr>
<td>B</td>
<td>5′-GAAGCGGCGCGTGAGATCCGTGAAACGGCGCTGACAATCG* and 5′-GCCGATAGTTAATGTACGGCCACCTGAGCGTTGCGAGAC*</td>
<td>T369G</td>
<td>1:10⁷ to 1:10⁸</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5′-GAACGCGCGCTGGACTGTAAGCGCGGTGACAATCC* and 5′-GGCGATAGTTAATGTACGGCCACCTGAGCGTTGCGAGAC*</td>
<td>T369C</td>
<td>2</td>
<td>1:10⁷ to 1:10⁸</td>
</tr>
</tbody>
</table>

*aIndicates the presence of a deoxyribofuranosyl at the 3′ terminus.  
*bDefined as the minimum copy number of the mutated template from which a detectable mutated product is generated when a mutation-specific Bi-PAP-A assay is used.  
*cThe ratio of the minimum copy number of the mutated template with detectable product to the maximum copy number of the wild-type template with undetectable product when a mutation-specific Bi-PAP-A assay is used.
two copies in each assay. For the titration, the stock solution (12–15 ng/µL) was serially diluted by 10-fold to 0.2 copies per reaction, with an estimated error of no more than ±17%–48% given a 2%–5% of error per dilution using a Pipetman® P10 (Rainin, Emeryville, CA, USA). If the average is two copies of the mutated λ DNA template per reaction, with an estimated error of no more than ±17%–48%, then a reaction is expected to contain at least one copy based on a Poisson distribution. Results were unchanged when Experiment II was repeated in the presence of large amounts of wild-type λ template (Experiment III) or large amounts of human genomic DNA (Experiment IV) (Figure 3A; data not shown for 369 T to G and 369 T to C). A dose response was observed between the template copy number and the signal intensity.

Measurement of Mutation Load in Mouse Tissues

To assess the utility of Bi-PAP-A for measuring ultra-rare mutations in mammalian cells, the 369 T to G mutation was analyzed in genomic DNA from the BigBlue mice. Two micrograms of mouse genomic DNA were amplified in a 25-µL reaction containing 1.2 × 10⁷ copies of the lacI gene (20 copies per genome). The mutation-specific Bi-PAP-A assay for 369 T to G (Table 1, assay B) was performed for 18 samples in duplicate (Figure 4A). Three categories of results were defined, each with similar numbers of samples. Six samples were positive twice, seven samples were positive once, and five samples were negative twice.

Two samples in each category were further studied (Figure 4, B and C) (Table 2). In the first category, for 5 and 12, the samples with the strongest amplified signals (Figure 4 A), a 4-fold dilution to 0.5 µg and a 16-fold dilution to 0.125 µg of mouse genomic DNA, were performed for further quantitation (Figure 4 B). The 369 T to G mutant frequency for each sample was estimated and varied 370-fold among the six samples (Table 2). The average 369 T to G mutant frequency of 2.9 × 10⁻⁷ was within 50% of the average 369 T to G mutant frequency of 2.1 × 10⁻⁷ that was measured directly using the BigBlue mutation detection system.

DISCUSSION

Selectivity Bi-PAP-A

The selectivity of Bi-PAP-A varies from 1:10⁹ to 1:10⁷, depending on the assay (Table 1). The approximately 100-fold difference in selectivity between the nucleotide positions 190 and 369 may derive from three sources. (i) Spontaneous mutations at position 369 are present at a frequency of 10⁻⁷ to 10⁻⁸ in the wild-type λ DNA template (27,28). (ii) The purity of P* is lower. There is a possible side reaction due to the impurity of P* contamination with unblocked oligonucleotide where the dideoxy terminus has not been added, although no unblocked oligonucleotide was detected. However, this selectivity may not be severely limited by small amounts of unblocked oligonucleotide because the product generated would be much more likely to be the wild-type rather than the specific mutation (1:3.3 × 10⁵). (iii) Fidelity of pyrophosphorolysis for a perfect match at the 3′ terminus and fidelity of DNA polymerase to incorporate a correct nucleotide may be associated with sequence context such that the Type II nonspecific amplification occurs at a frequency of 10⁻⁷ to 10⁻⁸. A 100-fold difference in selectivity could arise from a 10-fold difference in pyrophosphorolysis fidelity and a 10-fold difference in DNA polymerase fidelity with sequence context (29–32).

Measurement of Mutation Load in Mouse Tissues

Transgenic mouse mutation detection systems permit determination of the frequency and pattern of spontaneous or induced mutations in vivo. The BigBlue system uses transgenic mice that harbor chromosomally integrated λ phage DNA containing the E. coli lacI gene as the mutational target (33–35). The lacI gene is integrated within each mouse diploid genome in 40 tandemly repeated copies. The BigBlue mutation detection system is performed by isolating genomic DNA from transgenic mouse tissues and mixing it with λ packaging extracts. The packaged λ phage can infect E. coli. In the presence of X-gal
Table 2. Somatic Mutant Frequency Measured by Bidirectional Pyrophosphorolysis-Activated Polymerization Allele-Specific Amplification (Bi-PAP-A)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tissue</th>
<th>Age (Months)</th>
<th>Mouse Genomic DNA Source</th>
<th>2 µg DNA Frequency of Positive Amplification</th>
<th>0.5 µg DNA Frequency of Positive Amplification</th>
<th>0.125 µg DNA Frequency of Positive Amplification</th>
<th>Estimated Mutant Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Adipose</td>
<td>6</td>
<td>8/8</td>
<td>8/8</td>
<td>4/8 (0.69)</td>
<td>9.25 × 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Liver</td>
<td>25</td>
<td>8/8</td>
<td>8/8</td>
<td>5/8 (0.98)</td>
<td>1.31 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Liver</td>
<td>25</td>
<td>8/24 (0.41)</td>
<td></td>
<td></td>
<td>3.83 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Liver</td>
<td>25</td>
<td>13/24 (0.78)</td>
<td></td>
<td></td>
<td>6.50 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Liver</td>
<td>25</td>
<td>2/24 (0.09)</td>
<td></td>
<td></td>
<td>7.25 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Liver</td>
<td>25</td>
<td>1/24 (0.04)</td>
<td></td>
<td></td>
<td>3.52 × 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.91 × 10⁻⁷</td>
<td></td>
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</table>

a See Figure 4A.
b The ratio of the number of positive signals for the 369 T to G mutation relative to the total number of reactions. The frequency given in parentheses is the expected average number of 369 T to G mutants per reaction, which is estimated using a Poisson distribution (the frequency of zero mutants per reaction = e⁻¹, where x is the average number of mutants per reaction), assuming that the mutant distributes in the reaction according to a Poisson distribution, and if one or more mutants are in the reaction, the amplification is positive, and if zero mutants are in the reaction, the amplification is negative.
c The expected frequency of the 369 T to G mutant of the lacI gene in mouse genome per reaction, estimated assuming that the mutant distributes in mouse genomic DNA according to a Poisson distribution and that one or more mutants are positive in the detection. For samples 12 and 5, a total of approximately 6.0 × 10⁶ copies of the lacI gene are used for the estimate and for samples 3, 9, 7, and 10, approximately 2.9 × 10⁸ copies are used, assuming that 2 µg of the lacI mouse genomic DNA contains approximately 1.2 × 10⁸ copies of the lacI gene.

Figure 3. Selectivity of bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification (Bi-PAP-A) as assessed by nondenaturing single-stranded conformational polymorphism (SSCP) analysis of amplification products. (A) The mutation-specific Bi-PAP-A assay for 190 A to T. In Experiment I, the copy numbers of the wild-type λ DNA are as indicated (lanes 1–5). Lane 6 is a negative control with no DNA. For Experiment II, the copy numbers of the mutated λ DNA are indicated (lanes 7–11). Lane 11 (0.2 copy) is a negative control to support the dilution accuracy of copy number, and lane 12 is a negative control with no DNA. For Experiment III, the copy numbers of the mutated λ DNA in the presence of 2 × 10⁹ copies of the wild-type λ DNA are indicated (lanes 13–17). Lane 18 is a negative control with only the wild-type λ DNA. For Experiment IV, the copy numbers of the mutated λ DNA in the presence of 100 ng of carrier human genomic DNA are indicated (lanes 19–23). Lane 24 is a negative control only with the human genomic DNA. Lane C WT is the wild-type product control in which the wild-type P*s were used to amplify 2000 copies of the wild-type λ DNA. Lane C Mut is the mutated product control in which the mutated P*s were used to amplify 2000 copies of the mutated λ DNA. The wild-type and mutated products with unique mobilities are indicated. (B) The mutation-specific Bi-PAP-A assay for 369 T to G. (C) The mutation-specific Bi-PAP-A assay for 369 T to C. Exp., experiment; WT, wild-type; Mut, mutant; gDNA, genomic DNA.
substrate, \textit{lacI} mutants produce blue plaques on a background of colorless wild-type plaques. Observed mutants derive overwhelmingly from the mouse (36). The mutant frequency is determined by dividing the number of circular blue plaques by the total number of plaques. Thirty-one 369 T to G mutants have been found in a total of 149 \times 10^6 plaques screened from various ages, genders, and treatments in this laboratory (frequency = 2.1 \times 10^{-7}). A 370-fold variation in mutant frequency was observed in 5 samples at

![Figure 4. Somatic mutation load.](image)

(A) Selected samples. Eighteen genomic DNA samples of the \textit{lacI} transgenic mice were chosen. Two micrograms of genomic DNA from each sample were amplified using assay B to detect the 369 T to G mutations in replicates. Samples 1–10 are from the livers of 25-month-old mice. Samples 11–14 are from the heart (samples 11, 13, and 14) and adipose tissues (sample 12) of 6-month-old mice. Samples 15–18 are from the brains of 10-day-old mice. (B) Sample 12. Assay B was performed multiple times. Lanes 11–12, 13–16, and 17–20 contain 2, 0.5, and 0.125 \mu g, respectively, of the \textit{lacI} mouse genomic DNA. Lanes 1–10 are controls; the copy number of the mutated \textit{\lambda} DNA per reaction was reconstructed by 2-fold serial dilutions. In lanes 1–10 and 13–20, each reaction also contained 1 \mu g of the \textit{lacI} mouse genomic DNA carrier. (C) Samples 3 and 9. Assay B was also performed in these samples. In lanes 1–10 and 11–18, 2 \mu g of the \textit{lacI} mouse genomic DNA from sample 3 were used in each reaction. In lanes 15–18, 2 \mu g of the \textit{lacI} mouse genomic DNA from sample 9 were used in each reaction. Lanes 1–10 are controls; the copy numbers of the mutated \textit{\lambda} DNA per reaction are indicated. Each control reaction also contained 1 \mu g of the \textit{lacI} mouse genomic DNA carrier. P, positive control that amplified the mutated \textit{\lambda} DNA; N, negative control with no DNA; +, amplified product; -, no product; ss, single-stranded; ds, double-stranded; gDNA, genomic DNA.
25 months of age (Table 2). This large variation could be due to reproducible amplification from one copy of the template. To address this issue, each of the analyses was repeated at least twice with similar results. For example, in sample 9, 7 of 14 reactions with 2 µg DNA were positive in one experiment, 3 of 4 such reactions were positive in another experiment, and 2 of 4 such reactions were positive in a third experiment. For sample 7, there was one positive in 8 and one positive in 14 reactions. The product was sequenced to confirm the 369 T to G mutation after reamplification from the positive reaction. In addition, positive controls (2 µg of the lacI+ mouse DNA with approximately 10 copies of 369 T to G) and negative controls (mouse genomic DNA without the lacI target; that is, the lacI− mouse DNA) were performed. As additional positive controls, reconstruction experiments were performed so that the copy number of the mutated λ DNA per reaction was serially diluted by 2-fold in the presence of the lacI− genomic DNA carrier. Reproducible amplifications from as low as one copy of template were demonstrated (Figure 4, B and C).

The 370-fold variation in a mutation frequency by Bi-PAP-A is consistent with the reported other measurements by the BigBlue mutation detection system, which are compatible with variable amounts of clonal expansion from the original mutation (37,38). The clonal expansion is a major contributor to the hyper-Poisson variance of mutant frequencies observed in BigBlue and other systems (37,38).

Among six mice in which the interanimal variation was assayed using the BigBlue mutation detection system, the overall mutant frequency was 3- to 4-fold, with significant founder effects in one or a few of the mice. This variation, frequently in the range of 2 × 10^{-5} to 8 × 10^{-3}, is the sum of more than 1000 different mutations. Here only the 369 T to G mutation was assayed.

We anticipate that the great majority of the signal derives from duplex-mutated templates (36), but note that unresolved mismatch intermediates derived primarily from DNA replication or DNA repair would also generate a signal. Thus, the physical limit of sensitivity is actually one half of a duplex DNA molecule per reaction.

**Pyrophosphorolysis-Activated Polymerization Versus READIT® and Pyrosequencing™**

PAP and derivative methods are distinct from other methods such as READIT (US patent nos. 6,159,693 and 6,277,578) and Pyrosequencing (Pyrosequencing, Uppsala, Sweden). READIT is a method for detecting known nucleic acid sequences. DNA polymerase catalyzes pyrophosphorolysis of the 3′-terminal deoxynucleotide of an oligodeoxynucleotide probe in the presence of pyrophosphate and complementary target sequence to release dNTPs from the 3′ terminus. The pyrophosphorolysis is then measured by a “cassette” of two enzymes using light as output. READase™ kinase (Promega, Madison, WI, USA) generates an ATP from a released dNTP. The ATP produces chemiluminescence when luciferase is present. The READIT assay differs from PAP because (i) the 3′-terminal oligonucleotide is extendable in READIT, while PAP utilizes 3′-terminal blocked oligonucleotides; (ii) READIT detects pyrophosphorolysis using an “enzyme cassette” to generate a light signal, while pyrophosphorolysis and polymerization are serially coupled in PAP; (iii) in READIT, there is no dNTP added or polymerization, while pyrophosphate and dNTPs coexist as substrates in PAP; (iv) READIT is a detection method for known mutations, while PAP is a method for nucleic acid amplification that can be used for rare allele amplification and microarray-based scanning for unknown mutations.

Pyrosequencing is a method for nucleic acid sequencing (39). DNA polymerase catalyzes the incorporation of the deoxynucleotide triphosphate into the DNA strand if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate. ATP sulfurylase quantitatively converts pyrophosphate to ATP in the presence of adenosine 5′ phosphosulfate. The ATP then drives the conversion of luciferin to oxyluciferin, generating visible light in amounts that are proportional to the amount of ATP.

**3′-Terminal Dideoxynucleotide-Blocked Oligonucleotide**

Automated chemical synthesis of P* would be helpful because the enzymatic synthesis and gel purification of P* is still time-consuming. With this exception, the physical reaction of Bi-PAP-A is as easy and inexpensive as PCR. In theory, 3′ to 5′ chemical synthesis should generate 100% blocked oligonucleotide because the synthesis is initiated from the 3′ terminus dideoxynucleotide. DdC-blocked oligonucleotide is routinely available from Integrated DNA Technologies (Coralville, IA, USA) or Proligo (Boulder, CO, USA). Other types of blockers, such as acyclonucleotide, can be applied to PAP (40).

In conclusion, we demonstrate that Bi-PAP-A can analyze ultra-rare mutations at frequencies as low as 10^{-7} to 10^{-9}, depending on the assay. Bi-PAP-A can detect a single copy of the somatic mutation directly from mammalian genomic DNA. In mammalian DNA, the copy number of template is limited by the enormous genome size. Two micrograms of genomic DNA contain only 600,000 mouse haploid genomes, yet the reaction is viscous. Our analysis of the BigBlue mouse genomic DNA was facilitated by 20 copies of the lacI gene per haploid genome. To measure mutation load in humans, genomic DNA in one reaction could be increased at least 3-fold by reducing the viscosity (e.g., shearing the DNA into small segments by ultrasonic treatment) and another 4-fold by expanding the reaction volume to 100 μL. Genomic regions of interest can also be isolated and enriched prior to Bi-PAP-A. Mutation load in human genomic DNA might be facilitated by analyzing segments of virtually identical sequence in tRNA genes. For less complex genomes such as Caenorhabditis elegans, Drosophila, and the human mitochondria genome or chronic hepatitis B infections, single copy DNA is readily amplifiable with this protocol.

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REFERENCES


