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Notes & Tips

Preparation of heteroduplex enhanced green fluorescent protein plasmid for in vivo mismatch repair activity assay

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ABSTRACT

Preparation of heteroduplexes in large quantities with high purity is essential for the measurement of DNA mismatch repair (MMR) activity. Here we report a rapid, less labor-intensive method for the preparation of a heteroduplex plasmid that expresses the enhanced green fluorescent protein (EGFP) if the mismatch is repaired correctly. The method involves the use of a wild-type and a mutated EGFP expression plasmid and a few steps of enzymatic digestion. When the constructed heteroduplex EGFP plasmid was transfected into MMR-proficient and -deficient cell lines, the number of EGFP-expressing cells was much higher in the MMR-proficient cells than in the MMR-deficient cells, suggesting that the heteroduplex can be used for MMR activity assay in live model systems.

The DNA mismatch repair (MMR)[2] system is known to participate in maintaining genomic stability [1]. Loss of DNA MMR function can result in the development and progression of certain cancers [2,3]. Various assays have been developed for the measurement of DNA MMR activity in cell extracts or living cells. They all involve the preparation of a heteroduplex DNA with defined mismatched DNA sequences as the substrate for MMR enzymes [4]. A DNA heteroduplex is usually produced by annealing two near complementary synthetic oligonucleotides [5] or by annealing the single-stranded (ss) f1 phage/phagemid DNA to one strand of a linearized plasmid followed by extensive chromatographic purification [6]. Synthesis of a large amount of long oligonucleotides is costly and suffers from contamination with a certain percentage of inaccurate or incomplete oligonucleotide. On the other hand, it is technically demanding and time-consuming to prepare circular phage/phagemid ssDNA and to purify heteroduplex substrates with multiple preparation steps, often resulting in low yield [7].

Here we present a strategy for the rapid preparation of a heteroduplex enhanced green fluorescent protein (EGFP) plasmid with a large quantity and high purity that can be delivered into live cells for monitoring their MMR activity. This method was developed in connection with our ongoing investigation into the role of the MMR system during mutagenesis and tumorigenesis. In our previous work, we were the first to report an EGFP-based method to quantitatively measure MMR activity in live cells [8]. In that method, a heteroduplex EGFP substrate containing a G/G or T/G mismatch at the ATG start codon of the EGFP gene was prepared with either synthetic oligonucleotides or a phagemid ssDNA annealed with a linear double-stranded (ds) DNA. However, both approaches were tedious, laborious, costly, and inefficient as discussed above. Furthermore, they appear too technically demanding to be widely used by other research laboratories without well-trained technicians. As such, we have adopted an enzyme digestion approach using a newly generated mutant EGFP expression plasmid to produce a heteroduplex EGFP plasmid more rapidly. The mutant EGFP plasmid, named p189, was generated by introducing a premature stop codon ($TGG_{58} \rightarrow TAG$) to the EGFP gene in the plasmid pGEM5Z(+)–EGFP, which was created in our previous study [8]. The mutation was created with a polymerase chain reaction (PCR)-based site-directed mutagenesis method using pGEM5Z(+)–EGFP as template [9]. The creation of this premature stop codon completely eliminated fluorescence property of EGFP (data not shown), much more efficient than the mutation of its start codon, which still allowed a small percentage of EGFP expression in a start codon-independent manner as we reported previously [8].

The procedure for the preparation of the heteroduplex EGFP plasmid is depicted in Fig. 1A. First, a circular ssDNA of the coding strand (indicated as "+" and TGG strand in Fig. 1A) of the EGFP gene was generated with the nicking enzyme Nb.Bpu10I (Fermentas, Hanover, Germany). Nb.Bpu10I is a site- and strand-specific endonuclease artificially engineered from the restriction enzyme Bpu10I. It has the same recognition site as Bpu10I but cleaves only one strand

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[2] *Abbreviations used:* MMR, mismatch repair; ssDNA, single-stranded DNA; EGFP, enhanced green fluorescent protein; dsDNA, double-stranded DNA; PCR, polymerase chain reaction; PSAD, plasmid-safe ATP-dependent DNase; RFP, red fluorescent protein; PBS, phosphate-buffered saline.

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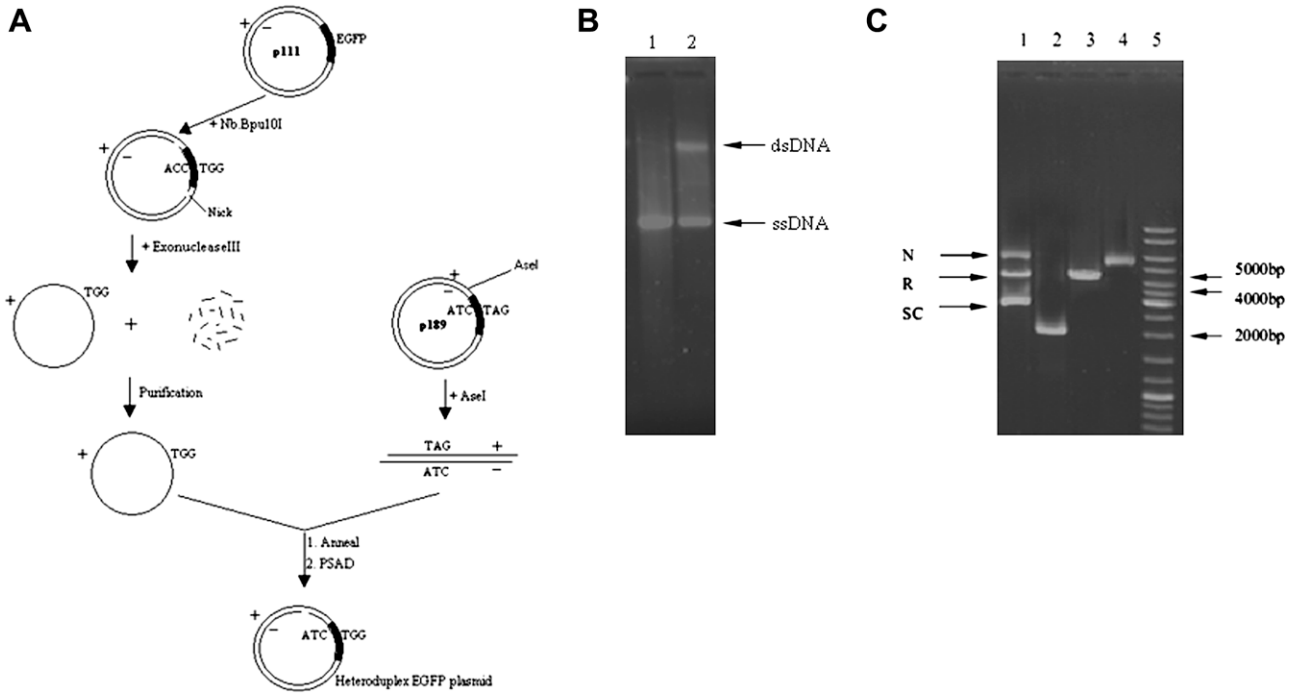

Fig. 1. Preparation of the heteroduplex EGFP plasmid. (A) Flow chart for preparation of the heteroduplex EGFP plasmid. pGEM5Z(+)–EGFP (also named p111) was nicked with Nb.Bpu10I in a 400-μl reaction volume containing 20 μg of pGEM5Z(+)–EGFP, 4 μl (20 u) of Nb.Bpu10I, 40 μl of 10 × buffer, and nuclease-free water at 37 °C for 1 h. After phenol/chloroform extraction and ethanol precipitation, the nicked plasmid was digested with 1200 u of Exonuclease III for approximately 10 min at 30 °C to degrade the nicked strand. p189 was linearized with restriction enzyme *Ase*I and mixed with the purified circular ssDNA at a ratio of 1.0:1.5 (heat at 90 °C for 3 min and cool down to room temperature by leaving the heater block on the bench) to generate a heteroduplex EGFP plasmid containing a G/T mismatch and a nick. The annealing mixture was treated with the plasmid-safe ATP-dependent DNase (PSAD) to degrade leftover ssDNA and linearized plasmid DNA. The heteroduplex EGFP plasmid with high purity was recovered using a DNA cleanup kit. (B) Comparison of the ssDNA generated with the enzyme digestion approach described in panel A (lane 1) and with a phagemid pGEM5Z(+)–EGFP (4.6 kb) using our previously described approach in Ref. [8] (lane 2). (C) Electrophoretic analysis of various DNA species: pGEM5Z(+)–EGFP isolated with an alkaline lysis method containing supercoiled (SC), relaxed (R), and nicked circular (N) DNA (lane 1), circular ssDNA (lane 2), linear form of p189 (4.7 kb) (lane 3), purified heteroduplex EGFP plasmid with a nick (lane 4), and DNA ladder (lane 5).

of the DNA within its recognition sequence on a dsDNA substrate. Therefore, Nb.Bpu10I can only cut the template strand (indicated as "–" and ACC strand in Fig. 1A) of the EGFP gene to introduce several nicks in pGEM5Z(+)–EGFP. A detailed protocol for the application of Nb.Bpu10I for the preparation of circular ssDNA from supercoiled double-stranded plasmids containing its recognition site can be found on the Fermentas website (http://www.fermentas.com/techinfo/modifyingenzymes/protocols/p_singlemol.htm). The nicked strand was then removed with Exonuclease III (Fermentas) digestion. The intact ssDNA of the remaining strand (coding strand) was recovered with phenol/chloroform extraction and ethanol precipitation. Because pGEM5Z(+)–EGFP can be easily obtained from *Escherichia coli*, using this approach we were able to obtain large quantities of circular ssDNA with high purity in less than 2 h. The yield of the ssDNA is usually approximately half of the double-stranded plasmid. In contrast, with the f1 phagemid-based ssDNA method that we used previously, owing to a low yield of f1 phagemid as well as multiple preparation steps, it would take us at least 2 days to produce approximately 50 μg of circular ssDNA from a 200-ml culture of helper phage-infected phagemid-producing bacteria. In addition, the circular ssDNA obtained with the enzyme digestion approach was much cleaner than the circular ssDNA obtained from f1 phagemid, which appeared to be contaminated with double-stranded plasmid due to lysis of the bacteria (Fig. 1B) and sometimes also contaminated with help phage DNA (data not shown).

To generate heteroduplex DNA, we linearized p189 by digesting it with *Ase*I (New England Biolabs, Beverly, MA, USA), which cut the plasmid upstream of the CMV promoter and 843 bp away from the mismatched TAG stop codon as indicated in Fig. 1A. The linearized p189 was purified with phenol/chloroform extraction and ethanol precipitation and annealed with the circular ssDNA of pGEM5Z(+)–EGFP (Fig. 1A). After annealing, the mixture was digested with a plasmid-safe ATP-dependent DNase (PSAD, Epicentre Biotechnologies, Madison, WI, USA) to degrade leftover linearized dsDNA and circular ssDNA. The heteroduplex EGFP plasmid was recovered using a DNA purification kit (Beyotime Biotechnology, Shanghai, China) and was found to be highly pure with agarose gel electrophoresis analysis as shown in Fig. 1C.

The heteroduplex EGFP plasmid contains both a G/T mismatch and a nick at the template strand, which can act as a strand discrimination signal for MMR assays in vitro [10]. Repair of the G/T mismatch to G/C in the heteroduplex EGFP plasmid should restore the expression of a functional EGFP with green fluorescence property. To demonstrate the usefulness of the heteroduplex EGFP plasmid prepared by this method for monitoring MMR activity in live cells, we transfected it into an MMR-proficient cell line (HeLa) and an MMR-deficient cell line (HCT116) with Lipofectamine (Invitrogen, Carlsbad, CA, USA). The cells were plated in 60-mm dishes at a density of 5×10^5 cells/dish. Transfection was performed the following day according to the manufacturer's protocols. Heteroduplex EGFP plasmid measuring 0.5 μg was cotransfected with 0.5 μg of a red fluorescent protein (RFP) expression plasmid, pDsRed1–N1 (Clontech, Palo Alto, CA, USA). pDsRed1–N1 was used to ascertain whether the transfection efficiencies were comparable between the two cell lines. Fluorescent images of the transfected cells were taken with a Nikon fluorescence microscope 24 h after transfection. The cells were then trypsinized and resuspended in phosphate-buffered saline (PBS) at a concentration of 5×10^5 cells/ml. A total of 20,000 cells were measured for their fluorescence intensity at wavelengths of 530 ±

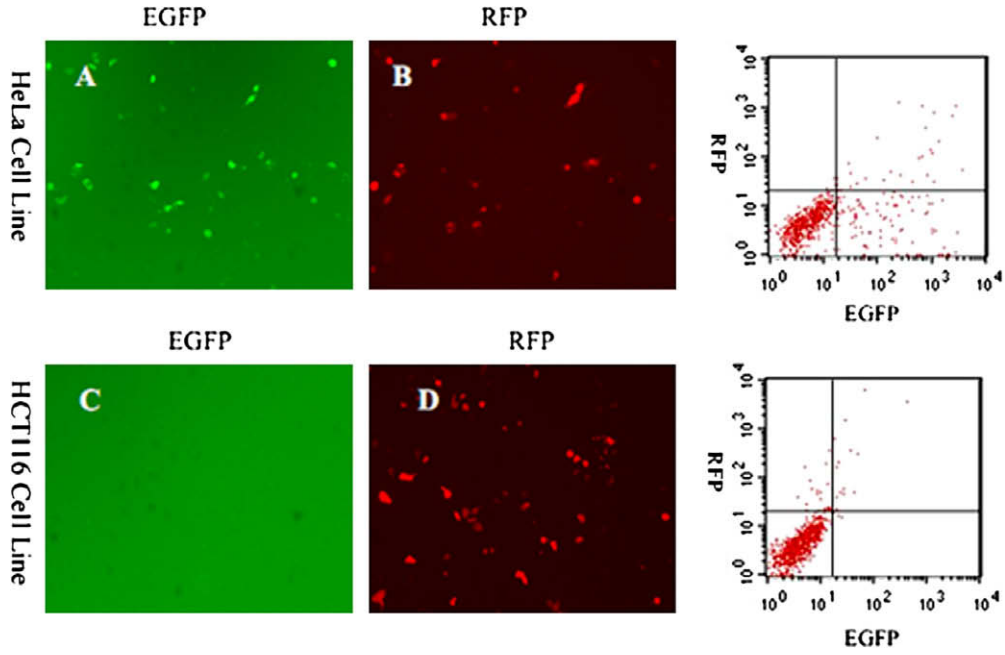

Fig. 2. Repair of heteroduplex EGFP plasmid in live cells. MMR-proficient HeLa cells (A,B) and MMR-deficient HCT116 cells (C,D) cotransfected with the heteroduplex EGFP plasmid and pDsRed1–N1. Flow cytometry results are shown on the right side.

30 nm for EGFP and above 670 nm for RFP using a FACSCalibur flow cytometer (Becton Dickinson). As shown in Fig. 2, although the number of RFP-expressing cells was comparable between HeLa and HCT116, indicating similar transfection efficiency, there were significantly more EGFP-expressing HeLa cells than HCT116 cells. These results indicate that the heteroduplex EGFP plasmid prepared by this method can efficiently distinguish MMR-deficient cells from MMR-proficient cells.

In summary, we have developed a simple but efficient method to prepare a DNA heteroduplex with a G/T mismatch using a novel mutant EGFP gene expression plasmid and demonstrated its application in monitoring MMR function. With minor modifications of the EGFP expression plasmid, this strategy should be amenable for generating various kinds of DNA heteroduplex with defined mismatches for the measurement of activities of different MMR enzymes either in vitro or in vivo.

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