Analytical Biochemistry 556 (2018) 35–39

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Modified parallel strategies for preparation of heteroduplex plasmids for *in vitro* mismatch repair assays

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ARTICLE INFO

*Keywords:*
Mismatch repair assay
Heteroduplex
DNA repair
Bacterial packaging cell line
Phagemid
ssDNA

ABSTRACT

We present efficient and reproducible parallel strategies for preparing large quantities of pure heteroduplex plasmids containing defined mismatches. The strategies described involve the use of synthetic oligonucleotides, the commercially available pGEM-T plasmid, and nicking enzymes to prepare prerequisite ssDNA. Alternatively, bacterial packaging cell lines containing an engineered phagemid construct to produce ssDNA without the need of a helper phage were utilized, hence providing added flexibility and choice. These integrated approaches help to construct different mismatch substrates of choice in large quantities, thus enhancing the usability of mismatch repair assays and extending their range and accessibility to wider research groups.

Maintaining the integrity of the genetic material is a fundamental cellular process, and hence cells exhibit a vast repertoire of DNA repair mechanisms for this very purpose. Mismatch repair (MMR) is a pivotal DNA repair mechanism that acts upon wrongly incorporated (mismatching) nucleotides during DNA replication, thereby increasing replication fidelity by 20–400-fold [1]. Moreover, MMR helps to avoid illegitimate recombination and its detrimental effects on the cell and the organism [2]. MMR system is also known to participate in repair of DNA adducts caused by various other biological as well as environmental sources including oxidative stress, alkylating agents, UV light and carcinogens [3,4]. Since MMR malfunction is linked to development of various types of cancers [5,6], it is important to gain a precise understanding of the pathways leading to mismatch correction, and of the proteins involved and their activities *in vitro* as well as *in vivo*.

*In vitro* DNA mismatch repair assays greatly facilitate the rapid assessment of MMR activity detection and/or its deficiency in various cellular/organellar extracts [7–9]. A typical MMR assay utilizes a DNA substrate possessing an overlapping restriction endonuclease site with a mismatch, which upon repair restores either of the sites available to be cleaved by one of the restriction enzymes, thus indicating a mismatch repair. The prerequisite for these assays is the availability of appropriate amounts of DNA substrate containing defined mismatched lesions. Several methods have been described so far to prepare different heteroduplex substrates for MMR assays. In general, they involve the annealing of a single-stranded f1 phage DNA to an identical double stranded plasmid containing a single base mutation [7,9]. This requires access to such specialized phages bearing a mismatch on a complementary strand of double stranded DNA plasmids, their culturing and maintenance. This is generally technically demanding and time consuming, involving multiple preparative steps, and may lead to low starting substrate yield. Another difficulty of using phage DNA is the lack of unique restriction sites, which limits its utility. These limitations restrict the usability of MMR assays to only specialized laboratories. Other methods involve the preparation of plasmid variants using several site-directed mutagenesis steps and the treatment of each batch of substrate with nicking endonucleases followed by a chromatographic and/or streptavidin bead-based purifications [10,11]. Consequently, difficulties are commonplace during the preparation of specific MMR substrates containing a single defined lesion and a single nick acting as a strand discrimination signal [12]. Yet these substrates are essential to assess the actual biological response of MMR proteins.

Here we describe two simple and efficient strategies for producing large quantities of G/T and A/C heteroduplex substrates with high purity and reproducibility for *in vitro* MMR assays. We used a commonly available and easily accessible high copy number plasmid pGEM-T with multiple cloning sites (MCS), for the development of these methods.

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https://doi.org/10.1016/j.ab.2018.06.020
Received 9 May 2018; Received in revised form 17 June 2018; Accepted 20 June 2018
Available online 22 June 2018
0003-2697/ 

These strategies can be used in parallel providing methodological choice and involve the use of routine standard laboratory methods and reagents (e.g. restriction enzymes). The first strategy is modified from Wang and Hays [10] and utilizes nicking endonucleases; whereas the second parallel strategy involves the use of "bacterial packaging cell lines" [13], which secrete the phagemid DNA in culture medium without the need of the helper phage, producing large amounts of circular single-stranded DNA (ssDNA) which can later be annealed to linear double-stranded plasmid DNA to obtain heteroduplex substrates of choice. Both these strategies are integrated and can be utilized interchangeably. Moreover, these strategies can also be easily adapted to produce various kinds of substrates with different lesions other than G/T and A/C used here, such as base/base mismatches, insertion/deletion substrates, and substrates with DNA loops, among others. Hence, these strategies are highly flexible to modifications and can be easily adapted according to specific requirements, thus increasing the accessibility of MMR assay to more laboratories.

Firstly, the pGEM-T plasmid was modified in order to incorporate the two desired oligonucleotide pairs (Fig. S1A) each with unique SmaI and SacI restriction sites, not available in the plasmid. This was an optional step dependent on the choice of restriction enzyme sites required for analysis. Choosing a different oligoduplex pair (e.g. HindIII-XhoI) would eliminate the requirement of this step (see Fig. S1B for alternate options). The use of another plasmid having an f1 origin but which is devoid of the restriction sites chosen for the MMR assay is equally appropriate [14]. In order to delete the pre-existing SacI site, 10 μg of the pGEM-T plasmid was double-digested overnight at 37 °C with NdeI and SacI (10U each). The resulting linear plasmid with overhangs was purified and treated subsequently with Klenow DNA polymerase (10U) to digest the 3′ overhang and to fill-in the 5′ overhang. The incubation was performed at 25 °C for 25 min, and was followed by the addition of EDTA to a final concentration of 10 mM, and the inactivation of the enzyme at 75 °C (20 min). This reaction resulted in removal of 10 nucleotides in total, and eliminated the plasmid's SacI site. The resulting linear plasmid with blunt ends was purified, self-ligated (re-circularized) using T4 DNA ligase and was transformed into DH5α cells for propagation. The absence of SacI site was confirmed by sequencing and the resulting plasmid was referred as pGSacI-minus.

Synthetic oligonucleotides containing one restriction site each for SacI or SmaI were mixed in equal amounts, heat denatured and allowed to re-anneal slowly. This process yielded a 39 bp double-stranded homoduplex DNA with 4 bp sticky ends complementary to the overhangs resulting from NcoI digestion (see Fig. S1A for the features of the synthetic oligos). These re-annealed homoduplexes were phosphorylated with T4 polynucleotide kinase (NEB) following the supplier's protocol to facilitate ligation into a linear plasmid with dephosphorylated overhangs (pGSacI-minus).

The pGSacI-minus plasmid from the previous step was digested with NcoI, purified and treated with calf intestinal alkaline phosphatase (CIP) following the manufacturer's protocol, in order to avoid re-circularization of the NcoI-digested plasmid. The re-annealed phosphorylated homoduplex DNA oligos with overhangs were then ligated to the dephosphorylated pGSacI-minus using T4 DNA ligase (Promega) as per manufacturer's instructions. This ligation reaction was transformed into chemically competent DH5α cells by heat shock. The clones obtained after 15 h culture at 37 °C were PCR amplified and sequenced to confirm the successful ligation of the desired homoduplexes into the plasmids. Two types of clones were selected for each plasmid construct: in case of SmaI construct, those having a G nucleotide (nt) on the top strand (pGSM-t) or at the bottom strand (pGSM-b). Similarly, for SacI constructs, we isolated clones containing plasmids pGSA-t or pGSA-b depending on the occurrence of a T nt on the top or bottom strand, respectively (Fig. 1). Bidirectional cloning with sticky NcoI ends allowed for a single cloning and transformation step to yield all four plasmids. Sequencing of these plasmids with M13 forward primer confirmed the presence of insertion of 43mer oligonucleotide with desired nucleotide at exact positions required to prepare heteroduplex DNA substrates (see Fig. S2 and S3).

The most crucial step in synthesis of heteroduplex is to obtain sufficient quantities of circular ssDNA. Two different strategies were applied to obtain it from either of the plasmids depending on the mismatch required. The first strategy depends on treatment with nicking endonucleases followed by Exonuclease III digestion. The second strategy, on the other hand, exploits the phagemid properties present in the parent plasmid (i.e. pGEM-T), as described below.

Nicking endonuclease + Exonuclease III based mismatch repair substrate production strategy is modified from the one described earlier by Zhou et al. [15]. Both pGSM and pGSA contain two unique nicking endonuclease sites, one site for Nt.BspQI is inherent to pGEM-T plasmid whereas the other nicking site, recognized by either Nt.BbvCI or Nb.BbvCI depending on the strand to be digested, was introduced by the oligo-homoduplex ligated to it. The addition of this recognition site adds flexibility by allowing nicking of either the top or the bottom strand. After nicking the plasmids, Exonuclease III digestion results in the complete removal of the nicked strand. The resulting circular single-stranded DNA can then be used for heteroduplex preparation as described below. This method is suitable when substrate requirement is limited.

The second strategy utilized bacterial packaging cell lines to produce ssDNA. The most frequently utilized approaches for the preparation of ssDNA employ phagemids, which are derived from the single-stranded bacteriophage M13, fd, or f1, and are capable of replicating as plasmids in bacterial hosts [16]. The pGEM-T has properties of a phagemid as it contains an f1 origin enabling ssDNA replication and packaging into phage particles. Therefore, it was chosen for the current study to produce ssDNA with the help of a phage display system. Additionally, a novel approach was utilized to overcome the need of a helper phage. DH5α cells were transformed with the plasmid M13cp making them "bacterial packaging cell lines" [13]. M13cp is an M13-based helper plasmid that contains the phage genome without its packaging signal/origin and possesses a chloramphenicol resistant gene. The absence of packaging signal makes it incapable of packaging its own DNA. However, when a phagemid (e.g. pGSM or pGSA) containing the f1 origin is transformed into M13cp containing cells, they replicate the phagemid, producing and packaging ssDNA in viral particles (See Chasteen, Ayriss, Pavlik and Bradbury [13] for more details). The M13cp transformed cells were grown overnight on plates containing chloramphenicol (25 μg/mL). These M13cp-cells were made chemically competent using the protocol described by Inoue, Nojima and Okayama [17], and used as bacterial packaging cell lines to produce large quantities of pure ssDNA from the phagemids pGSM and pGSA without the need of a helper phage.

For this purpose, M13cp-cells were transformed with the recombinant plasmids pGSM and pGSA using a heat shock transformation protocol and grown on LB agar-ampicillin-chloramphenicol plates. Colonies from each transformation were picked and grown overnight in LB-ampicillin-chloramphenicol media at 30 °C at 250 rpm. The bacterial cells were separated from the broth by centrifugation, and the supernatant was used for ssDNA isolation using a PEG-phenol-chloroform extraction followed by ethanol precipitation as described previously [18]. Simultaneously, a silica column-based protocol described previously was also employed [19]. Using this approach a large amount of specific circular ssDNA was produced. A 50 ml liquid culture of bacterial packaging cell line containing the M13-cp plasmid and one of the recombinant phagemids typically yielded ~7–15 μg of ssDNA. The four plasmid/phagemid constructs described above, namely pGSM-t, pGSM-b, pGSA-t, and pGSA-b, led to the production of circular ssDNAs containing C-, G-, A- and T-nucleotides at the target restriction site, respectively.

In order to synthesize the desired heteroduplex, 1 μg ssDNA prepared as described above was mixed with 350 ng of plasmid linearized using ScaI (or NaeI) and containing a non-complementary base at the