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DNA Reassociation using Oscillating Phenol Emulsions

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Abstract

Reassociating double-stranded DNA from single-stranded components is necessary for many molecular genetics experiments. The choice of a DNA reassociation method is dictated by the complexity of the starting material. Reassociation of simple oligomers needs only slow cooling in an aqueous environment, whereas reannealing the many single-stranded DNAs of complex genomic mixtures requires both a phenol emulsion to accelerate DNA reassociation and dedicated equipment to maintain the emulsion. We present a method that is equally suitable for reassociating either simple or complex DNA mixtures. The Oscillating Phenol Emulsion Reassociation Technique (OsPERT) was primarily developed to prepare heteroduplex DNA from alkali-denatured high molecular weight human genomic DNA samples in which hundreds of thousands of fragments need to be reannealed, but the simplicity of the technique makes it practical for less demanding DNA reassociation applications.
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Keywords: DNA reassociation; Heteroduplex DNA

Introduction

Many molecular genetic studies require the production and analysis of heteroduplex DNA fragments, that is, double-stranded DNA (dsDNA) where each strand originates from a different source. These heteroduplexes may result from annealing PCR amplicons from two individuals during diagnostic studies [1], or may arise from annealing two synthetic complementary oligomers for routine molecular biology applications. In these cases where the starting material is present as a low complexity mixture, DNA reassociation readily takes place in aqueous solution.

Such is not the situation where thousands of unique single-stranded DNA (ssDNA) molecules must find their proper complement, such as in subtractive hybridizations of genomic DNAs [2–4] or cDNAs [5–7], or in Genomic Mismatch Scanning (GMS) [8–11]. For these applications, the phenol emulsion reassociation technique [12] (PERT) is used as it allows DNA reassociation to proceed at a rate thousands of times faster than that achievable in aqueous solutions.

A modification of the original PERT method substituted thermal cycling for mechanical shaking as the means of maintaining the phenol emulsion [13]. Preservation of the emulsion is essential as it is thought that phenol microspheres adsorb and then present ssDNAs to potential pairing partners, leading to rapid formation of properly matched duplexes [14]. Another modified method, FPERT [15], introduced formamide into the emulsion thereby allowing an increased size range of reassociated dsDNAs. However, the addition of formamide also increased the amount of phenol (from 10% to ~25% of final volume) required to produce an emulsion.

Here, we present a technique that combines aspects of the PERT procedure and its modifications with the goal of: (1) maintaining the high molecular weight DNA product obtained with FPERT, (2) decreasing reaction volume (so as to decrease losses through handling), (3) keeping the phenol concentration (10%) of the original PERT procedure [12] which, with a smaller reaction volume, would eliminate chloroform extractions (another source of DNA loss), and (4) pursuing the PERT modification that uses thermal cycling [13] rather than agitation as the means of driving the DNA/phenol mixture through its

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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solution and emulsion phases. Our PERT modification, the Oscillating Phenol Emulsion Reassociation Technique (OsPERT), oscillates DNA samples between 25°C and 65°C in a sodium chloride-based phenol emulsion and allows reannealing of high molecular weight genomic DNA with a greater yield of reassociated DNA than FPERT. Although an OsPERT reaction of less than one hour will reassociate complementary strands from a simple mixture of DNAs (e.g. single-stranded plasmid DNAs), the intention of this paper is to demonstrate the ability of OsPERT reactions to reanneal both the long 23 kb *Hin*dIII fragment of bacteriophage λ and the high molecular weight DNAs present in human genomic DNA restriction digests.

Results

Overview of OsPERT

Double-stranded DNA is denatured, and the resultant ssDNAs dispersed in a sodium chloride-based phenol emulsion. Double-stranded DNA is then reassociated by cycling the ssDNA reactants hundreds of times using a high temperature step (15 s at 65°C) which dissolves the phenol into the aqueous phase, and a low temperature step (15 s at 25°C) which regenerates the phenol emulsion. Unreacted ssDNAs are removed by binding to nitrocellulose, and salts and phenol are removed from the reassociated dsDNA, which is then suitable for downstream applications.

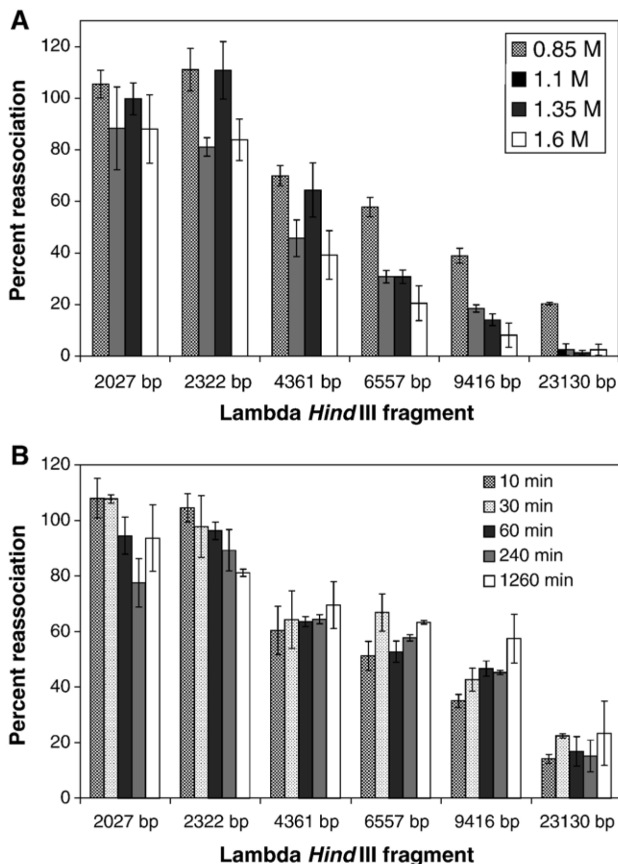

Fig. 1. Reassociation of denatured *Hind*III-digested λ phage DNA by OsPERT at (A) various concentrations of sodium chloride-based phenol emulsion and (B) various time points in 0.85M sodium chloride-based phenol emulsion.

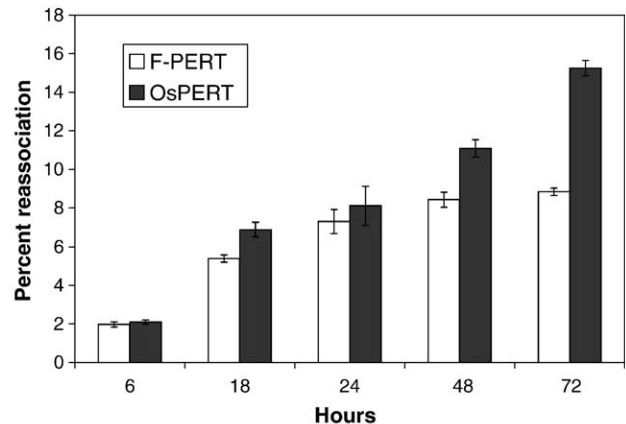

Fig. 2. Comparison of reassociation of denatured *Pst*I-digested human genomic DNA at various time points by FPERT and OsPERT.

NaCl concentration and time course of DNA reassociation

Preliminary trials of the OsPERT method showed that sodium thiocyanate (NaSCN), the preferred salt for optimal yield in phenol-enhanced DNA reassociations [12,13] yielded little or no reassociated DNA product, so NaCl was substituted. The effect of differing concentrations of NaCl on reassociation of denatured *Hind*III digested λ DNA was examined. Fig. 1A shows that a final NaCl concentration of 0.85 M gave nearly complete reassociation of the smaller λ *Hind*III fragments and a 20% reassociation of the large 23 kb λ fragment in a 16 hour OsPERT reaction.

Next, the optimal time necessary to reanneal λ DNA fragments in a 0.85 M NaCl-containing phenol emulsion was determined. Ten minutes of the oscillating phenol emulsion sufficed for reassociating most of the fragments present in this simple DNA mixture (Fig. 1B). Using the rate of reassociation for the 6.5 kb λ *Hind*III fragment, we found that the $C_ot_{1/2}$ in OsPERT is very similar to that achieved by Kohne and colleagues using PERT [12], which is many thousand fold higher than DNA reassociation in aqueous medium, confirming that phenol emulsions greatly enhance DNA reassociation.

For human genomic DNA digests where the complexity of the DNA mixture is much higher, longer times in the oscillating phenol emulsion were necessary (Fig. 2).

Formation of heteroduplex DNA

To compare the efficiency of FPERT and OsPERT to reassociate a complex human genomic DNA mixture, we mixed equal amounts of *Pst*I-digested DNA from one individual and methylated *Pst*I-digested DNA from a different individual. The DNA mixtures were denatured and allowed to reanneal by either FPERT or OsPERT. OsPERT provided more total reassociated dsDNA than did FPERT, and its production of reassociated dsDNA did not level off over time (Fig. 2).

Non-methylated and methylated DNAs were used so that hemimethylated heteroduplex DNA could be generated and isolated from the total dsDNAs reassociated. The resulting heteroduplex DNA can then be used as substrate for molecular analysis including DNA mismatch detection. The hemimethylated heteroduplex DNAs were isolated from the total reassociated DNA by their resistance to treatment with *Dpn*I and *Mbo*I, which preferentially digest completely methylated and unmethylated DNA, respectively. Table 1 shows amounts of reassociated total dsDNA (homoduplexes and heteroduplexes) recovered from 6, 18 and 24 hour FPERT and OsPERT reactions starting with five micrograms of non-methylated DNA and five micrograms of methylated DNA, and the amounts of hemimethylated heteroduplex DNAs recovered from the reassociated total dsDNAs.

The percentage of hemimethylated heteroduplex DNA in this total dsDNA was essentially equivalent for each reassociation technique (between 25 and 30 percent of total reassociated dsDNA). That a value closer to the 50 percent theoretical yield was not realized is due to several factors: 1) adventitious binding of dsDNA to nitrocellulose, 2) incidental losses from silica adsorption and desorption, and 3) digestion of some hemimethylated DNA by *Dpn*I, which preferentially digests methylated DNA, but will also slowly digest hemimethylated substrates (http://www.neb.com/nebecomm/products/faqproductR0176.asp#10).

Characterization of reassociated heteroduplex DNA

The size range of the fragments present in this hemimethylated heteroduplex DNA was analyzed by gel electrophoresis, and the authenticity of this hemimethylated heteroduplex DNA was evaluated by its resistance to another round of *Dpn*I and *Mbo*I digestion. Neither *Dpn*I nor *Mbo*I treatments appreciably reduced the fragment size of hemimethylated heteroduplex DNAs prepared from FPERT or OsPERT reactions, but did digest the non-methylated and methylated DNAs used as controls to confirm the activity of the *Dpn*I and *Mbo*I preparations employed in the analysis. Any minor activity of *Dpn*I on hemimethylated heteroduplex DNA was probably masked by the predominance of unaffected DNA in the hemimethylated heteroduplex DNA sample. The size range of the heteroduplex DNA largely reflects that of the initial *Pst*I digested genomic DNA, indicating that the FPERT or OsPERT techniques did not promote a selective bias for reassociation of particular size classes.

Table 1
Recoveries of reassociated double-stranded DNA

Reaction time (h)	FPERT [Ave ± SD (ng DNA)]		OsPERT [Ave ± SD (ng DNA)]	
	Total DNA	Heteroduplex	Total DNA	Heteroduplex
6	197 ± 12. 7	54 ± 7. 1	210 ± 11. 3	62 ± 1. 6
18	538 ± 19. 1	136 ± 1. 4	688 ± 37. 5	180 ± 17. 7
24	730 ± 61. 5	185 ± 1. 4	811 ± 100. 4	239 ± 65

Discussion

OsPERT is a general DNA reassociation procedure derived from the classical PERT method [12] that reanneals large DNA fragments present in complex genomic digests. Rather than using constant agitation to maintain the phenol emulsion, OsPERT employs hundreds of iterations of thermal cycling to rapidly and repeatedly oscillate the state of the DNA/phenol from emulsion to solution and then back again. Because a comparatively large amount of DNA (10 μgm) is dissolved in a small volume, the random reappearance of phenol microdroplets produced by every oscillation encourages contact between previously unreacted ssDNAs, augmenting the yield of reassociated dsDNA. Continuous shaking of the phenol emulsion in the standard PERT method is replaced by recurrent formation and dissolution of the emulsion with every heating/cooling cycle in the OsPERT procedure. Finally, the brief high temperature step, by melting out poorly matched duplexes, minimizes progressive sequestration of unreacted ssDNAs into unproductive complexes. These freed ssDNAs accordingly re-enter the reassociation reaction improving the final dsDNA yield.

The products of OsPERT are of the same high molecular weight size range as the starting material; therefore, it is a suitable procedure for reannealing large DNA fragments. As a practical matter, OsPERT DNA reassociation takes place in a reasonable time and does not require special laboratory equipment.

Materials and methods

Reassociation of λ DNA fragments

One μgm of *λ Hind*III digested DNA (New England BioLabs) was either denatured with NaOH, as in the typical OsPERT procedure (see below), or was not denatured so as to provide a control for DNA recovery. The DNA was then allowed to reassociate in an overnight OsPERT reaction in 0.85 M, 1.1 M, 1.35 M or 1.6 M NaCl. Following removal of ssDNA with nitrocellulose (see below), the samples were electrophoresed on a 0.7% agarose gel, and the image of the scanned EtBr-stained gel was analyzed using NIH Image, the ImageJ program (http://rsb.info.nih.gov/ij/).

Genomic DNA and enzymes

The DNAs used were *Pst*I digested human genomic DNA (“non-methylated DNA”) and dam methylase-treated *Pst*I digested DNA (“methylated DNA”). Concentrations of these *Pst*I digests were at least 500 μgm/ml. Restriction enzymes, dam methylase and Exonuclease III were from New England BioLabs.

FPERT procedure

Five μgm each of non-methylated DNA and methylated DNA were mixed and held for 15 min at room temperature in a final volume of 100 μl of 0.3 N NaOH. The reaction was neutralized with 15.5 μl of 3 M MOPS (free acid), then 32 μl formamide, 53 μl water and 200 μl of 4 M NaSCN, 20 mM Tris–HCl pH 8.0, 0.2 mM EDTA were added and mixed. After addition of 175 μl of Tris buffer-saturated phenol (Invitrogen, pH 7.49–7.79), the sample was vortexed briefly to generate an emulsion and placed on a rotating wrist-action shaker at RT to maintain the emulsion. Isolation of DNA from the FPERT reactions involved extraction with an equal volume of chloroform and back extraction of the chloroform phase with one-tenth

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volume of 10 mM Tris–HCl pH 8.0, 1 mM EDTA (TE). The chloroform extraction and TE back extraction were repeated, and the DNA in the aqueous phase was purified using the QIAprep spin miniprep protocol (Qiagen) followed by elution with 50 μl of Qiagen Buffer EB (10 mM Tris–HCl pH 8.5). Single-stranded DNAs in these eluates were removed with nitrocellulose as stated below.

OsPERT procedure

One μgm of λ *Hind*III digested DNA or 5 μgm each of nonmethylated and methylated DNAs were mixed and held for 15 min at RT in a final volume of 34.6 μl of 0.3 N NaOH. After neutralization with 5.4 μl of 3 M MOPS, 50 μl of 1.5 M NaCl, 2 mM EDTA was added and mixed. Ten μl of buffer-saturated phenol was added and mixed by pipetting. The sample was held at 65°C for 30 s to dissolve the phenol, quenched in ice-water to generate a phenol emulsion, and then placed in a thermal cycler programmed for a 15 s high temperature step of 65°C and a 15 s low temperature step of 25°C. At specified times, the reactions were removed from the thermal cycler and directly applied to nitrocellulose for ssDNA removal (see below). On the PTC-100 thermal cyclers (MJ Research), each complete cycle required about two min; a 16 h OsPERT reaction therefore consisted of about 480 cycles.

Removal of ssDNA using nitrocellulose

Eluates from the FPERT reaction were made 1 M in NaCl in a 100 μl volume; OsPERT reactions were used as is. These were spun (500 *g*, three min, RT) through Centrex MF 1.5 nitrocellulose filters (Schleicher and Schuell) (treated with 1 M NaCl, 10 mM Tris–HCl pH 8.0), and the filters washed with 25 μl of 1 M NaCl, 10 mM Tris–HCl pH 8.0. The eluted dsDNA was purified using QIAprep spin miniprep columns as above.

Removal of homoduplex DNA and preparation of heteroduplex DNA

Homoduplexes present in above dsDNA were removed by incubation of 250 ng of the dsDNA in a total volume of 40 μl of 1X NEBuffer #1 (New England BioLabs), 100 μgm BSA/ml with 0.1 U of *Dpn*I and 0.5 U of *Mbo*I for 30 min at 37°C. One μl of 0.1 U Exonuclease III was added and incubation at 37°C continued for 15 min. Enzymes were inactivated at 70°C for 20 min, the reaction brought to 1 M in NaCl in a 100 μl volume, and ssDNAs produced by Exonuclease III activity on *Dpn*I- and *Mbo*I-cleaved DNAs removed by centrifugation through nitrocellulose filters as above. This elute, containing hemimethylated heteroduplex DNAs, was purified using QIAprep spin miniprep columns as above.

Calculation of rate of reassociation

In their paper, Kohne et al [12] found the $Cot_{1/2}$ for sonicated *E. coli* DNA in a 1 M NaCl/ 10% phenol emulsion to be 7.8×10^{-4} mol s/ L. Using their conditions, a $Cot_{1/2}$ for a similarly sonicated 6,557 bp λ DNA fragment, which is about 700 times smaller than the *E. coli* genome, would be 1.1×10^{-6} mol s/ L.

We found that 50% reassociation of the 3.1×10^{-14} mol of the 6,557 bp λ DNA fragment in the 100 μL OsPERT reaction was complete in 10 minutes, yielding a $Cot_{1/2}$ of 1.9×10^{-7} mol s/ L, a somewhat faster reassociation than the rate of 1.1×10^{-6} mol s/ L calculated above, but not remarkably different when one considers differences in NaCl concentrations, buffer systems and complexity of the DNA mixtures in our study and that of Kohne et al [12].

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