Benchmarks

Tunable fractionation of nucleic acids

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We developed a method for selective purification of DNA using the cationic detergent, cetyltrimethylammonium bromide (CTAB), accompanied with urea and controlled high-salt (NaCl) concentration. This method is effective for rapid separation of DNA fragments from artifacts such as PCR primer dimers or ligation adapters. The CTAB-associated purification completely removed the short PCR artifacts and primers, as well as enzymes and buffer, while recovering a sufficient quantity of amplicons for subsequent experiments such as preparation of libraries. This method could also be applied to the fractionation of nucleic acids generated by other types of reactions.

Many molecular biology techniques require purification of cDNAs, DNAs, or DNA-RNA hybrids, and/or fractionation of nucleic acids from various biological sources and reactions to improve downstream performance. Generally, PCR amplicons with short primers (~20 bp) and no artifacts can be readily purified with commercially available PCR purification kits that have a maximum cutoff of 100 bp. However, in multi-step techniques requiring PCR, these purification kits often cannot remove artifacts longer than 100 bp; this is especially problematic when DNA libraries must be prepared (1–3). Next-generation flow-cell sequencers offer DNA sequencing directly from PCR, which is advantageous because subcloning is avoided, but it also requires the subsequent purification of the amplified products to increase the sequencing quality, which is proportional to the purity of the template (4). In cap analysis of gene expression (CAGE) technologies with deep sequencing or deepCAGE (2)—in which PCR is used as a key step to prepare libraries for flow-cell sequencing—sometimes artifacts <150 bp are generated (1,2). In a search for ways to remove these artifacts, we systematically investigated the effect of NaCl concentration on DNA fraction-

ation in the presence of cetyltrimethylammonium bromide (CTAB) and urea (5,6), using a glass fiber matrix in a spin column (illustra GFX Column; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The GFX kit exploits a glass fiber matrix in conjunction with chaotropic salt to capture nucleic acids (7,8). Here, to better modulate the fractionation of differently-sized nucleic acids as a substitute for chaotropic salt, we have introduced a CTAB-urea solution to capture the nucleic acids. Selective purification was performed with variable NaCl concentrations. CTAB has been used for decades to extract and purify DNAs from biological sources (5,6,9–11). It has recently been shown that CTAB along with urea can improve the purification of RNAs by removing polysaccharides and other contaminants (6). We have also found that RNAs shorter than tRNAs are not precipitated efficiently under certain conditions (6). With these problems in mind, we have explored various options to optimize this purification method.

To set up the methodology, we started with 500 ng 50-bp DNA ladder marker (Invitrogen, Carlsbad, CA, USA) or PCR reaction (5 μ L). The DNAs were mixed with 1% CTAB-urea solution (4M urea, 50

mM Tris-HCl pH 7.0, and 1 mM EDTA pH 8.0). CTAB forms positive micelles in solution with nucleic acids: it interacts with nucleic acids through the phosphates and its quaternary ammonia, and the long chain of the detergent localizes away from the DNA groove to reduce contact with the surrounding water. It does this by forming aggregates and thereby promotes condensation (10,12). We applied this property of CTAB to capture the DNAs in the glass fiber. The exact mechanism of this capture is currently uncharacterized, but several possibilities are that (i) micelles are absorbed to the glass filter matrix, (ii) CTAB coats the fiber and makes it positively charged, or (iii) the CTAB-DNA complex is simply retained very efficiently to the surface. The interaction of CTAB and nucleic acids was controlled by adding 5 M NaCl solution to final concentrations of 0.4–0.6 M. Then, the mixture was shaken briskly and heated at 65°C for 10 min. Following incubation, samples were kept at room temperature for 10 min and applied to the GFX columns. Each column was centrifuged at 16,000 $\times g$ for 1 min. Flow-through was collected and eluted DNA was recovered by ethanol precipitation for later control electrophoresis. To remove the CTAB, we washed the column with 600 μ L wash buffer (60% ethanol, 0.3 M NaCl, 100 mM Tris-HCl pH 7.5, and 5 mM EDTA pH 8.0). The column was washed a second time with 600 μ L 80% ethanol, which removes the CTAB while keeping the DNA precipitated onto the matrix. The column was replaced with a new 1.5-mL siliconized tube and DNA was eluted with 50 μ L nuclease-free H₂O, then concentrated by ethanol precipitation. DNA obtained in the first flow-through and final elution was analyzed on 6% polyacrylamide gel or 1.5% agarose gel. Gels were stained either with ethidium bromide or GelStar (Lonza, Rockland, ME, USA) and visualized with BioDocIt (UVP, Upland, CA, USA). We estimated how much DNA was recovered by comparison with a fragmented ladder and quantified by measuring the concentration with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The overall recovery was ~65%; recovery was ~30% for smaller fragments just above the 150-bp cutoff, and ~80% for larger fragments (data not shown).

To verify the effectiveness and to search out the different cutoffs, we tested various salt concentrations (Figure 1, A and B). In general, the cutoff value increased with increased salt concentration. For instance, with a 0.6 M salt concentration (used later for

Figure 1. Selective purification of DNA by CTAB-NaCl method. (A and B) Effect of NaCl concentrations on selective separation of DNA using DNA ladder markers: 50 bp on GFX (A) and 10 bp/50 bp on PureLink (B) matrixes. Electrophoresis was carried out on 6% polyacrylamide gel at 100 V for 3 h in TA buffer. M1/M2/M3/M4: 50/100/25/10 bp DNA ladder markers (Invitrogen); 1: First flow-through; 2: Final elution; GFX: Purification according to the manufacturer's instructions. (C) Application of CTAB-NaCl method for purification of CAGE libraries. Four different CAGE libraries (lanes 1–4; left half of the image) made of 5' cDNAs of reverse-transcribed PCR (total RNA from human HepG2 cell line) with random primer that produces some artifacts (up to 150 bp as prominent in library number 3 and 4) were purified with 0.6 M NaCl. It is evident that the artifacts could be completely removed (right half of the image) from the PCR products. Electrophoresis was carried out on 1.5% agarose gel at 100 V for 10 min in TAE buffer. M1, FastRuler DNA Ladders Middle Range; M2, GeneRuler 1-kb DNA Ladders (Fermentas); NC, minus template PCR control without purification.

the purification of PCR products; see Figure 1C), DNA up to 150 bp could be removed but was accompanied by less recovery of larger-sized DNAs. Even though the recovery of larger DNA fragments was not high, it was more than sufficient for subsequent steps for library preparation methods that require removal of <150-bp fragments, such as in the CAGE protocol (Figure 1C). Nevertheless, a much higher salt concentration can be used if a higher cutoff is required (Figure 1B). As we expected (Figure 1A), fractionation of DNA does not occur at ≤ 0.4 M (9). However, under more stringent conditions at higher salt concentrations, the CTAB-DNA interaction is lost gradually and proportionally to the salt concentration; CTAB may work as an anion exchanger with some PCR molecules releasing the bound DNA. We targeted a salt concentration in the range of ~ 0.55 – 0.60 M and observed that minute fractional variation can act as a specific molecular sieve (data not shown). This protocol is optimized for the conditions described herein. Therefore, we emphasize that deviation from the recommended salt concentrations needs recalibration when using different conditions.

With little modification and adjustments to the salt concentration, this purification protocol was applied to a fractionation of nucleic acids from other reactions such as purification of reverse-transcribed cDNA (0.5 M NaCl, incubation at room temper-

ature for 10 min and centrifugation for binding and washing at $4300\times g$). This was done to remove primers from PCR amplicons <100 bp (0.3 M NaCl) and to recover DNA cleavage fragments <50 bp from restriction enzyme digestion samples; we omitted NaCl and the heating step, incubated samples on ice or at 4°C for 1 h, and used a low-salt wash buffer (150 mM NaCl, 1.5 mM EDTA). All of these purifications and fractionations can be performed routinely with sufficient recovery of the desired products (data not shown). Some optimization may be required if glass matrix spin columns other than GFX are used, although in principle the method should be universally applicable to all matrixes. We compared the purification with the PureLink column (Invitrogen), which showed some deviation in the purification range. However, with minor recalibration (Figure 1B), we believe that the protocol can be broadly used on various silica-based supports.

The reproducibility of the method was excellent on the samples/libraries when tested to purify various CAGE libraries. However, the buffer conditions may affect the reproducibility. We found that minor changes in concentrations or pH levels of buffer components were acceptable. Incubation of sample at room temperature following the heating step with binding buffer should be 10 min, since longer incubation causes lower recovery. For better reproducibility,

we recommend that the protocol be strictly followed and the buffers freshly prepared. The DNA ladders and CAGE libraries used to validate this protocol are composites of DNA populations of different sequences and sizes. Therefore, it is difficult to determine if there is sequence bias. However, within the range of our experience by analyzing CAGE libraries prepared with this method, we have not noticed any issues related to CG content or any other effect deriving from the sequence composition of the sequenced tags (not shown). Since we have yet to see evidence of this, we speculate that the method is sequence-independent. Indeed, purification based on CTAB has long been used for extraction and purification of many kinds of nucleic acids and there are no reports of bias (5,6,9–11).

Though laborious and time-consuming DNA extraction and size fractionation methods have been extensively studied, less research has been conducted on selective purification methods (13–15). The method we have developed is a new approach for selective purification of nucleic acids with CTAB-urea and NaCl. It has numerous advantages over existing methods that use chaotropic salt to purify nucleic acids. Chaotropic salt is not only a hazardous material but also sometimes imposes restriction on downstream experiments due to its carryover to the eluted nucleic acids. One disadvantage of our method is the moderate recovery of purified nucleic acids, but we observed that recovery can be improved by coating the glass fiber matrix with tRNA (20 $\mu\text{g}/\text{column}$) and eluting the DNA twice with pre-warmed (65°C) water. However, our method still compares favorably with commercially available kits such as NucleoSpin ExtractII (Macherey-Nagel GmbH & Co KG, Duren, Germany): in the case of selective purification of nucleic acids, this kit shows less recovery of large fragments above the cutoff (www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/DNA%20clean-up/UM_PCRcleanup_Gelex_NSExII.pdf). Additionally, our method uses chemicals that are more affordable than chaotropic salts. We also significantly reduce the cost by reusing the glass fiber matrix (www.biotechniques.com/multimedia/archive/00004/BTN_A_05392AF01_O_4525a.pdf). Thus, the simple and rapid purification protocol described here might be very useful for molecular biology applications where purification and/or fractionation is a necessity, such as single nucleotide polymorphism (SNP) genotyping, cDNA

labeling, rolling circle amplification methods, and preparation of libraries for next-generation DNA sequencers.

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Competing interests

The authors declare no competing interests.

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