Benchmarks

A convenient and efficient purification method for chemically labeled oligonucleotides

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We developed an efficient, cost-effective, and rapid purification method for chemically-labeled oligonucleotides that requires less time than conventional procedures such as ethanol precipitation or size-exclusion chromatography. Based on the hydrophilic and hydrophobic properties of DNA and amine-reactive fluorophores, we show that *n*-butanol saturated with distilled water may be used to remove unreacted fluorophores by sequestering them in the organic phase, while labeled DNA remains in the aqueous phase. This phase extraction method is simple, fast, and allows for processing multiple samples simultaneously, a necessity for high-throughput labeling strategies.

Chemical labeling of DNA has been of great interest in life science fields, including molecular biology, cell biology, and molecular diagnostics. Chemical conjugation is often done by introducing chemically reactive nucleophilic groups, such as primary amines and sulfhydryls to specific sites on DNA molecules. Fluorophore dyes or probes functionalized by electrophilic groups may then be conjugated to defined sites on DNA via specific chemical reactions and subsequently analyzed by various methods (1). For primary amine-mediated conjugation, a fluorophore-containing amine-reactive group such as active ester, isothiocyanate, or aldehyde, is chemically conjugated to the target amine group on the DNA molecule. Once labeled, the reaction mixture must be further purified to remove free dye and unlabeled DNA.

Conventional primary amine-based DNA labeling is usually performed in alkaline conditions (typically at pH 8.0–9.0) since this allows the deprotonated form of the primary amine to be readily available for covalent conjugation. For optimal labeling efficiency, an excess of the amine-reactive dye is used, therefore requiring subsequent removal of unreacted dye. Conventionally, this elimination step is carried out by ethanol precipitation, size-exclusion chromatography, and dialysis (2), methods that are time-consuming, tedious, and inefficient. To circumvent these problems, we developed and tested a phase extraction method (3) to separate free dye from labeled DNA.

We found that several ATTO dyes (ATTO-TEC GmbH, Siegen, Germany), including ATTO 390, ATTO 550, and ATTO 647N, are hydrophobic enough to separate into organic phase solvents, particularly *n*-butanol saturated with distilled water (Supplementary Figure S1A). The partition ratio of ATTO 647N ($\kappa_{partition} = [S_{organic}]/[S_{aqueous}] = 62.2$) was determined to quantitatively analyze the equilibrium constant for the distribution of the hydrophobic dye in two immiscible solvents (4). This ratio clearly implies that the dye favors the *n*-butanol phase. We then applied this simple concept to eliminate free dye following a chemical labeling reaction (Figure 1B), depending upon the highly hydrophilic nature of the oligonucleotide (5) to cause immiscibility in the organic solvent, and leave labeled DNA in the aqueous phase. We assessed the efficiency of phase extraction and the loss of labeled DNA by analyzing the butanol and aqueous phases with UV/VIS spectrophotometry (Supplementary Figure S1B). A representative chart of the analysis is shown in Figure 1C. Further information, including results from other dyes, is given in Supplementary Figures S1–S3 and Supplementary Tables S1–S3. After a single extraction, the majority of the free dye partitioned to the butanol phase; labeled and unlabeled DNA remained in the aqueous phase throughout the extraction process. We also tested another water-saturated solvent, isoamyl alcohol, and found that it worked equally well for

Method summary:

We developed an efficient, cost-effective, and rapid purification method for chemically-labeled oligonucleotides that requires less time than conventional procedures. Using *n*-butanol saturated with distilled water, excess amine-reactive fluorophores are sequestered in the organic phase, while labeled DNA remains in the aqueous phase.

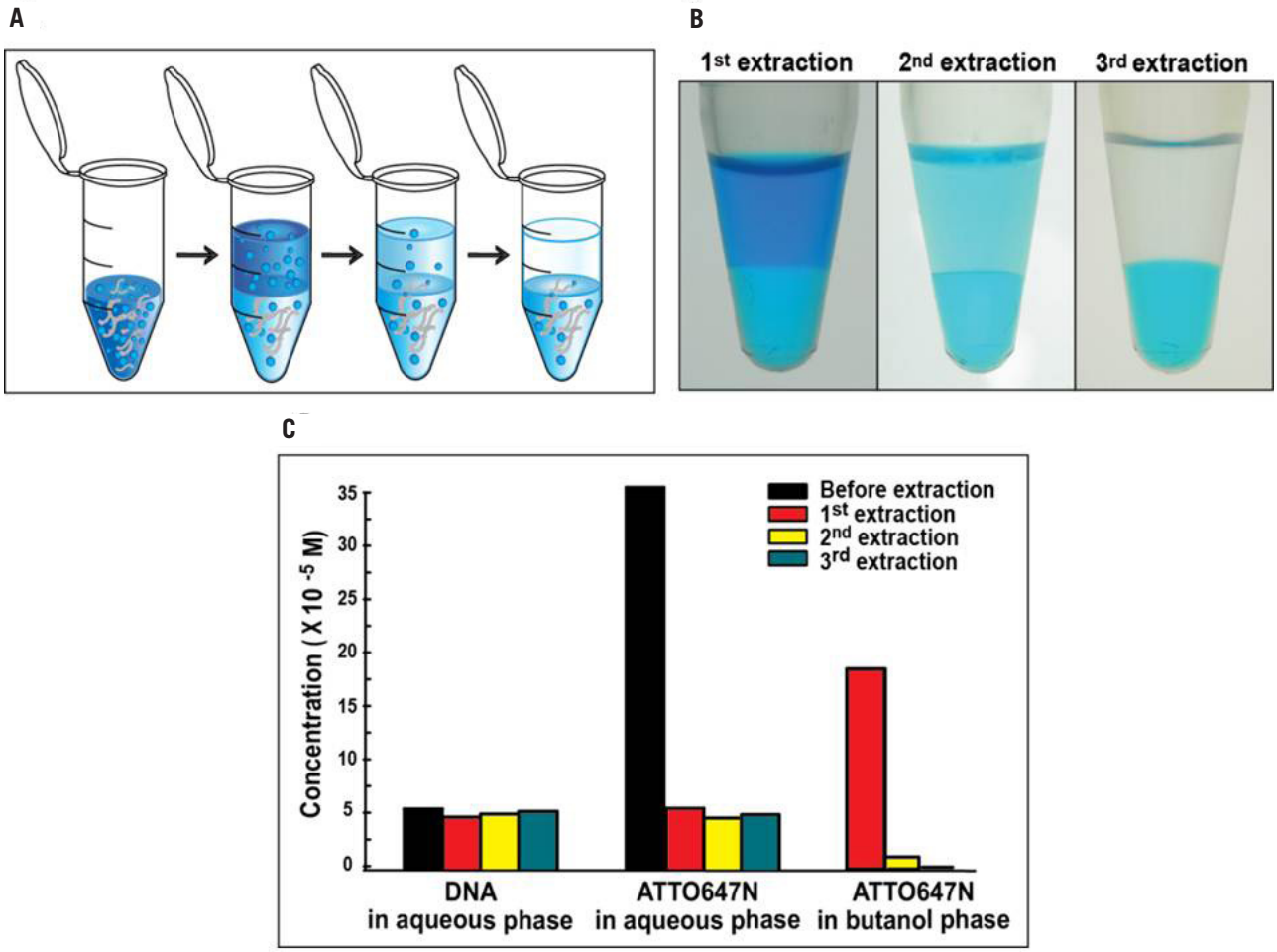

Figure 1. Purification strategy for chemically labeled oligonucleotides. (A) Schematic representation of free dye removal using water-saturated butanol. Multiple extractions successfully removed free dye from the labeling reaction mixture. The gray line and blue dots denote oligonucleotide and dye, respectively. (B) Extraction of unreacted ATTO 647N dye from the DNA labeling reaction mixture. After labeling DNA with amine-reactive ATTO 647N, 2–4 volumes of water-saturated butanol was added and vigorously mixed for 10 s. The two phases were separated by centrifugation at 4000× g for 10 s, and the upper organic phase was carefully removed. The extraction was repeated two more times to ensure the complete removal of free dye. A dramatic color contrast change in the upper butanol phase indicated that most of the free dye was removed in the first extraction step. (C) Efficiency assessment of free dye removal. DNA concentrations in the aqueous phase remained consistent, indicating that DNA molecules were not partitioned into the butanol phase. In addition, the concentration of ATTO 647N in the aqueous phase proves the effectiveness of dye removal after the first extraction. The concentration of ATTO 647N in the butanol phase supports the observed color contrast shown in (B).

phase extraction of labeled DNA (data not shown).

To determine whether the organic solvent altered any chemical or spectroscopic properties of either the DNA or the fluorophore, we annealed the labeled DNA to its complementary sequence and analyzed the result by gel electrophoresis. As shown in Supplementary Figure S4, we found no functional difference in the annealing properties of DNA extracted by ethanol precipitation or *n*-butanol phase extraction. The fluorescence intensity of butanol-extracted labeled DNA was compared with that of labeled DNA prepared using the conventional procedure. Supplementary Figure S5 shows that the fluorescence intensities are nearly identical, indicating that the method is suitable for purifying labeled DNA without causing any significant side effects.

After removal of free dye, unlabeled DNA must also be removed. In our hands, as long as high quality amine-modified oligonucleotides were used, the amine-based conjugation efficiency was high enough that no further purification was necessary. However, if the labeling efficiency is low, further purification will be required. This purification is typically done using gel electrophoresis or reverse phase or ion exchange chromotography. We also established a simple purification procedure for this step based on the hydrophobic nature of the ATTO dyes. To remove unlabeled DNA with reverse phase chromatography, the stringency of the washing conditions needs to be controlled to guarantee maximum yield and purity (6). When labeled dyes are relatively hydrophilic, one must sacrifice either the yield or the purity in order to maximize the other. On the other hand, DNA labeled with ATTO dyes showed a significant increase in hydrophobicity compared to unlabeled free DNA when analyzed by HPLC reverse phase chromatography (Figure 2A). Thus, a highly stringent wash condition can be applied without compromising the balance between yield and purity. We

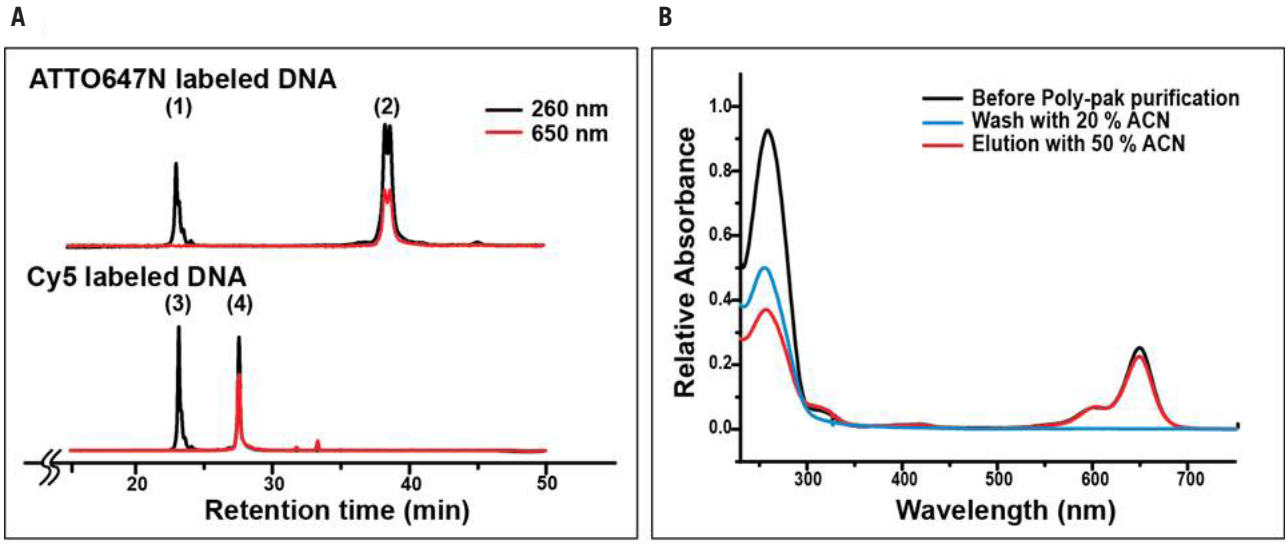

Figure 2. Effect of hydrophobic fluorophores on reverse phase chromatography. (A) Assessment of hydrophobicity for ATTO 647N (ATTO-TEC GmbH) and Cy5 (GE Healthcare, Uppsala, Sweden) dyes. Dye-labeled oligonucleotides were analyzed by high performance liquid chromatography (HPLC) with a µRPC C2/C18 ST 4.6/100 reverse phase column (GE Healthcare). The running conditions were 100% buffer A up to 10 min, followed by gradually increasing buffer B to 50% for 40 min. The flow rate was 1 mL/min, with Buffer A and B – 0.1M triethylammonium acetate (TEAA) and 100% acetonitrile (ACN), respectively. Peaks 2 and 4 are Cy5 labeled and ATTO 647N labeled DNA, respectively, while peaks 1 and 3 correspond to unlabeled DNA in the labeling reaction mixture. The difference in hydrophobicity between Cy5 and ATTO 647N is evident in the chromatogram where the separation between ATTO 647N and unlabeled DNA is much greater than between Cy5 and unlabeled DNA. (B) Absorbance spectra after PolyPak (Glen Research) purification for the removal of unlabeled DNA. Butanol-extracted samples were loaded onto PolyPak columns, which were washed twice with 3 mL 20% acetonitrile and eluted with 2 mL 50% acetonitrile. Each fraction was analyzed by UV-VIS absorbance. DNA is represented by absorbance at 260 nm, while ATTO 647N DNA is represented by absorbance at 650 nm. Labeled DNA was successfully obtained with high purity.

optimized purification using disposable reverse phase columns (PolyPak, Glen Research, Inc., Sterling, VA). As shown in Figure 2B, 20% acetonitrile solution ensures the removal of free DNA during washing without eluting the labeled DNA from the column. The UV/VIS spectrum of the eluted product demonstrated the high purity of labeled DNA (Figure 2B).

This report describes a simple, efficient, cost-effective, and rapid method for isolating labeled DNA with high purity. Hydrophobic ATTO dyes are particularly suited for this approach, but it may be possible to find a variety of hydrophobic dyes across the UV and visible wavelength range for optimization with this procedure. Although we demonstrated the method mainly with amine-based DNA-fluorophore conjugation, it can also be applied to other types of labeling chemistries such as thiol- and click chemistry-based coupling (7,8), as supported by Supplementary Figure S6, which shows the results of a thiol-based labeling experiment. Importantly, this extraction technique requires less than 5 min without resulting in any noticeable loss compared with other conventional methods, which are typically accompanied by lower recovery and take 30–60 min for disposable size-exclusion chromatography, several hours for dialysis, or up to a full day for electrophoresis-based gel extraction (9). The simplicity and cost-effectiveness of this approach should facilitate high-throughput labeling of DNA as well.

Acknowledgments

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Competing interests

The authors declare no competing interests.

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