SHORT TECHNICAL REPORTS

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Elimination of Residual Natural Nucleotides from 3′-*O*-Modified-dNTP Syntheses by Enzymatic Mop-Up

BioTechniques 25:814-817 (November 1998)

ABSTRACT

*Here, we describe a novel strategy called enzymatic "Mop-Up" that efficiently removes contaminating dNTPs from reverse-phase, high-performance liquid chromatography (RP-HPLC) purified 3¢-*O*-modified dNTP syntheses. Enzymatic mop-up takes advantage of the high selectivity of DNA polymerases for the former nucleoside triphosphates over the latter nucleotide analogs. We demonstrate the selective removal of contaminating dATP and dTTP from RP-HPLC purified 3¢-*O*-methyl-dATP and 3¢-*O*-(2-nitrobenzyl)-dTTP syntheses, respectively. These data highlight the importance of natural nucleotide contamination when interpreting enzymatic incorporation data and provide an alternative hypothesis for the observed property of catalytic editing of DNA polymerases. Moreover, the effective removal of natural nucleotides from 3¢-*O*-modified analogs addresses the important issue of nucleotide read-through for stop-start DNA sequencing strategies, such as the base addition sequencing scheme (BASS).*

INTRODUCTION

Characterization of the behavior of deoxyribonucleoside triphosphate (dNTP) analogs as terminators of DNA syntheses has greatly increased our understanding of DNA polymerases. In general, DNA polymerases show restricted selectivity and specificity for dNTP analogs that have been particularly useful for the development of antiviral compounds. While these analogs potentially have broad use for molecular biology applications, in practice however, large screening assays must be used to identify specific DNA polymerases that show the desired activity. Moreover, the study of 3′-*O*-modified-dNTPs has been particularly challenging because the starting material for the chemical syntheses of these compounds is the natural 2′-deoxyribonucleoside, which can be carried-over into the chemical phosphorylation step. The subsequent use of chromatography is typically inadequate for the complete removal of the contaminating dNTPs from 3′-*O*-modified-dNTP synthesis.

Previously, we demonstrated the feasibility of a stepwise DNA sequencing strategy, called the base addition sequencing scheme (BASS), by the synthesis and enzymatic incorporation of 7 different 3′-*O*-modified-dATPs and 3′-*O*-methyl-dTTP (3). However, a major obstacle in BASS has been the presence of contaminating natural dNTPs carried over in the chemical synthesis of 3′-*O*-modified-dNTPs (2,3). Although reverse-phase, high-performance liquid chromatography (RP-HPLC) can purify 3′-*O*-modified-dNTPs $\geq$99.5%, the level of contamination remains sufficiently high that the DNA polymerase selectively incorporates the preferred natural dNTP over the 3′-*O*-modified-dNTP analog. Incorporation of the natural dNTP at the desired termination base, referred to here as natural-nucleotide read-through, can significantly result in decreased signal intensities and higher background signals in subsequent base addition steps, thus making the interpretation of enzymatic incorporation data more difficult. To overcome this problem, we have developed an enzymatic "Mop-Up" strategy that specifically removes dNTP contamination from RP-HPLC-purified 3′-*O*-modified-dNTPs.

MATERIALS AND METHODS

Reagents

Nucleotides, Klenow fragment of DNA polymerase I and avian myeloblastosis virus reverse transcriptase (AMV-RT) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). *Bst* DNA Polymerase was purchased from Bio-Rad (Hercules, CA, USA). Streptavidin-coated magnetic Dynabeads® M-280 were purchased from Dynal AS (Oslo, Norway). DNA synthesis reagents were purchased from PE Applied Biosystems (Foster City, CA, USA), except for the biotin phosphoramidite, which was purchased from Glen Research (Sterling, VA, USA). All oligonucleotides were synthesized trityl-on using a Model 394 DNA Synthesizer (PE Applied Biosystems) and purified using Nensorb™ 20 columns, according to the manufacturer's protocol (NEN Life Science Products, Boston, MA, USA).

Mop-Up Assay

Biotinylated primer (500 pmol) (5′-CAGAGCCAAGGCTCTACCTAGCAGTGTAAAACGACGGCCAGT-3′) in 100 µL of reaction buffer (10 mM Tris-

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Mop-Up Assay

Biotinylated primer (500 pmol) (5′-CAGAGCCAAGGCTCTACCTAGCAGTGTAAAACGACGGCCAGT-3′) in 100 μL of reaction buffer (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$) was added to 100 μL of prewashed streptavidin-coated magnetic Dynabeads, incubated for 1 h in 10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 1 M NaCl at ambient temperature and washed twice with reaction buffer by magnetic separation. Five hundred picomoles of the mop-up template [5′-$(\mathrm{T})_{26}$ACTGGCCGTCGTTTTACA-3′) in 100 μL of reaction buffer were added to the primer-captured beads and annealed by heating to 80°C for 5 min. The reaction was slowly cooled to ambient temperature and washed twice with reaction buffer. Incorporation of the natural nucleotide was performed according to the enzymatic conditions described for *Bst* DNA polymerase (4 U) or Klenow fragment (10 U) (3) at 65° or 37°C for 1 h, respectively. The mop-up solution containing the 3′-*O*-modified-dNTP was isolated by magnetic separation and directly used in the incorporation assay. For enzymatic mop-up of dTTP analogs, the following template was used: [5′-$(\mathrm{A})_{26}$CTGGCCGTCGTTTTACA-3′].

RESULTS AND DISCUSSION

Here, the mop-up assay is described by which the contaminating natural nucleotide is enzymatically removed from a 3′-*O*-modified-dNTP solution by

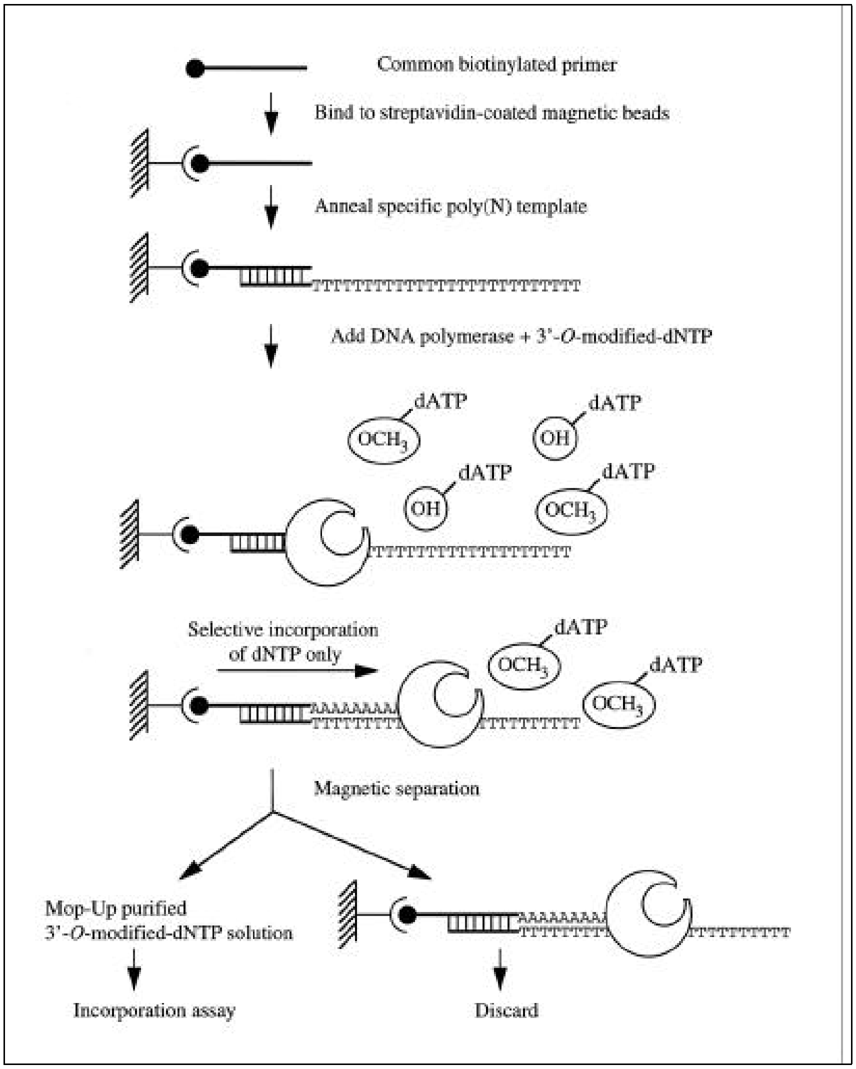

Figure 1. Enzymatic mop-up assay to remove contaminating dNTPs from a 3′-*O*-modified-dNTP solution. A common biotinylated primer is attached to streptavidin-coated magnetic beads, washed and annealed to a specific poly(dN) template. The mop-up polymerase, buffer and 3′-*O*-modified-dNTP are incubated to allow the mop-up enzyme selectively to remove the contaminating dNTP by DNA synthesis of the solid-phase bound primer-template complex. Once completed, the mop-up purified 3′-*O*-modified-dNTP is isolated from the primer-template complex by magnetic separation and directly used in the incorporation assay.

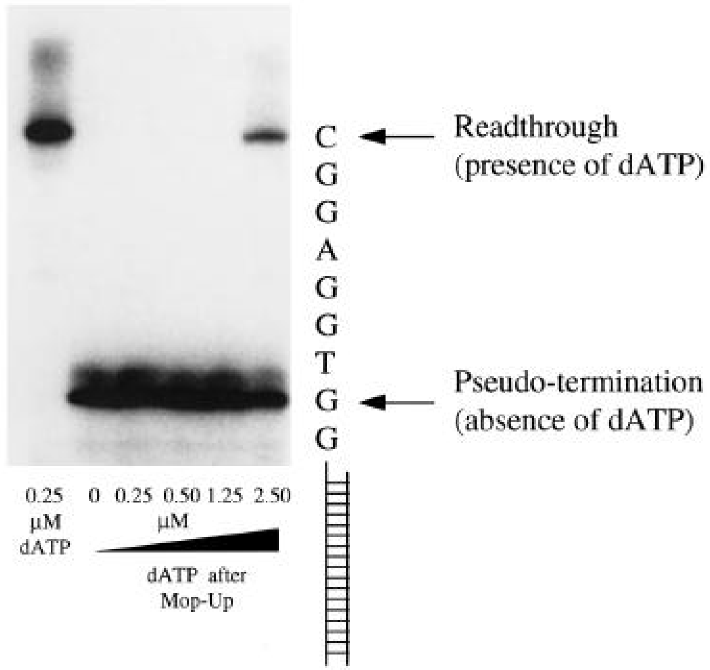

Figure 2. Capacity of the mop-up assay. Enzymatic mop-up was performed on a range of dATP concentrations (0, 5, 10, 25 and 50 μM) using *Bst* DNA polymerase and assayed (final concentrations: 0, 0.25, 0.50, 1.25 and 2.5 μM, respectively) using AMV-RT. Conditions for the AMV-RT incorporation assay have been described previously (3) and are ^{32}P-labeled universal primer-annealed to the oligo-template in AMV-RT buffer. All reactions contained dCTP, dTTP, ddGTP and the compound listed below each lane.

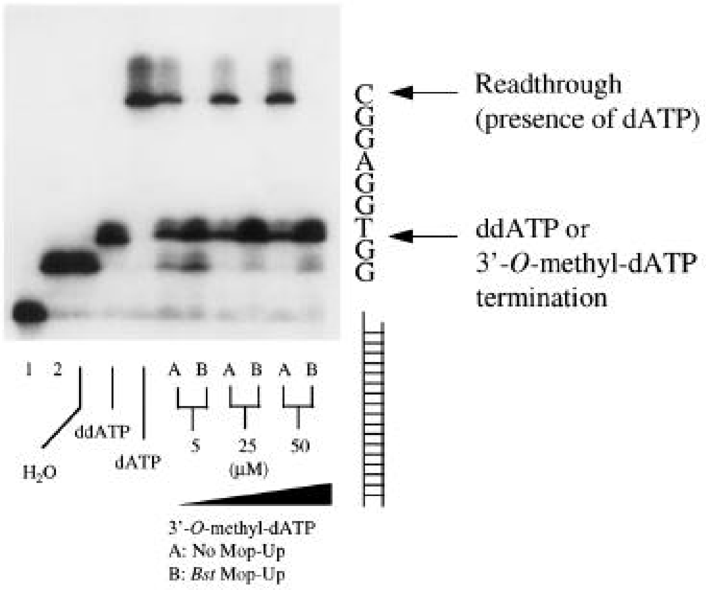

Figure 3. Complete termination of 3′-*O*-methyl-dATP by AMV-RT. As shown, various concentrations of 3′-*O*-methyl-dATP are assayed in the absence (lanes A) or after enzymatic mop-up (lanes B). All reactions contained ^{32}P-labeled universal primer-annealed to the oligo-template and AMV-RT in reaction buffer (lane 1). In addition, lane 2 contained dCTP and ddGTP. All other reactions contained dCTP, dTTP, ddGTP and compounds listed below each lane.

DNA synthesis and the extension of a solid-phase bound primer-template complex containing a complement oligo(dN) 5′-end overhang (Figure 1). The mop-up strategy takes advantage of DNA polymerases that show high affinity for natural nucleotides and minimal specificity for 3′-*O*-modified-dNTPs by standard-template incorporation assays. Once enzymatic removal of the natural nucleotide is complete, the purified 3′-*O*-modified-dNTP solution is isolated by magnetic separation and can be directly used for the incorporation assay. Heat inactivation or chemical extraction of the mop-up polymerase can be performed before the template assays, although experiments omitting this step have resulted in similar incorporation data.

To test the capacity of enzymatic mop-up, a range of dATP concentrations (0, 5, 10, 25 and 50 μM) were tested using *Bst* DNA polymerase as the mop-up enzyme and assayed (final concentrations: 0, 0.25, 0.50, 1.25 and 2.5 μM, respectively) using AMV-RT for the presence of nucleotide read-through (Figure 2). Under these conditions, *Bst* DNA polymerase efficiently mopped-up dATP concentrations between 25 and 50 μM. Similar results have been obtained for the enzymatic mop-up of dTTP (data not shown). Typically, 3′-*O*-modified-dNTPs have been evaluated at a final concentration range of 12.5–50 μM in incorporation assays (References 2 and 3; data not shown), corresponding to a capacity to efficiently mop-up a 2.5%–20% level of natural-nucleotide contamination.

Previously, the incorporation of RP-HPLC purified 3′-*O*-methyl-dATP by AMV-RT was shown to cause both termination and natural nucleotide read-through by the oligo-template assay (3) (Figure 3, lanes A). *Bst* DNA polymerase that was previously shown not to incorporate 3′-*O*-methyl-dATP (3) was used to selectively remove dATP contamination from an RP-HPLC purified 3′-*O*-methyl-dATP solution. Following enzymatic mop-up, the incorporation of 3′-*O*-methyl-dATP by AMV-RT shows complete termination without natural nucleotide read-through (Figure 3, lanes B). Klenow fragment has also been used to efficiently mop-up both contaminating dATP from a 3′-*O*-methyl-dATP solution before incorporation by human immunodeficiency virus type 1 (HIV-1)-RT (data not shown) and contaminating dTTP from a 3′-*O*-(2-nitrobenzyl)-dTTP solution before incorporation by *Bst* DNA polymerase (data not shown). Thus, these data show that dNTP contamination of RP-HPLC 3′-*O*-modified-dNTPs can be effectively removed by the enzymatic mop-up assay.

Canard et al. (1) have provided evidence for the hydrolysis of 3′-*O*-ester linkages by a mechanism of catalytic editing of DNA polymerases. Both 3′-*O*-methyl and 3′-*O*-(2-nitrobenzyl) protecting groups described in the current study are attached to the sugar ring by ether linkages. The oligo-template assay used here and elsewhere (3) has been designed to differentiate between the incorporation of the natural dNTP (read-through) and the 3′-*O*-modified-dNTP (true termination) (Figures 2 and 3). Using this assay, we have observed that the majority of RP-HPLC purified 3′-*O*-modified-dNTPs contains minimal, but significant levels of dNTP contamination. Previously, we evaluated 7 different 3′-*O*-modified-dNTPs, 3 of which contained ester linkages that were not incorporated by 8 different DNA polymerases (3). One of these 3′-*O*-ester linked analogs, 3′-*O*-acyl-dATP, can terminate terminal deoxynucleotidyl transferase (TdT) synthesis in a template-independent manner (data not shown). The dATP contamination for 3′-*O*-acyl-dATP was estimated at 0.4% by TdT nucleotide-extension assays. Following chain termination of 3′-*O*-acyl-dATP by TdT, DNA synthesis could not be reinitiated after a dATP chase suggesting that the presence of contaminating natural nucleotides, not catalytic editing of DNA polymerases, is a major cause for nucleotide read-through past the desired termination base. Moreover, the levels of natural nucleotide contamination can vary significantly for different base substituents containing the same 3′-*O*-protecting group resulting in no read-through [3′-*O*-(2-nitrobenzyl)-dATP] (3) or read-through [3′-*O*-(2-nitrobenzyl)-dTTP] (data not shown). Taken together, our data highlight the importance of natural nucleotide contamination of the 3′-*O*-modified-dNTPs in the characterization and interpretation of enzymatic-incorporation data.

Here, we have demonstrated that enzymatic mop-up is a viable strategy that uses conventional reagents for the purification of novel RP-HPLC-purified 3′-*O*-modified-dNTP analogs. The development of enzymatic mop-up also addresses the important issue of nucleotide read-through in stop-start DNA sequencing strategies such as BASS.

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This work was supported in part by Grants 1 R01 HG01459 and 1 R01 AI33334 and grants to K.B. from the Texas Advanced Technology Program and National Institutes of health HG/GM01745. Address correspondence to Dr. Michael Metzker, Department of Human Genetics, Merck Research Laboratories, West Point, PA 19486, USA. Internet: michael_metzker @merck.com

Received 23 March 1998; accepted 15 July 1998.

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