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TECH-100 ver.1
Technical Product Information Sheet

Using Untagged Aminoxy Reversible Terminators with DNA Polymerases

Firebird Reversible Terminators:

Terminator	Description	Catalog Numbers
Ready to Use (Untagged)	3'-ONH2. Does not require deprotection prior to use	TONH2-171, CONH2-172, AONH2-173, GONH2-174, IONH2-175
Oxime Protected (Untagged)	3'-acetoxime. Requires deprotection with methoxylamine	TONH2-101, CONH2-102, AONH2-103, GONH2-104, IONH2-105

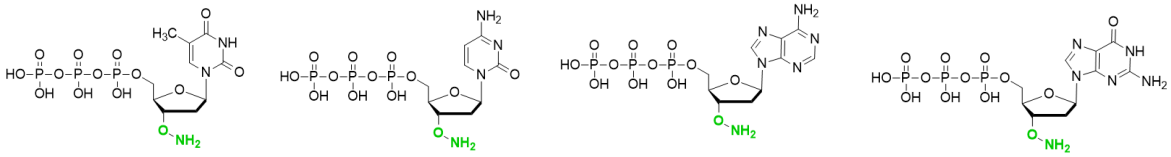
May 2019
This Report replaces Technical Report NONH2-21 for use of untagged reversible terminators

OVERVIEW

The reagents

3'-Aminoxy triphosphates have the hydrogen on their 3'-hydroxyl (-O-H) group replaced by an $–NH_2$ group (3'-O-NH_2). These triphosphates can be used by many enzymes to add a nucleotide with a 3'-ONH_2 moiety to a primer in either a template-directed fashion (as with DNA polymerases and reverse transcriptases) or without template direction (as with terminal transferases). This Technical Information sheet concerns the use of 3'-aminoxy nucleoside triphosphates with DNA polymerases.

Figure 1. Aminoxy terminated triphosphates



Thymine Ready to Use
TONH2-171

Cytosine Ready to Use
CONH2-172

Adenine Ready to Use
AONH2-173

Guanine Ready to Use
GONH2-174

The polymerases

One preferred polymerase for incorporating 3'-aminoxy triphosphates is Therminator™, sold by New England Biolabs (Cat#: M0261S). For those wishing to use aminoxy triphosphates in competition with irreversibly terminating 2',3'-dideoxynucleoside triphosphates (for example, to detect SNPs), Firebird's POL475 polymerase (Cat # POL475) is recommended.

Resuming primer extension after incorporation of the 3'-Aminoxy nucleotide

Once an aminoxy terminated nucleotide is added to the 3'-end of a DNA strand, primer extension terminates. The 3'-ONH_2 group can then be reacted with other functional groups. Alternatively, the 3'-ONH_2 group can be cleaved to generate a natural 3'-OH group, at which point the DNA strand can be used like standard DNA, including for further cycles of extension with 3'-aminoxy terminators.

The preferred reagent for cleavage of the 3'-ONH2 group to generate a 3'-OH is sodium nitrite buffered in aqueous acetate at pH 5.5 at room temperature. This cleavage reaction is complete in a few minutes. While these reaction conditions also cause deamination of the standard nucleobases C, A and G, that side reaction is at least 1,000 times slower than the rate of cleavage of the 3'-ONH2 group (data shown below, Page 3).

Cautions

The 3'-ONH_2 group forms oximes (R-O-N=CR_2) with aldehydes and ketones, which are poor polymerase substrates. Therefore, care must be taken not to expose the reagents to acetone, formaldehyde, or other aldehydes and ketones, which are widespread in many laboratories. Since standard Milli-Q water may contain small organic species, we recommend HPLC-grade water or water distilled from potassium permanganate for the preparation of all buffers or solutions. Care should also be taken not to use glassware rinsed with acetone. Conversely, the oxime protected untagged species must have the oxime deprotected prior to use, as described further on Page 6.

One way to manage ketone/aldehyde contamination is to add excess methoxylamine ($MeONH_2$) to the reaction mixture. This scavenges any aldehyde or ketone, wherein the methoxylamine forms an oxime with the aldehyde or ketone (MeO-N=CR_2). This protects the reversible terminator from conversion to its oxime. The aminoxy reversible terminators obtained from their acetoximes by our *in- situ* protocol (Page 6) already contain a sufficient excess of methoxylamine. Methoxylamine does not generally interfere with polymerase activity. It must, however, be removed before cleavage of the 3'-O-NH_2 with buffered sodium nitrite. If the primer-template complex is immobilized, this is done by washing away solutions containing methoxylamine.

Additionally, care must be taken to remove any methoxylamine from solution prior to storage of unused reversible terminator, especially for cytosine terminators, as methoxylamine at high concentrations will degrade the cytosine nucleobase over extended periods of time. Other cautions reflect those routinely taken with triphosphates in general: store frozen, avoid contamination by bacteria, avoid heating.

MODEL REACTIONS

Experimental data, shown below, helps the informed practitioner better understand the performance characteristic of the aminoxy triphosphates.

Cleavage of 3'-O-aminothymidine with buffered aqueous HONO

A procedure on the nucleoside itself illustrates the scope of the cleavage reaction, whose rate is sensitive to pH. To show this, cleavage reagent (50 µL, 350-700 mM $NaNO_2$ in 1 M aqueous sodium acetate buffer) was added to an aqueous solution of 3'-O-aminothymidine (20 mM, 2 µL total volume) at a range of pH's. The resulting pH was measured with a microelectrode. Parallel reactions were stopped after incubation at room temperature for 1-2 min by raising the pH through the addition of potassium phosphate buffer (1 M, 200 µL, pH 7.0). The products were analyzed by analytical reversed-phase HPLC (Waters NovaPak column C-18 4µm, 3.9x150, with guard column Waters NovaPak C-18 4µm, 3.9x15mm, eluent A = 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 3% B to 13% B in 20 min, flow rate = 0.5 mL/min, R_t = product: 8 min; starting material: 11 min). The amount of cleavage was determined by integrating the UV absorbance peaks (267 nm) of the remaining 3'-O-aminothymidine and the product (thymidine). Results are shown in Table 1.

Table 1. Amounts of cleavage of 3'-O-aminothymidine

mM conc of	actual pH (± 0.02)	product after 1 min	product after 2 min
350	5.50	ND	90%
700	5.50	98%	>99%
700	5.65	80%	96%

ND – Not Determined

Testing for deamination of exocyclic amino group

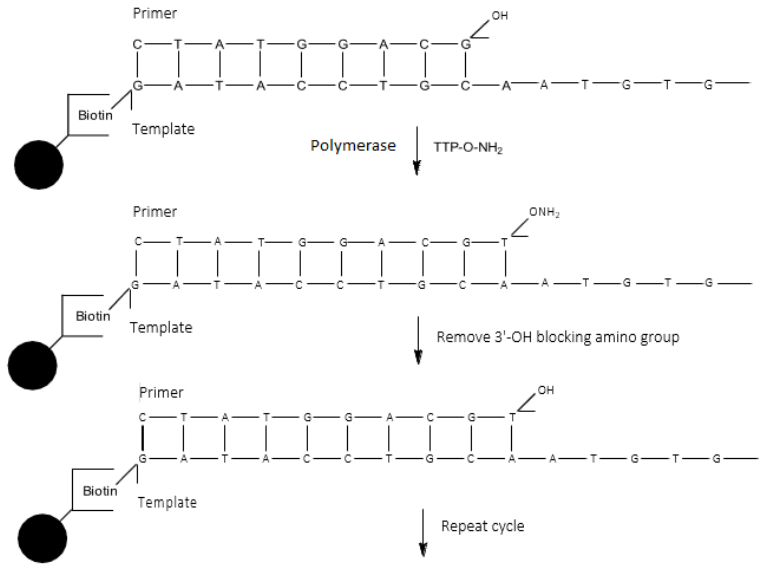
To estimate the extent of deamination of various nucleobases under cleavage conditions, aqueous solutions of 2'-deoxyguanosine, 2'-deoxyadenosine and 2'-deoxycytidine (20 mM, 30 µL) were separately treated with cleavage buffer (700 mM $NaNO_2$, 1 M aqueous sodium acetate buffer, pH 5.5, 500 µL) at room temperature for 72 h. Aliquots (50 µL) were removed, neutralized by the addition of potassium phosphate buffer (1 M, 200 µL, pH 7), and analyzed by analytical reversed-phase HPLC (Waters NovaPak C-18 column 4µm, 3.9x150 mm, with guard column Waters NovaPak C-18 4µm, 3.9x15mm. eluent A = 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 0% B to 3% B in 10 min, then to 30% B in 20 min, flow rate = 0.5 mL/min, R_t = dG: 14 min, dA: 18 min, dC: 8 min). The amount of deamination was determined by integrating the UV absorbance peaks (260 nm) of the remaining starting material (nucleoside) and the product(s). The results are shown in Table 2.

Table 2. Extent of deamination (exocyclic amino group) caused by cleavage reaction after 72 hours

nucleoside	byproducts after 72 h, UV quantitated at 260 nm
dG	20%
dA	13%
dC	15%

PRIMER EXTENSION USING AMINOXY NUCLEOSIDE TRIPHOSPHATES

Experimental data shown below used the aminoxy triphosphates to extend with termination a primer-template complex that is immobilized on magnetic beads. This permits a wash cycle to remove reagents in solution from DNA on the bead.



Cycle of polymerase extension with termination followed by cleavage on a support

Reagents and substrates

(a) dNTP-ONH_2 stock solutions: Solutions of the triphosphates (5 mM) are prepared *in-situ* following the cleavage protocol on Page 6.

(b) Thermopol® Buffer: The 10X solution (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100, pH 8.8 at 25 °C), diluted to 1X (final) in HPLC grade H_2O.

(c) Dynabeads® M-270 Streptavidin: Magnetic beads carrying streptavidin were purchased from Invitrogen (Cat number 653-06). Biotinylated nucleic acids were immobilized on the beads following the manufacturer's procedure (Dynabeads product insert).

(d) Cleavage Reagent: This reagent is prepared fresh as an aqueous solution of $NaNO_2$ (700 mM) in an aqueous sodium acetate buffer (1 M, pH 5.5).

(e) Quench Buffer: 10 mM EDTA in HPLC grade H_2O.

(f) 1% $MeONH_2$ stock solution: Dilute 100 µL of methoxylamine-hydrochloride solution (25-30% in water, e.g. Alfa Aesar Cat number L08415) with 2.6 mL of water (HPLC grade or better) and adjust pH to ca. 7 with aqueous NaOH solution (10 M, ca. 30-50 µL).

(g) Primary Wash Buffer: 50 mM Tris pH 7.5, 500 µM EDTA, 1 M NaCl, 0.1% $MeONH_2$.

(h) Post Extension Wash buffer: 50 mM Tris pH 8.5, 0.1% $MeONH_2$.

(i) Therminator™: Purchased from New England Biolabs (Cat #: M0261S); 2 U/µL.

Polymerase Extension Reaction

The reaction mixture contains:

(a) 5'-Biotinylated template in water (30 pmol)
(b) ^{32}P labeled primer in water (2.5 pmol)
(c) Unlabeled primer in water (20 pmol)
(d) Thermopol Buffer (1X)
(e) dNTP-ONH_2, diluted from stock to 100 µM (if multiple dNTPs are used, they are diluted into the same mixture to a final concentration of 100 µM each), adjusted as desired.
(f) Therminator 0.5 U per assay, adjusted as desired.
(g) ddH_2O to bring the final reaction volume up to 10 µL

In this example, the biotinylated template is bound to magnetic beads, permitting extension and wash steps that employ magnetic separation to change the buffer. In a 10 µL reaction volume, 5'-biotinylated template (30 pmol), complementary 5'-^{32}P-labeled primer (2.5 pmol) and unlabeled primer (20 pmol) are annealed by incubation at 96 °C for 5 min, followed by slow cooling (over 1 hour) to room temperature. The duplex is then immobilized onto Dynabeads following the manufacturer's protocol (Dynabeads M- 270 Streptavidin, Invitrogen). The beads carrying the immobilized DNA are resuspended in Thermopol buffer (1X) containing 0.5 Unit of Therminator and incubated (72 °C, 30 sec). The dNTP-ONH_2 triphosphate (100 µM final of each) is added to initiate the reaction, with incubating at 72 °C for 2 min. Extension is stopped with EDTA (3.33 mM, final concentration).

Cleavage Reaction to Reverse Termination

The magnetic beads are recovered with a small, hand-held magnet, and the quench buffer is largely removed by pipette. Two aliquots of Primary Wash Buffer (500 µL each) are sequentially added and removed, and the beads are washed with two aliquots of Post Extension Wash Buffer (500 µL each).

The Post Extension Wash Buffer is then removed. Cleavage Reagent (100 µL) is added, and the mixture is incubated at room temperature for 5 min. This step is typically repeated.
Following cleavage, the beads are recovered, the Cleavage Reagent is removed, and the beads are washed with two aliquots of Primary Wash Buffer (500 µL each).

To prepare the bead-bound primer-template complex for another round of extension, the beads are washed with two aliquots of 1X Thermopol buffer (500 µL each).

Clean-up of 3'-OH contamination

All 3'-aminoxy triphosphates contain traces (< 0.2%) of their natural analog carrying a free 3'- OH that cannot be removed by physical purification methods such as HPLC. Since polymerases prefer these natural triphosphates over their 3'-aminoxy counterparts, even trace levels of contamination can lead to a significant degree (several percent) of unblocked extension ("phasing") during each elongation cycle.

This preference of polymerases for natural triphosphates (i.e. the "contaminant") over the 3'-aminoxy analogs led us to develop an easy *in-situ* clean-up procedure that completely prevents phasing: prior to the start of the biological assay, each 3'-aminoxy triphosphate is incubated under extension conditions with polymerase and a small amount of "trapping duplex". This trapping duplex consists of a primer and template where the template enables the repeated incorporation of that particular nucleobase. As an example, to clean-up 3'-aminoxy thymidine triphosphate, the template would contain an oligo-A overhang:

5'-GCG TAA TAC GAC TCA CTA TGG ACG-3'
3'-CGC ATT ATG CTG AGT GAT ACC TGC AAA AAA AAA AAA-5’

During this incubation, the polymerase will exhaust the entire trace of 3'-OH contaminant, leaving behind a concentrated stock solution of the clean 3'-aminoxy triphosphate, ready to use.

The following protocol produces 15 µL of a 1 mM stock solution of clean TTP-ONH_2:

TTP-ONH_2 (10 mM, "contaminated")	1.5 µL	15 nmol
Trapping duplex (25 µM, annealed)	2.0 µL	50 pmol
Thermopol buffer (10X)	1.5 µL	
ddH_2O	8.5 µL	
POL475 polymerase	1.5 µL	Incubate

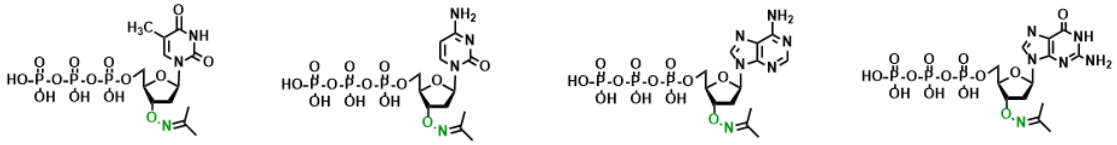
at 72 °C for 2 min. Keep on ice until ready to use.

Since this clean-up procedure focuses on the incorporation of natural triphosphates, a different polymerase could be used, as long as it is compatible with the Thermopol buffer and does not interfere with the subsequent assay.

USING AMINOXY REVERSIBLE TERMINATORS DELIVERED AS OXIMES

As an alternative to ready-to-use terminators, we also offer reversible terminators with the 3'-ONH_2 group protected as the acetoxime (TONH2-101, CONH2-102, AONH2-103, GONH2-104), which are more suitable for longer term storage (years). These are not incorporated by polymerases, however, and must be treated with methoxylamine (Me-ONH_2) according to the protocol below to deprotect the oxime and yield aminoxy triphosphates that can be used directly in the polymerase extension reaction.

Figure 2. Aminoxy terminated triphosphates protected as acetoximes



Thymine Oxime
TONH2-101

Cytosine Oxime
CONH2-102

Adenine Oxime
AONH2-103

Guanine Oxime
GONH2-104

Deprotection of terminator oximes *in-situ* for subsequent polymerase incorporation.

The triphosphate-oximes (TONH2-101, CONH2-102, AONH2-103, GONH2- 104) must be deoximated before they can be used as polymerase substrates. First, a sample of the lyophilized terminator-oxime (5 µmol) is dissolved in 500 µL of water (HPLC grade or better) to give a 10 mM stock solution. This stock solution may be stored at -20°C.

A buffered aqueous $MeONH_2$ solution is prepared as follows (the following recipe yields about 1.9 mL and should be prepared fresh daily): 200 µL of a commercial methoxylamine-hydrochloride solution (25-30% in water, e.g. Alfa Aesar Catalog number L08415) is diluted with 1.2 mL of water (HPLC grade or better); the pH of this solution will be below 1. To this is added aqueous sodium hydroxide solution (10 M) in portions until the pH is about 5-6, approximately 60-90 µL (depending on the exact concentration of the particular batch of $MeONH_2$•HCl solution). This solution is buffered by the addition of 400 µL of aqueous sodium acetate solution (1 M, pH 5.5).

Immediately before use, an aliquot of the terminator-oxime stock solution (10 mM) is mixed with an equal volume of the buffered aqueous $MeONH_2$ solution, and the mixture is incubated at room temperature for 1 h.

The resulting solution is 5 mM dNTP-ONH_2, in ca. 200 mM in $MeONH_2$, ca. 200 mM in NaCl, and 100 mM in sodium acetate buffer, with a pH of 5.5. The solution can be used directly with polymerases (after appropriate dilution to the desired triphosphate concentration). To prevent degradation of the nucleobases by the high concentration of methoxylamine, this solution should not be stored as-is but rather diluted to the final terminator concentration and used within 24 h; alternatively, the excess methoxylamine can be removed by freeze-drying followed by re-dissolving the terminator in water. The resulting solution can then be stored frozen at -20 °C for several months.