EXPRESSIONS

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IN THIS ISSUE

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The effect of nucleic acid modifications on digestion by DNA exonucleases

by Greg Lohman, Ph.D., New England Biolabs, Inc.

New England Biolabs offers a wide variety of exonucleases with a range of nucleotide structure specificity. Exonucleases can be active on ssDNA and/or dsDNA, initiate from the 5´ end and/or the 3´ end of polynucleotides, and can also act on RNA. Exonucleases have many applications in molecular biology, including removal of PCR primers, cleanup of plasmid DNA and production of ssDNA from dsDNA. In this article, we explore the activity of commercially available exonucleases on oligonucleotides that have chemical modifications added during phosphoramidite synthesis, including phosphorothioate diester bonds, 2´-modified riboses, modified bases, and 5´ and 3´ end modifications. We discuss how modifications can be used to selectively protect some polynucleotides from digestion *in vitro*, and which modifications will be cleaved like natural DNA. This information can be helpful for designing primers that are stable to exonucleases, protecting specific strands of DNA, and preparing oligonucleotides with modifications that will be resistant to rapid cleavage by common exonuclease activities.

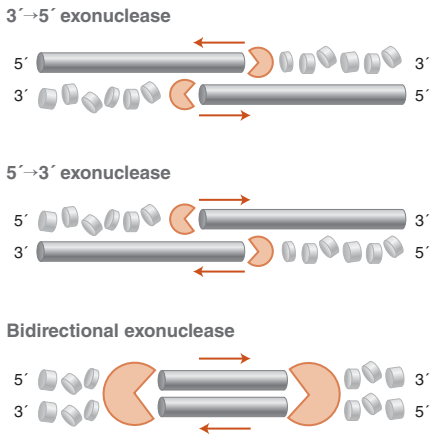
The ability of nucleases to hydrolyze phosphodiester bonds in nucleic acids is among the earliest nucleic acid enzyme activities to be characterized (1-6). Endonucleases cleave internal phosphodiester bonds, while exonucleases, the focus of this article, must begin at the 5´ or 3´ end of a nucleic acid strand and cleave the bonds sequentially (Figure 1). Exonucleases may be DNA or RNA specific, and can act on single-stranded or double-stranded nucleic acids, or both. Double-strand specific exonucleases may initiate at blunt ends, nicks, or short single-stranded 5´ or 3´ overhangs, though most exonucleases are active on a subset of these structures. For a summary of the substrate specificity of exonucleases available from NEB, view our newly-updated selection chart, Properties of Exonucleases and Non-specific Endonucleases, at **go.neb.com/ExosEndos**.

A variety of DNA exonucleases have been characterized from many different organisms; *in vivo*, these enzymes play critical roles in polynucleotide repair, recycling, error correction, and protection from exogenous DNA (6-8). *In vitro*, exonucleases are used in many applications where it is desirable to remove certain nucleic acids. For example, Exonuclease V (RecBCD) (Exo V, NEB #M0345) is often used to remove contaminating linear ssDNA and dsDNA from plasmid preparations (4,9); T7 Exonuclease (T7 Exo, NEB #M0263) can be used to generate 3´ overhangs in DNA (4, 10, 11); Exonuclease I (Exo I, NEB #M0293), Thermolabile Exonuclease I (NEB #M0568) or Exonuclease VII (Exo VII, NEB #M0379) can be used to eliminate ssDNA PCR primers, leaving double-stranded products undigested (12, 13), and Lambda Exonuclease (Lambda Exo, NEB #M0262) can be used to convert dsDNA to ssDNA for a variety of applications (14-16). More information on common applications of exonucleases available from NEB can be found in our selection chart, Common Applications of Exonucleases and Non-specific Endonucleases, at **go.neb.com/ExosEndos**, (also see page 8).

What about cases where you only want to degrade some of the ssDNA in a reaction? Or, when you want to make ssDNA from a dsDNA substrate, but which strand is degraded matters greatly? What about cases where the ends of your nucleic acids are modified—will exonucleases still digest the substrate, or cleave the modification? Several methods depend on selective protection of polynucleotides, such as protection of primers from degradation by polymerase exonuclease domains (17), selective protection of one strand of a DNA duplex for the production of ssDNA (14-16), and the protection of polynucleotides from degradation by serum nucleases, as in the case of RNA interference drugs (18, 19). In each of these cases, it is critical to understand the influence of modifications on exonuclease activity—which modifications inhibit nucleotide cleavage and which do not.

Recently, researchers at NEB have worked to characterize the interaction between exonucleases and modified polynucleotides, as part of a broader effort to gain deeper insight into the sequence and structural determinants of nuclease activity and specificity. In an effort to catalog the modifications that inhibit exonuclease digestion, we treated polynucleotides containing a range of modifications (including non-standard bases, sugars and backbone chemistries) with exonucleases under the recommended *in vitro* reaction conditions. This article will summarize data from the literature, as well as the key results from NEB's work related to understanding the activity of exonucleases on chemically modified polynucleotides. We will focus on the most widely used—and most successful—method for blocking nuclease activity, the phosphorothioate bond (20-23), but will also discuss the use of other modifications to inhibit nuclease activity, as well as which modifications have little to no effect on exonuclease digestion.

Figure 1:
Examples of exonuclease directionality

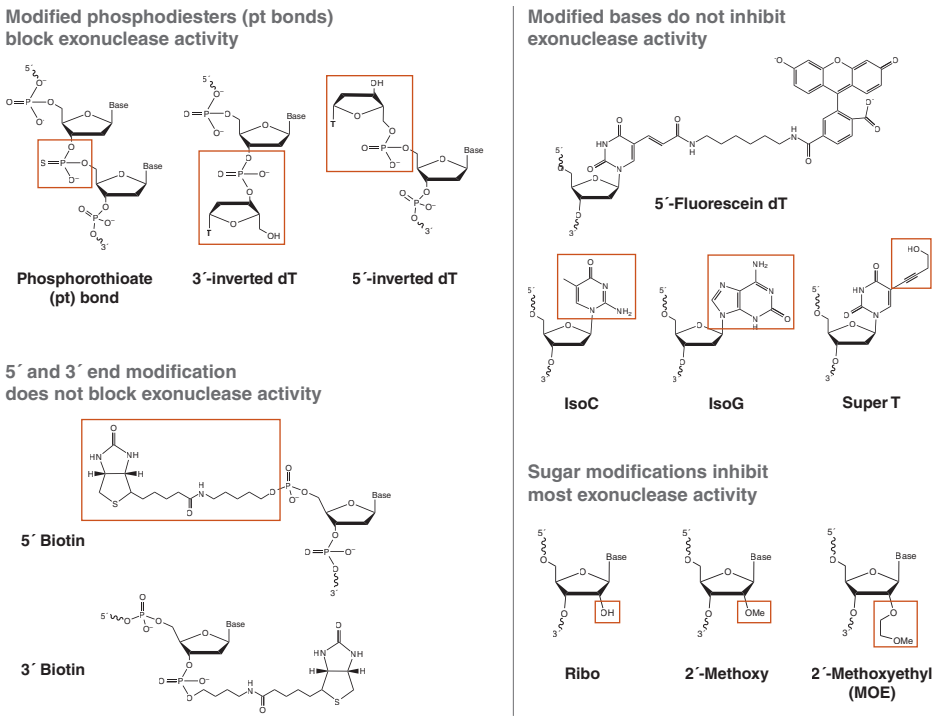


Pictured are double stranded exonucleases with a 3´ to 5´ polarity (top), a 5´ to 3´ polarity (middle), and a bidirectional nuclease (bottom).

Phosphorothioate linkages

A phosphorothioate (pt) bond is a phosphodiester linkage where one of the two non-bridging oxygens has been replaced by a sulfur (Figure 2). This modification has been used for decades to inhibit nuclease phosphodiesterase and phosphoryl transferase activities, as well as for gaining mechanistic insights into these enzymes (20, 23). Chemically, the substitution of oxygen with sulfur does not dramatically change the reactivity of the bond, and pt-containing polynucleotides can still function in many enzymatic reactions. In a

Figure 2:
Examples of common nucleotide modifications and their effect on exonuclease activity



typical phosphodiester bond, the two non-bridging oxygens are chemically equivalent. When one of these oxygens is replaced by sulfur, however, the phosphorus is now connected to four distinct groups, rendering it a chiral center with two possible configurations referred to as "S_P" and "R_P" (Figure 3). It is this key feature that confers resistance for the majority of nuclease enzymes studied; one configuration will react at rates similar to a phosphodiester, while the other is significantly inhibitory or completely unreactive. Isomer reactivity varies from enzyme to enzyme, and different pt isomers can inhibit enzymes that catalyze the same reaction (e.g., phosphoryl transfer). For example, DNA Polymerase I (DNA Pol I, NEB #M0209) can incorporate deoxynucleotide triphosphates with a pt ester at the α phosphate (dNTPαS), allowing formation of pt-bonded polynucleotides. However, it can only react with S_P configured dNTPαS molecules, and does so with inversion of the stereocenter to form exclusively R_P-configured pt bonds in the product. Conversely, the 3´→ 5´exo activity of this polymerase cleaves R_P, but not S_P configured bonds (20). Alternatively, the 3´→ 5´ exo activity of *E. coli* Exonuclease III (Exo III, NEB #M0206) cleaves S_P, but not R_P configured pt bonds (24). Therefore, DNA created from the incorporation of dNTPαS by DNA Pol I is highly resistant to exonuclease cleavage by Exo III (25).

Phosphorothioates can block many, but not all, exonucleases. To block exonuclease cleavage, the pt bonds must be placed at the end(s) where the enzyme initiates, e.g., the 5´ end for Lambda Exo and the 3´ end for Exo III. It is important to note that a single pt bond is insufficient to fully protect an oligonucleotide from exonuclease digestion. When the pt bond is installed via an oxidation step during phosphoramidite synthesis, a nearly equal amount of each isomer (S_P and R_P) is formed at each pt linkage (20). Since most enzymes can cleave one of these isomers, a single chemically installed pt will protect only half the molecules from digestion by a given exonuclease. Thus, it is typically recommended that 3–6 pt bonds be used to block exonuclease digestion, to prevent this read-through. One might expect that because each bond is a 50:50 mixture of isomers, when presented with 5 consecutive isomers, a given enzyme could cleave the first bond on half the molecules, then half of the molecules that had the first bond hydrolyzed would have the second hydrolyzed, and so on, such that there would be a range of partially degraded products. In practice, it has been reported (and confirmed by recent results at NEB) that five consecutive pt bonds completely block all exonuclease activity at all pt bond positions (16). The exact reasons for this are not currently known, but it is likely that exonucleases engage multiple bases at once, and the net effect of the isomeric mixture somehow prevents the active site from properly organizing around bonds that are the normally cleavable pt isomer.

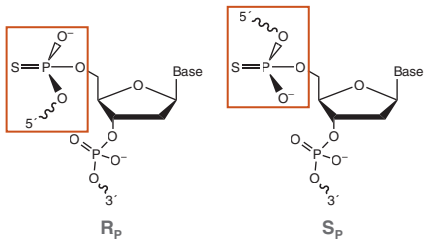
There are several commonly used exonucleases that are not blocked even by 5 consecutive pt bonds; for example, Exo V, Exo VII and T5 Exonuclease (T5 Exo, NEB #M0363) all can cleave, leaving short oligos instead of cutting at every bond in a series, and thus can digest DNA by skipping over termini blocked by multiple pt bonds and cleaving at the first phosphodiester (5, 26). Importantly, any enzyme with endonuclease activity, like DNase I, will simply ignore the ends and degrade the polynucleotides from the inside out (unless every phosphodiester bond is replaced by a phosphorothioate). Keeping these important exceptions in mind, phosphorothioate bonds remain the most generally applicable (and relatively inexpensive) way to protect oligonucleotides from digestion by exonucleases. For a complete list of DNA exonucleases and their interaction with pt bonds, view our selection chart, Activity of Exonucleases and Non-Specific Endonucleases, at **go.neb.com/ExosEndos**.

2´-modified nucleosides

Generally, DNA exonucleases do not digest RNA portions of oligonucleotides, though RNA is itself susceptible to RNases and nonspecific hydrolysis. We have further found that hybridizing RNA to DNA strands does not block the activity of dsDNA exonucleases on the DNA strand. Hybridization of ssDNA to RNA will block the activity of ssDNA exonucleases as effectively as hybridization to dsDNA. Additionally, certain 2´-O-modified riboses, are both stable to spontaneous hydrolysis and offer strong resistance to exonuclease activity (27). 2´-O-methyl and 2´-O-methoxyethyl (MOE) nucleosides, which contain bulky substituents off the sugar ring, have been shown to grant strong resistance to nucleases and additionally increase the strength of annealing to complementary DNA and RNA. These features have found utility in antisense nuclease strategies, to make oligonucleotides that are both resistant to degradation and able to bind tightly to target RNAs.

These sugar modifications also work *in vitro* to block exonuclease activity quite strongly. Our studies have found that, while a single terminal MOE nucleoside only weakly inhibits exonuclease activity, three successive MOE modifications provide enhanced resistance to many exonucleases,

Figure 3:
Chirality of phosphorothioate bonds



continued on page 4...

including Exo I, Exo III, Lambda Exo, $RecJ_f$ (NEB #M0264) and polymerase exonucleases, such as that of DNA Polymerase I, Large (Klenow) Fragment (NEB #M0210). Similar to pt bonds, several exonucleases can digest through these regions, notably T5 Exo, T7 Exo, Exo V, Exo VII and Exo VIII. Overall, exonuclease inhibition by MOE is quite strong, but pt bonds are more effective and are typically cheaper to prepare and incorporate. However, if for some reason the pt chemistry is not desired, 2´-O-modified ribose moieties are a viable alternative.

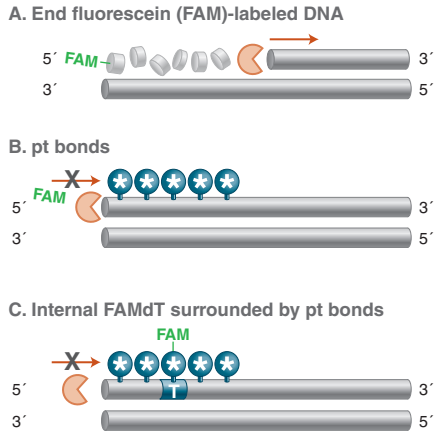
Other 5´/3´ end modifications

Several other modifications, such as the inverted deoxythymidine bases and dideoxynucleotides (Figure 2) have been reported to suppress serum nuclease activity when appended to the end of synthetic oligonucleotides (27). Many other modifications may be attached through "linkers" at either the 5´ or 3´ end, including fluorescent tags, biotin or other affinity labels, or reactive groups for attachment to beads or surfaces. These linkers are typically connected to the 5´ or 3´ end via a phosphodiester, but what is the interaction of these modified ends with exonucleases?

We have surveyed a range of these modifications in typical *in vitro* exonuclease assays. In general, while many provide modest inhibition as compared to a 5´-phosphate, all exonucleases tested could cleave all modifications connected through phosphodiester bonds. Interestingly, this poor inhibition held true for the inverted dT modifications, which have been reported to grant extra stability versus degradation by serum exonucleases for aptamers and other modified oligonucleotides. In our hands, 3´-inverted dT blocked only the relatively weak 3´→ 5´ exonuclease activity of DNA Polymerase I, Large (Klenow) Fragment (NEB #M0210) and Exonuclease T (Exo T, NEB #M0265), but did not block more active exonucleases such as in T7 DNA Polymerase (NEB #M0274), Exo I or Exo III. Similarly, 5´-inverted dT partially inhibited only Lambda Exo activity, which is known to require a 5´-phosphate for efficient initiation. Other 5´→ 3´ exonucleases were not significantly inhibited by this modification, showing complete digestion after a one-hour incubation under the recommended usage conditions.

We do not recommend 5´/3´ end modification as a good strategy for producing nucleotides resistant to exonuclease degradation *in vitro*. Researchers should be aware that these modifications will be cleaved by the majority of exonucleases, potentially leading to the loss of fluorescent labels and affinity tags. If a modification stable to exonuclease activity is needed, a better strategy is to use internal labels connected to the 5-methyl position of dT (e.g., Fluorescein dT, Figure 2). If these modified dT bases are used near the end of an oligo, they can be protected with surrounding pt bonds (Figure 4). The linkage to the base is not susceptible to enzymatic cleavage, and the pt bonds will protect the backbone from digestion, as described above.

Figure 4: **Designing oligonucleotides with nuclease-resistant modifications**



(A) End fluorescein (FAM) labeled-DNA is rapidly degraded by exonucleases. (B) pt bonds between nucleotides prevent the DNA strand from being degraded, but the end label can still be cleaved. (C) An internal FAMdT surrounded by pt bonds will prevent the exonuclease from removing the label.

Base modifications

None of the exonucleases available from NEB were significantly inhibited by modified bases under the conditions we tested. Modifications tested included 5-methyl-substituted dT (e.g., Fluorescein dT), deoxyuridine, the Tm-enhancing "super T," and the non-natural base pair isoG:isoC (Figure 2) (28). All modified substrates were digested completely by all the exonucleases tested. Some modifications showed weak blockage, pausing at the modification site before completely degrading the substrate. For several exonucleases tested, modified dT bases with large modifications off the 5-methyl position (Figure 2) showed a buildup of partially-digested intermediates, apparently stalling just before the modification; in no case did this resistance approach the inhibition seen for 2´ MOE sugars or pt linkages.

Conclusion

We have evaluated a variety of chemical modifications for their inhibition of exonuclease activity at the 5´ and 3´ ends of oligonucleotides. Broadly, the phosphorothioate modification, one of the more well-known used modifications to block nuclease cleavage, remains the most effective choice to protect oligonucleotides from degradation. However, one must be careful to use multiple pt bonds, place them at the correct end of the oligonucleotide to match the polarity of the exonucleases used, and be aware that several exonucleases can read-through or bypass terminal pt bonds; your choice of nucleases is as important as the modifications used. Aside from pt bonds, MOE nucleotides are the next best choice for providing nuclease resistance *in vitro*, with similar caveats to pt bonds. The vast majority of end modifications, including affinity tags and fluorophores, as well as internal non-standard bases, provide little, if any, nuclease resistance, and will be cleaved completely *in vitro*.

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Something to chew on.

Exonucleases and endonucleases are used in many of today's molecular biology workflows and applications. Did you know that NEB offers the largest supply of these important tools, and has a team of experts studying the function and optimization of these enzymes? We also offer several helpful tools to help you find the best enzyme to facilitate your work, including selection charts, recommended applications, usage guidelines and more.

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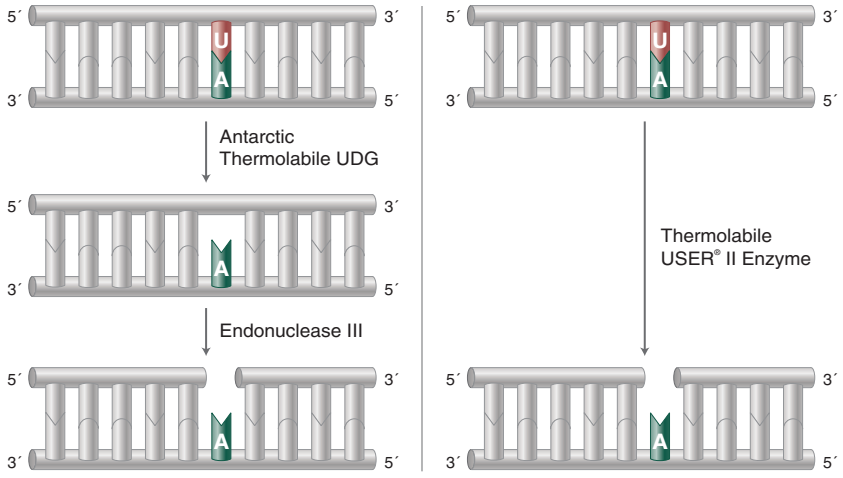

Antarctic Thermolabile Uracil DNA glycosylase (UDG) catalyzes the excision of a uracil base, forming an abasic (apyrimidinic) site while leaving the phosphodiester backbone intact. The lyase activity of Endonuclease III breaks the phosphodiester backbone at the 3′ and 5′ sides of the abasic site. In addition to generating a different 3′-terminus than USER Enzyme (a 3′-phospho-α, β-unsaturated aldehyde versus the 3′phosphate left by USER Enzyme, NEB #M5505), Thermolabile USER II Enzyme (NEB #M5508) can also be completely heat inactivated after 10 minutes at 65°C.

Applications include:

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- USER cloning

Ordering information:

Product	NEB #	Size
Thermolabile USER II Enzyme	M5508S/L	50/250 units

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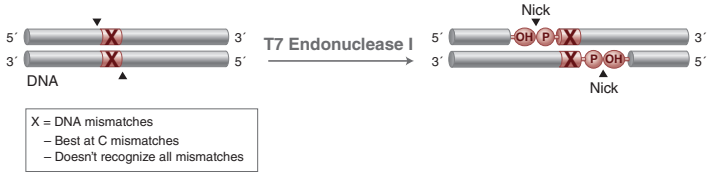
go.neb.com/ExosEndos

Featured DNA Repair Enzymes and Structure-specific Endonucleases (cont.)

T7 Endonuclease I

T7 Endonuclease I recognizes and cleaves non-perfectly matched DNA, cruciform DNA structures, Holliday structures or junctions, heteroduplex DNA and more slowly, nicked double-stranded DNA. The cleavage site is at the first, second or third phosphodiester bond that is 5´ to the mismatch. The protein is the product of T7 gene 3.



Application:

- Determining genome targeting efficiency (access protocol via the protocol tab at www.neb.com/M0302)

Ordering information:

Product	NEB #	Size
T7 Endonuclease I	M0302S/L	250/1,250 units

Thermostable FEN1

Thermostable Flap Endonuclease 1, FEN1, catalyzes the cleavage of 5´ DNA flaps from branched double stranded DNA substrates, creating a 5´ phosphate terminus. FEN1 products can be ligated by DNA ligase to create double-stranded DNA. *In vivo*, FEN1 is an essential component of the Okazaki fragment maturation pathway, and also plays a role in base excision repair.

Applications:

- Base excision repair

Ordering information:

Product	NEB #	Size
Thermostable FEN1	M0645S	1,600 units

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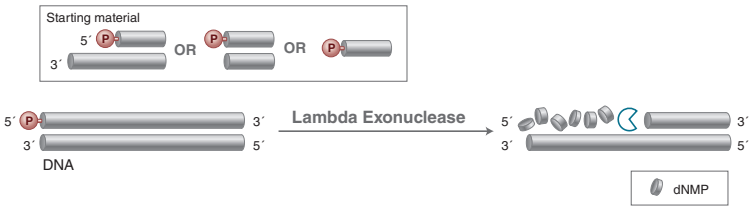
Featured Exonucleases

Lambda Exonuclease

Lambda Exonuclease catalyzes the removal of nucleotides from linear or nicked double-stranded DNA in the 5′ to 3′ direction. This enzyme is highly processive. The preferred substrate is 5′-phosphorylated double-stranded DNA, although non-phosphorylated substrates are degraded at a greatly reduced rate.



Application:

- Conversion of linear double-stranded DNA to single-stranded DNA via preferred activity on 5′-phosphorylated ends

Ordering information:

Product	NEB #	Size
Lambda Exonuclease	M0262S/L	1,000/5,000 units

Thermolabile Exonuclease I

Thermolabile Exonuclease I catalyzes the removal of nucleotides from linear single-stranded DNA in the 3′ to 5′ direction. Unlike Exonuclease I (NEB #M0293), Thermolabile Exonuclease I can be heat inactivated at 80°C in one minute.

Applications:

- Removal of single-stranded primers in PCR reactions prior to DNA sequencing or SNP analysis
- Removal of single-stranded primers for nested PCR reactions
- Removal of linear single-stranded DNA, leaving behind double-stranded DNA

Ordering information:

Product	NEB #	Size
Thermolabile Exonuclease I	M0568S/L	3,000/15,000 units

Common Applications for Exonucleases and Endonucleases

Not sure which exonuclease or endonuclease to choose?
Find the right enzyme for your application using the table below.

Application	Recommended Enzyme(s)	NEB #
Removal of 3´ overhangs	Quick Blunting Kit	E1201S/L
5´ overhang fill-in treatment	Quick Blunting Kit	E1201S/L
Removal of ss primers for nested PCR reactions	Thermolabile Exonuclease I	M0568S/L
Removal of primers post PCR prior to DNA sequencing or SNP detection	Exonuclease I Thermolabile Exonuclease I (1) Exonuclease VII (2)	M0293S/L M0568S/L M0379S/L
Mapping positions of introns in genomic DNA	Exonuclease VII	M0379S/L
Removal of primers with or without 3´ or 5´ terminal phosphorothioate bonds	Exonuclease VII	M0379S/L
Generating ssDNA from linear dsDNA: If 5´ → 3´ polarity required If 3´ → 5´ polarity required	 Lambda Exonuclease (3) Exonuclease III (4)	 M0262S/L M0206S/L
Preparation of nested deletions in double-stranded DNA	Exonuclease III (*E. coli*) plus Exonuclease VII	M0206S/L M0379S/L
Site-directed mutagenesis	Exonuclease III (*E. coli*) (5) T7 Exonuclease (6)	M0206S/L M0263S/L
Nick-site extension	T7 Exonuclease	M0263S/L
Degradation of denatured DNA from alkaline-based plasmid purification methods for improving DNA cloning	T5 Exonuclease	M0363S/L
Removal of chromosomal/linear DNA in plasmid preparations	T5 Exonuclease (7) Exonuclease V (RecBCD) (8)	M0363S/L M0345S/L
Removal of unligated products (linear dsDNA) from ligated circular double-stranded DNA	T5 Exonuclease (9) Exonuclease V (RecBCD) (10)	M0363S/L M0345S/L
Removal of residual gDNA after purification of low copy plasmid	Exonuclease V (RecBCD)	M0345S/L
Removal of contaminating DNA from RNA samples	DNase I	M0303S/L
Conversion of single-stranded DNA or RNA to 5´-mononucleotides	Nuclease P1	M0660S
Analysis of base composition, potential damage and modification of nucleic acids	Nuclease P1	M0660S
Progressive shortening of both ends of double-stranded DNA	Nuclease BAL-31	M0213S
Preparation of double-stranded DNA fragments with 5´-OH and 3´-phosphate	Micrococcal Nuclease	M0247S
Degradation of nucleic acids (both DNA and RNA) in crude cell-free extracts	Micrococcal Nuclease	M0247S
Preparation of rabbit reticulocyte	Micrococcal Nuclease	M0247S
Chromatin Immunoprecipitation (ChIP) analysis	Micrococcal Nuclease	M0247S

Notes:

1. *Rapid heat inactivation versus Exonuclease I*
2. *For 3´ chemically modified primers*
3. *Strand targeted for removal requires one 5´ end with phosphate*
4. *Strand targeted for removal requires a 5´ overhang, a blunt end, or a 3´ overhang (with less than 4 bases)*
5. *Removes nicked-strand DNA from 3´ to 5´*
6. *Removes nicked-strand DNA from 5´ to 3´*
7. *Degrades linear ss + dsDNA, nicked DNA*
8. *Degrades linear ss + dsDNA: preferred as Exo V will save nicked plasmids resulting in higher yields especially for low-copy number plasmid prep*
9. *Only the unnicked form of ligated circular double-stranded DNA remains*
10. *Both nicked and unnicked form of ligated circular double-stranded DNA remains*

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– **Sarah Fankhauser**
Oxford College of Emory University,
Oxford, GA, USA

"Through this work, I hope to inspire a solution that would liberate science in Africa, as well as create the platform that would permit scientists like myself to return home and do good science."

– **Mahmoud Bukar Maina**
University of Sussex, England, UK

This past May, New England Biolabs held its third Passion in Science Awards to recognize those within the scientific community who share the same core values as NEB and are working to solve many of today's challenges. We were pleased to welcome 12 award winners to the NEB campus in Ipswich, Massachusetts to celebrate their dedication to science mentorship, humanitarianism, environmental stewardship and artistic creativity, and to learn how scientists can create unique opportunities to explore their passions.

Scientific Mentorship and Advocacy

Sarah McAnulty – University of Connecticut, Willimantic, CT, USA
Daniel Heid – University of Heidelberg, Heidelberg, Germany
Steven Farber – Carnegie Institution for Science, Baltimore, MD, USA
Sarah Fankhauser – Oxford College of Emory University, Oxford, GA, USA

Humanitarian Duty

Mahmoud Bukar Maina – University of Sussex, England, UK
Nathan Schoepp – Caltech, Pasadena, CA, USA

Environmental Stewardship

Malali Gowda – TransDisciplinary University, Bangaluru, India
Samantha Romanick – University of Nevada Reno, Reno, NV, USA

Arts and Creativity

William Ward Murta – Theater Bielefeld, Bielefeld, Germany
Garfield Kwan – University of California, San Diego, CA, USA
Kyle McClary – University of Southern California, Los Angeles, CA, USA
Bryan Welm – University of Utah, Salt Lake City, UT, USA

Learn more about the recipients and watch their inspiring presentations at **www.neb.com/PassionInScience**

Using Exonuclease V (RecBCD) to Eliminate Residual Genomic DNA When Purifying Low Copy Plasmids with the Monarch® Plasmid Miniprep Kit

Peichung Hsieh, Ph.D., New England Biolabs, Inc.

Materials

- Endonuclease V (RecBCD) (NEB #M0345)
- NEB 10-beta Competent *E coli* (High Efficiency) (NEB #C3019)
- Antibiotic, typically Chloramphenicol
- LB Media
- Monarch Plasmid Miniprep Kit (NEB #T1010)

Introduction

The use of low and/or single-copy plasmids to clone large pieces of DNA (up to 200 kb) or to drive expression of slow folding or toxic proteins in *E.coli* is a commonly used strategy. Purification of low-copy plasmids or bacterial artificial chromosomes (BACs) presents some challenges that are not evident when working with higher copy number plasmids, such as pUC19. The ratio between bacterial gDNA and plasmid DNA is higher, thereby reducing yield of the desired plasmid produced by typical plasmid miniprep protocols. Additionally, elevated levels of host gDNA are often co-purified, reducing the accuracy of quantitation by UV absorbance or dsDNA specific dyes. Neither method can distinguish the contribution from gDNA to the overall nucleic acid content. Co-purification of host gDNA also affects the appearance of the sample when resolving by gel electrophoresis and adds unwanted contaminating DNA for any amplification-based application.

Exonuclease V (RecBCD, NEB #M0345) is an exonuclease that degrades both linear ss- and dsDNA, while keeping the circular DNA intact. Treatment of miniprep DNA samples of low copy plasmids with this exonuclease degrades the contaminating gDNA, restoring purity and ease of use in downstream applications.

Protocol

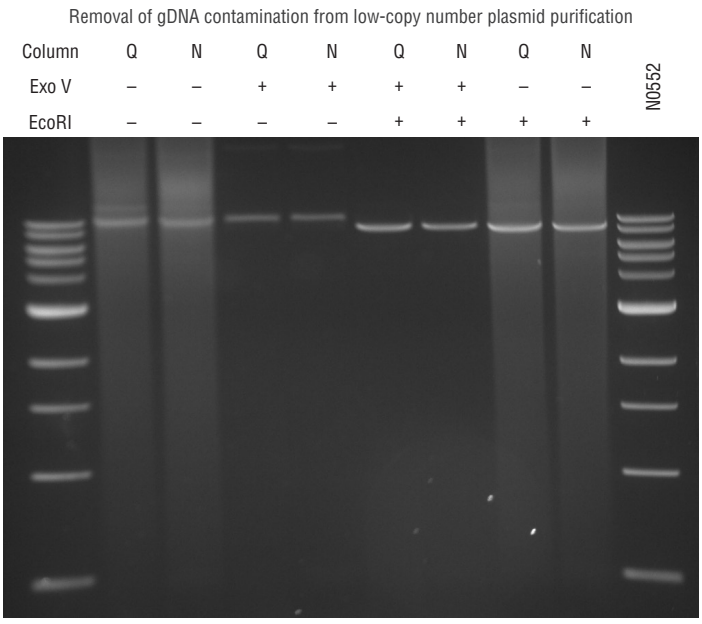
1. Transform an *endA-* strain (e.g. NEB 10-beta, NEB #C3019) with the BAC plasmid DNA and plate outgrowth onto a media plate with appropriate antibiotic. Incubate overnight at 30°C. BACs with Cam^R require reduced stringency selection. Chloramphenicol levels should be maintained between 10-15 µg/ml on the selective plate.
   Note: strains with an F' plasmid are not compatible with BACs or miniF plasmids.
2. Pick a colony, inoculate 10 ml LB + antibiotic, and incubate overnight at 30°C (200-250 RPM).
3. Check OD600 nm (usually it will be around 4 O.D./ml of cells).
4. Harvest 3 ml of the overnight culture and purify the plasmid DNA using the Monarch Plasmid Miniprep Kit (NEB #T1010) following the recommended protocol.
5. In the final elution step, elute the DNA with 30 µl of Monarch DNA Elution Buffer (pre-heated to 50°C).
6. To the eluted DNA, add 4 µl of NEBuffer 4 (10X), 4 µl of 10 mM ATP, and 2 µl of Exonuclease V (RecBCD). Mix reaction and incubate at 37°C for 1 hr.
7. Heat-inactivate the Exonuclease V by incubating at 70°C for 30 min. The plasmid DNA is now ready for restriction enzyme digestion, PCR or transformation.
   Note: Typically, 30-60 ng of single-copy plasmid can be purified from 3 ml of an overnight *E.coli* culture with (O.D. 600 nm = 4 O.D/ml)

Results:

Three milliliters of an overnight culture of NEB-10 beta competent *E. coli* cells transformed with pBAC were processed using the Monarch Plasmid DNA Kit and an equivalent Miniprep kit from another vendor. After isolating the DNA, samples were treated with Exonuclease V (RecBCD) and then digested with EcoRI. Samples were run on an agarose gel to assess the quality of the isolated DNA, and whether or not the Exonuclease V-treated DNA could be digested to completion. The Exonuclease V-treated samples showed no gDNA contamination (#3-6) while the untreated samples exhibited a significant amount of gDNA as seen by the smear observed in those samples (#1,2,7,8).

These results indicate that Endonuclease V can be used to efficiently degrade contaminating gDNA from plasmid purification steps, including those of low copy number.

pBAC samples exhibit no bacterial gDNA contamination after treatment with Exonuclease V (RecBCD)



Miniprep plasmid DNA samples isolated with the Monarch Plasmid Miniprep kit (N) and a similar kit from a competitor (Q) were either treated (+) or not treated (-) with Exonuclease V, and then digested with EcoRI. The samples treated with Exonuclease V showed no contaminating gDNA and they were correctly cut with EcoRI.

It's Not Easy Being a Right Whale

- Emily Greenhalgh, New England Aquarium

411: The predicted number of right whales remaining in the North Atlantic population.

These behemoths of the sea were once called the "right whales to hunt", because they swam close to shore, produced high yields of whale oil and baleen, and—thanks to their thick blubber—floated when killed. But now, rather than whalers, the population faces threats from humans in an ever-increasing urbanized ocean. Due primarily to human impacts, the population of these endangered whales has been in decline since 2010. Entanglement in fishing gear, changes in food distribution due to climate change, busy shipping lanes, and ocean noise are just some of the challenges facing the species.

It was humans who nearly destroyed the right whale population, and it is humans who are striving now to save it from extinction. In 1980, New England Aquarium scientists discovered a group of 25 North Atlantic right whales in Canada's Bay of Fundy. At the time, the species was thought to be almost extinct. In the nearly four decades since that startling discovery, dedicated researchers have been fighting to save this iconic species.

"The species is resilient. We know they can rebound if we just stop killing them."

– Dr. Scott Kraus, Vice President of the New England Aquarium's Anderson Cabot Center for Ocean Life

For nearly 40 years, members of the Aquarium's right whale research team have been dedicated to ensuring these whales have the opportunity to survive. Fieldwork, including 39 years of uninterrupted surveys in the Bay of Fundy, has provided invaluable data about right whale behavior, habitat use, and human impacts on the population. The Anderson Cabot Center Right Whale Program also oversees the North Atlantic Right Whale Catalog (**rwcatalog.neaq.org**), a tremendous collaborative effort of more than 300 individuals and organizations.

Right whales are identifiable by callosities, the natural patches on the top of their heads, as well as scars or markings on their bodies. Scientists can recognize individual whales from these marks in thousands of photographs, connecting important information about the population, such as location, mortality, health, and reproductive success. The Right Whale Catalog, which is linked with human impact studies, visual health assessments, and genetic and hormone analysis, is the cornerstone of right whale research.

Genetic samples from biopsies have also helped scientists estimate the original size of the right whale population before commercial hunting, and even shed light on how few calving-age females there were during the population's lowest point. About 80% of the right whale population has been sampled, and between the genetic database and the Right Whale Catalog, scientists know entire family histories for many of these whales.

This cornerstone has allowed dedicated scientists and researchers to build an array of methods to try to save this species. Since 1999, researchers from the Aquarium's Anderson Cabot Center have been collecting right whale fecal samples. By examining the levels of glucocorticoid hormones in these samples, Senior Scientist Rosalind Rolland, D.V.M. and her team were able to determine the stress levels of living whales for the first time. From 1999 to 2014, scientists examined samples from 125 different right whales – 113 healthy whales, six chronically entangled in fishing gear, one that was live-stranded, and five killed quickly by vessel strikes.

The robust samples allowed Rolland and her team to create a baseline of hormone levels in normal, healthy whales and compare those levels to animals under stress. The researchers found "sky-high hormone levels" in whales entangled in fishing gear. "These levels showed stress from extreme physical trauma," said Rolland. "It's an animal welfare issue."

How many of the right whales are undergoing this trauma? A total of 83% of endangered North Atlantic right whales show signs of entanglement, and 59% have been entangled more than once. Entanglements now surpass ship strikes as the main threat to the right whale population.

According to Anderson Cabot Center scientists, only a third of severely entangled female whales survive, and those that do survive are less likely to have calves. When in good condition, a right whale can give birth every three years. But with all the threats they are facing now, the birth rate for the population has dropped to an average 10 years between births - births are simply not outpacing deaths.

"Because they're a long-lived species, the right whales can weather short-term events. We have to give them the opportunity."

– Philip Hamilton, Research Scientist

Rolland and other scientists on the Aquarium's Marine Stress and Ocean Health team built on their pioneering fecal hormone research by studying stress hormone levels in whale blow, baleen, blubber and blood. The data collected from these samples include hormone levels, DNA, marine biotoxins and pathogens, to name just a few—and this helps paint a picture of the overall health of each animal.

Knowing how humans are affecting right whale populations is a key factor to protecting them in the future.

"We just have to keep from killing them, both directly and indirectly," said Rolland.

Scientists are working with partners across industry, scientific institutions, and the U.S. and Canadian governments to try to save the species from the brink of extinction. From supporting the implementation of ropeless fishing technology to speaking against offshore oil and gas development on our coasts, the right whale community is fighting hard to ensure these iconic whales not just survive, but thrive.

LEFT: "FDR" was photographed in the Bay of Fundy in 2016, entangled in a large amount of fishing gear. He was successfully disentangled by the Campobello Whale Rescue Team. Photographed by Jerry Conway, Campobello Whale Rescue Team. Permit: Collected under Permits issued by Canadian DFO under the Species at Risk Act.

ABOVE: "Caterpillar" was struck by a vessel, which left large propeller cuts on her body. Caterpillar was photographed in the Bay of Fundy on August 5, 2007 by Amy Knowlton, ACCOL, New England Aquarium. Permit: Collected under Permits issued by Canadian DFO under the Species at Risk Act.

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