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Excess primer degradation by Exo I improves the preparation of 3’ cDNA ligation-based sequencing libraries

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ABSTRACT
RNA sequencing library construction using single-stranded ligation of a DNA adapter to 3’ ends of cDNAs often produces primer–adapter byproducts, which compete with cDNA–adapter ligation products during library amplification and, therefore, reduces the number of informative sequencing reads. We find that Escherichia coli Exo I digestion efficiently and selectively removes surplus reverse transcription primer and thereby reduces the primer–adapter product contamination in 3’ cDNA ligation-based sequencing libraries, including small RNA libraries, which are typically similar in size to the primer–adapter products. We further demonstrate that Exo I treatment does not lead to trimming of the cDNA 3’ end when duplexed with the RNA template. Exo I digestion is easy to perform and implement in other protocols and could facilitate a more widespread use of 3’ cDNA ligation for sequencing-based applications.

METHOD SUMMARY
For 3’ cDNA ligation-based RNA sequencing libraries, we demonstrate that Exo I treatment after reverse transcription reduces primer–adapter contamination without causing trimming of the cDNA 3’ end. Our approach makes 3’ cDNA adapter ligation more broadly applicable to library preparation, including small RNA-seq libraries, which typically are impossible to separate from adapter-primer contamination based on size selection.

KEYWORDS
cDNA ligation • Exonuclease I • RNA-seq • sequencing library preparation

Massive parallel sequencing is a useful tool in transcriptomics and genomics and has been applied to the study of a multitude of different biological research questions over the last decade [1,2]. Sequencing of cDNA is now being used for gene expression analysis and for many other applications such as the study of small RNAs, protein–RNA interactions, RNA–RNA interactions, RNA structures and ribosome profiling.

Most sequencing methods, including the Illumina platform, require addition of adapter sequences to the ends of the DNA fragments [3] and many different strategies for adding these exist. For sequencing of double-stranded DNA, adapters can be added either by fragmentation of the DNA followed by ligation [4] or by transposon-based introduction of the adapters [5]. These strategies can be used to prepare libraries from cDNA-derived double-stranded DNA; however, for some applications this is not convenient or possible. Instead, adapter sequences can be ligated to the ends of RNA before reverse transcription, included in the reverse transcription primer or ligated to the cDNA 3’ end. Alternatively, the terminal transferase activity of M-MLV reverse transcriptase can be used in combination with its ability to switch template to add a 3’ cDNA adapter sequence during reverse transcription [6], or reverse transcription can be terminated stochastically with 3’ azido labeled dideoxynucleotides followed by click chemistry-mediated adapter addition [7].

One of the most straightforward strategies for constructing cDNA sequencing libraries is to use a primer with an adapter overhang for reverse transcription and subsequently ligate a DNA adaptor to the 3’ end of the cDNA with either CircLigase or T4 DNA ligase. This 3’ cDNA ligation strategy has successfully been used for RNA-Seq [8,9] and sequencing of transcriptome-wide RNA structure probing [10–13]. The 3’ cDNA ligation reaction is known to be biased, but the use of T4 ligase significantly reduces the bias [13], meaning that 3’ cDNA ligation is an attractive strategy for sequencing library preparation. However, widespread use has been limited by primer–adapter contamination arising from the adapter ligating to the 3’ end of the reverse transcription primer. During PCR amplification of the sequencing library, the primer–adapter product is amplified more efficiently than the intended cDNA product because of its short length. If a large amount of RNA starting material is used, it is typically possible to produce cDNA sequencing libraries with low primer–adapter product contamination, while lower amounts of starting material produce libraries with considerable amounts of primer–adapter product, which will reduce the number of informative sequence reads obtained from the sequencing [3,13–15].

To avoid contaminating primer–adapter products in 3’ cDNA ligation-based libraries, several different strategies can be applied. First, an increased amount of RNA starting material can in some cases minimize primer adapter contamination. Second, a reduction in the amount of primer used in the reverse transcription reaction reduces primer–adapter contamination, but the reduction also affects the efficiency of cDNA synthesis, meaning that this strategy requires optimization and may not work in all cases. Third, in many applications, the cDNAs of interest are longer than the primer–adapter, which makes
For many years, the ability of *Escherichia coli* Exo I to catalyze degradation of single-stranded DNA without targeting double-stranded DNA [20] has been exploited for cleanup of PCR products. Excess primer can be degraded by Exo I and the resulting dNTPs dephosphorylated by alkaline phosphatase, thereby preparing PCR products for downstream applications [21]. While the lack of Exo I activity on DNA duplexes is well characterized [20], Exo I’s activity on DNA in RNA–DNA duplexes has to our knowledge not been formally investigated and there are only a few examples of Exo I being used to remove reverse transcription primers after reverse transcription [22–25]. In two similar single-cell RNA-seq protocols, reverse-transcribed mRNA was treated with Exo I before poly-A tailing of the 3’ cDNA end in the subsequent step [22,23]. Likewise, in a recent optimization of the CLIP-Seq protocol, Exo I and alkaline phosphatase treatment were introduced to remove reverse transcription-primers before ligation of an adapter to the 3’ end of cDNA with T4 RNA ligase [24]. Finally, in a recent RNA-seq protocol, Exo I was used together with selection on streptavidin beads to remove surplus reverse transcription primer [25]. None of these studies included data on the effect of Exo I treatment in library preparation or investigated if inclusion of Exo I in their protocol caused trimming of the 3’ end of the cDNA.

Here, we demonstrate that Exo I treatment of reverse transcription reactions before cDNA ligation dramatically reduces the amount of primer–adapter contamination in RNA-seq libraries, including small RNA sequencing libraries, and does not result in 3’ end trimming of the cDNA.

**MATERIALS & METHODS**

**RNA purification**

*E. coli* growth conditions and RNA extraction are described in detail in the Supplementary methods. For small RNA library preparation, an equal mixture of five chemically synthesized 5’ phosphorylated and HPLC-purified RNA oligonucleotides (Eurofins) with a mutated version of the let-7 mRNA (mut-let-7a, b, c, e, f) (Supplementary Table 1) sequence were used.

**Fragmentation**

For fragmentation, 560 ng *E. coli* RNA was incubated in 50 mM Tris-HCl (pH 8) and 5 mM MgCl2 at 95°C for 3 min and 20 s before placing on ice and adding EDTA to a final concentration of 10 mM in a final volume of 70 μl.

**RNA & cDNA purifications between enzymatic reactions**

For *E. coli* RNA-seq libraries, fragmented RNA was purified using Agencourt RNAClean XP beads. After subsequent steps (reverse transcription, Exo I digestion, adapter ligation and PCR), DNA was purified with Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63987 and A63881) using 1 volume of the nucleic acid solution to 1.8 volume of beads according to manufacturer’s protocol. The size-selected sample was subjected to an additional round of Ampure purification. For preparation of microRNA libraries, samples were purified after all steps (polyadenyl-
Figure 2. Bioanalyzer electropherogram traces of final *Escherichia coli* RNA sequencing libraries. (A) RNA sequencing library prepared with a standard protocol. (B) Standard protocol, but with a 5× reduction in amount of reverse transcription primer. (C) Standard protocol, but with size selection using additional Ampure XP bead cleanup following reverse transcription. (D) Standard protocol, but treated with Exo I following reverse transcription to remove leftover primer. (E) As (B), but treated with Exo I. (F) Percentage of primer–adapter reads in the five libraries corresponding to percentage of read pairs that were 22 nt after trimming adapter sequences (see supplemental material and methods).

*Primer adapter peak (147 bp).
**Primer adapter concatemer.
M: Standard lower or upper size marker used for size estimation.
Figure 3. Bioanalyzer electropherogram traces of miRNA sequencing libraries. (A) MicroRNA sequencing library prepared without Exo I treatment. M: Standard lower or upper size marker used for size estimation. (B) MicroRNA sequencing library treated with Exo I in the Exo I buffer. (C) MicroRNA sequencing library treated with Exo I in the RT buffer. (D) Percentage of primer–adapter reads in the three libraries (A–C) corresponding to processed reads that were too short to incorporate miRNA sequences (see Supplementary materials and methods). (E) Distribution of alignment start positions from the reference miRNA sequences of the three libraries (A–C). (F) Denaturing acrylamide gel of 5′ end 32P-labeled poly-dT primer (Supplementary Table 1) in RT of polyadenylated miRNAs, treated with Exo I (+) and not treated with Exo I (−). The position of full-length cDNA (61 nt) and the non-elongated poly-dT primer (41 nt) in the gel is indicated.

*Primer–adapter peak (147 bp).
M: Standard lower or upper size marker used for size estimation; RT: Reverse transcription.
Polyadenylation
Samples containing 510 ng miRNA oligonucleotide were polyadenylated with 5 U *E. coli* Poly(A) polymerase (New England Biolabs, cat. # M0276) in 1× *E. coli* Poly(A) polymerase buffer and 1 mM ATP in a total volume of 20 μl and incubated for 30 min at 37°C.

First-strand cDNA synthesis
Reverse transcription was carried out by mixing the purified fragmented RNA with either 1 μl of 10 μM or 1 μl of 50 μM reverse transcription primer for random priming (**E. coli** libraries; Supplementary Table 1) or by mixing polyadenylated RNA with 4 μl 10 mM poly-dT primer (miRNA libraries; Supplementary Table 1). Reverse transcription primer and RNA mixtures were denatured by incubation at 65°C for 5 min before placing on ice. Reverse transcription was then carried out using PrimeScript Reverse Transcriptase (Takara Bio, cat. # 2680A) in 20 μl reactions of 1× reverse transcription buffer and 0.5 mM dNTP. For **E. coli** libraries, 0.5 M sorbitol and 0.1 M trehalose was added and samples were incubated at 25°C for 30 sec, 30°C for 10 min, 42°C for 30 min, 50°C for 10 min, 56°C for 10 min, 60°C for 10 min before placing on ice. For miRNA libraries, the samples were incubated 30°C for 10 min, 42°C for 60 min, 70°C for 10 min before placing on ice.

Exo I treatment
Purified reverse transcription reactions were digested with 20 U Exo I (New England Biolabs, cat. # M0293) in a total volume of 20 μl in 1× Exo I buffer or in 1× reverse transcription buffer, for 30 min (miRNA libraries) or 45 min (**E. coli** libraries) at 37°C.

Reactions contained 0.6 μM forward PCR primer (Supplementary Table 1), 0.5 μM reverse PCR index primer (Supplementary Table 1), 1× Phusion HF buffer, 0.2 mM dNTPs and either 1 U (miRNA libraries) or 2 U (**E. coli** libraries) Phusion® High-Fidelity DNA Polymerase (New England Biolabs, cat. # M0530). The PCR reactions were denatured for 3 min at 98°C, followed by four cycles of 98°C for 80 s, 64°C for 15 s and 72°C for 1 min, and finally two (**E. coli** libraries) or nine (miRNA libraries) cycles of 98°C for 80 s and 72°C for 1 min, and finally 72°C for 5 min.

### Blot-to-blot consistency.
**Time after time.**

To achieve meaningful comparison of expressed proteins from blot-to-blot, trustworthy data is essential. **BlotCycler™** is designed to eliminate slight variations in fluid delivery, timing and washing that may occur when protocols are performed manually. With rigorous precision, **BlotCycler** delivers an affordable solution to busy labs demanding reproducible results.
2P end labeling of primer & denaturing acrylamide gel electrophoresis

Primer degradation and trimming of cDNA by Exo I were assessed by gel electrophoresis of radioactively labeled cDNA and primers. For 2P 5’ end labeling of primer, 20 pmol reverse transcription poly_dT primer (Supplementary Table 1) was labeled with 100 pmol [32P] ATP (6000 Ci/mmole) by 10 U T4 Polynucleotide kinase (New England Biolabs, cat. # M0201) in 1x T4 PNK buffer for 30 min at 37°C. Labeling was terminated by adding ethylenediaminetetraacetic acid to a final concentration of 25 mM and incubating at 75°C for 10 min prior to purification by Oligo Clean & Concentrator™ (Zymo Research, cat. # D4061). Polyadenylated miRNAs were reverse transcribed with 2P poly_dT primer and either treated with Exo I or not, as described above. Samples were then run on a 7 M urea 16% acrylamide gel.

RESULTS & DISCUSSION

Exo I treatment reduces primer–adapter contamination

Exo I selectively catalyzes the degradation of single-stranded DNA in the 3’ to 5’ direction, whereas double-stranded DNA is protected from degradation. In 3’ cDNA ligation-based sequencing library preparation, the contamination with primer–adapter products is a recurrent issue [13]. We reasoned that treatment with Exo I could potentially degrade excess reverse transcription primers without affecting the cDNAs in complex with the template RNA (Figure 1). To test this strategy, we prepared five different sequencing libraries, each from the same amount of E. coli total RNA, using random primed reverse transcription and 3’ cDNA ligation. The library prepared using the standard protocol shows a prominent contamination with a 147-bp PCR product, which corresponds with the expected length of the adapter being ligated directly to the reverse transcription-primer and PCR amplified in the subsequent PCR (Figure 2A). In addition, we observe a broad peak of approximately 210 bp length, which predominantly consists of longer PCR concatemers of the primer–adapter byproduct (Figure 2A). Some of these byproducts contain the Illumina adapter sequences and will therefore reduce the number of informative reads obtained by sequencing. Neither reduced reverse transcription-primer concentration (Figure 2B) nor two rounds of size selection using solid phase reversible immobilization beads (Figure 2C) significantly reduce the primer–adapter contamination in the libraries. In contrast, Exo I treatment for 45 min or Exo I treatment in combination with reduced reverse transcription-primer concentration significantly reduce the level of primer-adapter byproducts present in the final libraries (Figure 2D & E). Sequencing and subsequent analysis of the sequence reads obtained from the different libraries confirm the qualitative interpretation from the bioanalyzer traces. The library prepared according to the standard protocol contains 75.2% primer–adapter reads, whereas reduction of reverse transcription-primer concentration or inclusion of additional bead-based cleanup result in 78.5 and 63.5% primer–adapter contamination, respectively (Figure 2F). In contrast, the libraries prepared with Exo I treatment or Exo I treatment in combination with reduced reverse transcription-primer contain 4.7 and 1.6% primer–adapter sequence reads (Figure 2F), demonstrating that RNA–cDNA duplexes are protected from Exo I, whereas the unprotected reverse transcription-primer is degraded. The removal of surplus reverse transcription primers by Exo I treatment is easy to perform as the cDNA–RNA duplexes from reverse transcription can be purified with solid phase reversible immobilization beads or phenol-chloroform extraction followed by precipitation without disrupting the duplexes.

Application of Exo I treatment to small RNA sequencing libraries

When sequencing small RNAs, such as miRNAs, primer–adapter contamination is especially problematic, because it is difficult to separate the library products from the adapter contamination products [16] and to our knowledge, no published study has so far employed 3’ cDNA ligation to prepare small RNA sequencing libraries. Instead, small RNA library preparation is typically based on ligation of adapters to the 3’ and 5’ ends of the small RNA. Exo I treatment has the potential to alleviate the need for size selection of cDNA products in 3’ cDNA ligation-based small RNA library preparation. To test this possibility, we used a mix of five chemically synthesized miRNA controls of 22 nt in length for library preparation with or without Exo I treatment. A polyA tail was added to the miRNA controls using Poly(A) polymerase I to facilitate reverse transcription with a poly-dT primer, ending in a degenerate VN sequence to direct priming to the miRNA-polyA junction. As expected, the small RNA-seq library prepared using the standard procedure displays abundant primer–adapter contamination (Figure 3A & Supplementary Figure 1A & D). In contrast, primer–adapter contamination is less prominent in the library having an Exo I treatment step included in the library preparation protocol after cDNA ligation and prior to library amplification (Figure 3B & Supplementary Figure 1B & E). We also tested the effect of including Exo I treatment immediately after reverse transcription without changing the buffer. Again, we observe a reduction in the amount of primer–adapter contamination in the library (Figure 3C & Supplementary Figure 1C & F), although not as large as observed with Exo I treatment in the optimal buffer. Sequencing of the libraries and analysis of the resulting sequence reads demonstrate that the rate of primer–adapter contamination is 72.2 % in the library prepared with the standard protocol as compared to 5.2 and 18.9% in the two Exo I treated libraries (Figure 3D). The relative numbers of mapped sequence reads in each of the three libraries are highly similar between each of the five different control miRNAs with an approximately fourfold increase in mapped sequence reads in the Exo I-treated libraries when compared to libraries based on the standard protocol (Supplementary Figure 2). This demonstrates that Exo I treatment can increase the proportion of informative sequencing reads in small RNA sequencing libraries based on 3’ cDNA ligation by degradation of the reverse transcription primer. Therefore, small RNA sequencing protocols based on 3’ cDNA ligation and including Exo I treatment are an alternative to the currently used methods.

A potential risk of treatment with Exo I is that breathing of the cDNA 3’ ends in the cDNA–RNA duplexes allows exonuclease trimming by Exo I, which would limit the potential application of Exo I treatment. We plotted the start positions of the sequence reads mapping to the five miRNAs for each
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