Ask Frank

By **Fr**ed and H**ank**

ExactSTART™ Platform Technology

Q. There are four different ExactSTART products: Full-Length cDNA Cloning Kit, Small RNA Cloning Kit, 5′- and 3′-RACE Kit, and End-Tagged Double-Strand cDNA Synthesis Kit for Small RNA. What is the difference among these products?

A. The basic strategy of the ExactSTART platform is the same for the four kits—preparing cDNAs containing tagging sequences at both 5′ and 3′ ends, from a selected class of RNA molecules (Fig. 1). Differences in the kits arise in the processes for tagging the desired class of RNA and subsequent cDNA synthesis, e.g., in the choice of RNA modifying enzymes that are employed, and size enrichment for small RNAs.

Q. Have any of the ExactSTART kits been used for next-generation sequencing technologies? How does this work?

A. Yes. The ExactSTART Full-Length cDNA Cloning Kit has been adapted for use in 454 pyrosequencing applications (see p. 4 in this issue). The kit was adapted for next-generation sequencing by simply substituting the appropriate sequence tags required for 454 sequencing for the ExactSTART RNA Acceptor Oligo and the cDNA Synthesis Primer.

Q. How does the ExactSTART Small RNA Cloning Kit select for small RNAs?

A. The kit includes the ExactSTART Small RNA Enrichment Solution, which selectively precipitates large RNAs (greater than ~200 nucleotides)—such as 18S and 28S rRNA, and most mRNAs—from the total RNA sample. Small RNAs less than ~200 nucleotides, such as miRNAs, other small noncoding RNAs, tRNAs, and small ribosomal RNAs, remain in solution and are recovered. Gel electrophoresis size selection may be performed as well, but care must be taken to avoid exposure of RNA to ultraviolet light and ethidium bromide during the procedure.

Q. The ExactSTART Kits use either RNA Polyphosphatase or Tobacco Acid Pyrophosphatase at the beginning of the procedure. What is the difference between these two enzymes?

A. RNA 5′ Polyphosphatase, discovered by EPICENTRE scientists, converts RNAs with a 5′-triphosphorylated end to a 5′-monophosphorylated end. RNAs with a 5′-cap structure or a 5′-monophosphorylated end are not affected by treatment with RNA 5′ Polyphosphatase. Tobacco Acid Pyrophosphatase (TAP) is commonly used to remove the 5′-cap structure that is present on most eukaryotic mRNAs. TAP will also convert the 5′-triphosphorylated end of RNA to a 5′-monophosphorylated end. Thus, TAP can be used to tag RNAs containing either a 5′-capped or 5′-triphosphorylated end, while RNA 5′ Polyphosphatase will facilitate tagging those RNAs that have a 5′-triphosphorylated end, but not those with a 5′-cap structure.

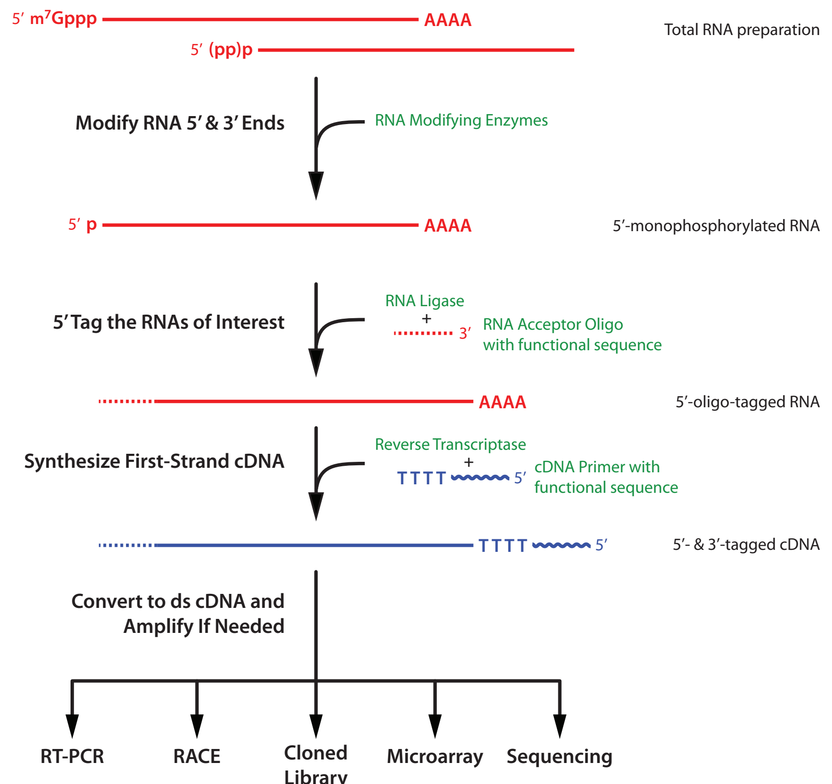

Figure 1. Overview of the ExactSTART™ transcript discovery and analysis process. The actual RNA modifying enzymes used and their order of use is dictated by the class of RNA that you wish to tag. The tagging sequence included in the RNA Acceptor Oligo and the cDNA Primer is dependent on the intended downstream application.

Q. For the ExactSTART 5′- and 3′-RACE Kit, can I use gene-specific primers in conjunction with the ExactSTART PCR RACE Primers to identify a specific cDNA?

A. Yes, you can. We recommend that you convert the first-strand cDNA to double-stranded cDNA (ds cDNA) by PCR, and then use the appropriate primer combinations (ExactSTART primers and your gene-specific RACE primers for the 5′ or 3′ ends). This eliminates any possibility of PCR failures due to differences in annealing temperatures and primer-dimer issues, and ensures that you have a representative cDNA library before performing RACE.

Q. When creating a full-length cDNA library, will the relative expression profile of the tissue/cell RNA be maintained?

A. As long as the input RNA is of good quality (for example, Bioanalyzer™ analysis generates a RNA integrity number [RIN] of at least 7.0) you should obtain a cDNA library that retains the expression profile of the original RNA sample.

Q. Why are only 16-20 cycles used for amplification of the single-strand cDNA created with the ExactSTART process, rather than a full 30-35 cycles as suggested by other cDNA cloning and amplification kits?

A. The primary reason for the reduced number of PCR cycles is to maintain amplification in the linear range. Due to the long PCR primers provided with the ExactSTART Kits, a higher number of cycles can lead to the production of PCR artifacts and misprimed cDNAs, eventually resulting in a population of truncated ds cDNA species that are not full-length. However, if the amount of input total RNA being used in an ExactSTART reaction is 10 µg or less, it will be necessary to optimize the number of PCR cycles so as not to obtain an over-representation of small PCR products.

Q. Can you make a cDNA library from RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissue samples?

A. Using the ExactSTART process, a full-length cDNA library, derived from the mRNA contained in a total RNA sample, requires that the RNA have either a 5′-capped or 5′-triphosphorylated end *and* a 3′-poly(A) tail. FFPE samples are notorious for yielding degraded RNA. Thus, it is highly unlikely that that you will be able to construct a full-length mRNA–derived cDNA library from an FFPE sample. However, small RNAs (e.g., miRNAs) are less likely to be degraded in an FFPE sample. In fact, we have successfully constructed small noncoding RNA libraries from FFPE samples, using the ExactSTART process.

Q. What are the sequences of the ExactSTART RNA Acceptor Oligo, cDNA Synthesis Primer, and PCR Primers?

A. The sequences will vary depending on the kits:

ExactSTART Full-Length cDNA and RACE Kits

ExactSTART RNA Acceptor Oligo:
5′-rGAGCGGCCGCCUGCAGGAAA-3′

cDNA Synthesis Primer:
5′-TAGACTTAGAAATTAATACGACTCA CTATAGGCGCGCCACCGGTG-d(T)$_{18}$-3′

PCR Primer 1:
5′-TCATACACATACGATTTAGGTGACA CTATAGAGCGGCCGCCTGCAGGAAA-3′

PCR Primer 2:
5′-TAGACTTAGAAATTAATACGACT CACTATAGGCGCGCCACCG-3′

ExactSTART Small-RNA Kits

ExactSTART RNA Acceptor Oligo:
5′-rGAGCGGCCGCGAAGAUCAGA-3′

cDNA Synthesis Primer:
5′-CTATAGGCGCGCCACCGGTG-(dT)$_{18}$-VN-3′ (V=A,C,G)

PCR Primer 1:
5′-GAGCGGCCGCGAAGATCAGA-3′

PCR Primer 2:
5′-CTATAGGCGCGCCACCGGTGTTTT-3

Q. How much input RNA will be required for using the ExactSTART Kits?

A. The ExactSTART Kits are optimized for use with 10-200 µg of total RNA. Enriched mRNA may be used, but less input RNA will be required as the mRNA component of total RNA will be between 1% to 5% based on the tissue or cell type. Thus, the use of enriched mRNA will require only between 500 ng and 10 µg of input RNA.

Q. Can the ExactSTART Kits be used to prepare a prokaryotic cDNA library?

A. Yes. Processed bacterial transcripts, which have a 5′-monophosphorylated RNA, can be used in a typical ExactSTART procedure after they are tailed with Poly(A) Polymerase.

Q. When making a small-RNA library, how do you identify the microRNAs (miRNA) in the library?

A. In general, miRNAs can be identified by sequencing of the ds cDNAs created with the ExactSTART Small RNA Cloning Kit or the ExactSTART End-Tagged Double-Strand cDNA Synthesis Kit for Small RNA. These sequences can be compared to databases of known miRNAs, e.g., miRBase (http://microrna.sanger.ac.uk).

Q. What is the key step in creating a high-quality, full-length cDNA library using the ExactSTART Kits?

A. See the previous question. Having high-quality, highly purified RNA with high RIN numbers and having a well-optimized PCR for amplification of the single-stranded cDNA are the most important criteria for obtaining a full-length cDNA library.

Q. Can you make cDNA libraries from plant species using the ExactSTART Kits?

A. As a general rule, plant mRNAs will generate excellent full-length cDNA libraries since they contain the requisite 5′ cap and 3′-poly(A) tails. However, small noncoding plant RNAs (e.g., miRNAs) frequently contain a 2′-methoxy group at the 3′end, which prevents 3′-poly(A)-tailing…a requirement for the ExactSTART small-RNA cDNA synthesis and tagging procedure.

Q. The ExactSTART™ Full-Length cDNA Cloning Kit claims that the clones obtained will be full-length cDNAs. How does this work?

A. The key feature of the ExactSTART process is generating cDNA tagged at both the 5′ and 3′ ends (Fig. 1). Tagging both ends of the cDNA requires that the mRNA contain either a 5′ triphosphate (i.e., uncapped primary transcript) or a 5′-cap structure, *and* a 3′-poly(A) tail. As a result, only full-length eukaryotic mRNAs are converted to 5′- and 3′-tagged cDNA. Further, the cDNA is converted to ds cDNA and amplified using PCR primers that anneal to the 5′- and 3′-tagging sequences. Thus, only full-length mRNAs are converted to amplified ds cDNA for cloning or other applications.

ExactSTART Kits are covered by issued and/or pending patents; see www.EpiBio.com/legal