BioTechniques

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/ibtn20

Reverse Transcriptase Template Switching: A SMART™ Approach for Full-Length cDNA Library Construction

Y.Y. Zhu, E.M. Machleder, A. Chenchik, R. Li & P.D. Siebert

To cite this article: Y.Y. Zhu, E.M. Machleder, A. Chenchik, R. Li & P.D. Siebert (2001) Reverse Transcriptase Template Switching: A SMART™ Approach for Full-Length cDNA Library Construction, BioTechniques, 30:4, 892-897, DOI: 10.2144/01304pf02

To link to this article: https://doi.org/10.2144/01304pf02

PRODUCT APPLICATION FOCUS

. . . a forum for manufacturers to describe the current and potential applications of new research instruments or products.

Reverse Transcriptase Template Switching: A SMART™ Approach for Full-Length cDNA Library Construction

Y.Y. Zhu, E.M. Machleder, A. Chenchik, R. Li, and P.D. Siebert
CLONTECH Laboratories, Palo Alto, CA, USA

BioTechniques 30:892-897 (April 2001)

ABSTRACT

Here, we describe a fast, simple method for constructing full-length cDNA libraries using SMART™ technology. This novel procedure uses the template-switching activity of Moloney murine leukemia virus (MMLV) reverse transcriptase to synthesize and anchor first-strand cDNA in one step. Following reverse transcription, three cycles of PCR are performed using a modified oligo(dT) primer and an anchor primer to enrich the cDNA population for full-length sequences. Starting with 1 μg human skeletal muscle poly(A)$^+$ RNA, a cDNA library was constructed that contained 3 × 10^6 independent clones with an average insert size of 2 kb. Sequence analysis of 172 randomly selected clones showed that 77% of cDNA clones corresponding to known genes contained intact open reading frames. The average length of complete open reading frames was 2.4 kb. Furthermore, 86% of the full-length clones retained longer 5′ UTR sequences than the longest 5′ end deposited in the GenBank® database. cDNA libraries generated using this method will be useful for accelerating the collection of mRNA 5′ end sequence information, which is currently very limited in GenBank.

INTRODUCTION

The generation of full-length cDNA libraries is indispensable for characterizing the structure and function of newly discovered genes. Conventional cDNA library construction methods (15) suffer from several major shortcomings. First, the majority of cDNA clones are not full-length, especially for mRNAs longer than 2 kb. This loss of sequence is typically due to premature termination of reverse transcription (22) or 5′-terminal sequence loss caused by cDNA blunt-end polishing before cloning (12). As a result, cDNA 5′ ends are significantly underrepresented in cDNA libraries. Second, an adaptor-mediated cloning process is still a common approach for cDNA library construction (24). Thus, the resulting cDNA libraries can be comprised of up to 20% undesirable ligation byproducts (chimeras) and inserts of non-mRNA origin (e.g., genomic DNA, mitochondrial DNA, ribosomal RNA, and adaptor dimers) (25). Additionally, current library construction methods for directional cloning suffer from their reliance on methylation, a process that is often incomplete in protecting internal restriction sites and is also inefficient for cloning (23).

Several protocols for cDNA library construction have been described that exploit the mRNA cap structure (14) to enrich for full-length sequences. Leading technologies in this field include the "oligo-capping" method (17,22), CAPture (13), and CAP-trapper (7). Each of these methods requires complicated, multi-step manipulations of the cap structure and mRNA and cDNA intermediates, which are difficult to control and often result in degradation of mRNA or cDNA-RNA duplexes. This likely explains why the average size of the resulting clones is

less than 1.5 kb—considerably shorter than the average mammalian mRNA size of about 2.2 kb (21). Moreover, because of the low efficiency of the enzymatic enrichment steps, these methods are not suitable in cases where starting material is limited because they require 20–100 µg mRNA.

More recently, oligo-capping has been used in conjunction with low-cycle PCR and *Sfi*I directional cloning for library construction (26). Low-cycle PCR provides selective enrichment of long cDNAs; *Sfi*I cloning eliminates the need for methylation because *Sfi*I sites are very rare in the mammalian genome. Although this method typically improves the average size of full-length clones to 1.9 kb, the multiple enzymatic steps still require 5–10 µg starting mRNA.

Here, we describe a simple cDNA library construction method that increases the lengths of cDNA clones by simultaneously employing two intrinsic properties of Moloney murine leukemia virus (MMLV) reverse transcriptase: reverse transcription and template switching (8,20). This procedure, termed SMART™ (switching mechanism at the 5′ end of the RNA transcript), anchors both ends of the first-strand cDNA by adding a distinct *Sfi*I site to each end during reverse transcription. First-strand 5′ ends are anchored when reverse transcriptase is primed by a modified oligo(dT) primer that contains an *Sfi*IB restriction site. The 3′ ends are anchored by using a template-switching oligonucleotide containing the *Sfi*IA site. This oligonucleotide serves as an extended template for reverse transcriptase after it reaches the end of the RNA molecule. Since the template-switching phenomenon is most efficient when reverse transcriptase has reached the end of the RNA template (8), prematurely terminated cDNAs usually lack the *Sfi*IA site and are selectively eliminated during the cloning process. This novel integration of the template-switching phenomenon with *Sfi*I-based anchoring and directional cloning ensures the construction of cDNA libraries that have high yields of representative, full-length clones when starting with as little as 1 µg poly(A)$^+$ RNA. This report marks the first detailed side-by-side comparison of the quality of SMART and conventional cDNA libraries.

MATERIALS AND METHODS

Preparation of RNA

Total RNA was prepared from human skeletal muscle by the acid guanidinium thiocyanate-phenol-chloroform method (9). Poly(A)$^+$ RNA was prepared from skeletal muscle total RNA using two cycles of chromatography on oligo(dT)-cellulose (24).

Phagemid Vector

The multiple cloning sites (MCS) of the λTriplEx-2(*Sfi*I A&B) phagemid vector is located within an embedded plasmid, pTriplEx-2(*Sfi*I A&B), which is flanked by *loxP* sites at the λ junctions. The MCS of pTriplEx-2(*Sfi*I A&B) was modified from λTriplEx-1 (CLONTECH Laboratories, Palo Alto, CA, USA; GenBank® accession no. U39779) by the insertion of an *Sfi*IA site (5′-GGCCATTA'TGGCC-3′) between the *Eco*RI and *Pst*I sites, an *Sfi*IB site (5′-GGCCGCCT'CGGCC-3′) between *Bam*HI and *Sal*I, and the deletion of a *Sfi*IC site: (5′-GGCCGACT'TGGCC-3′) between *Sac*I and *Msc*I. The λTriplEx-2(*Sfi*I A&B) arms were prepared for directional cloning by digestion with *Sfi*I enzyme (New England Biolabs, Beverly, MA, USA) and dephosphorylation with calf intestinal alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN, USA) in accordance with the manufacturers' protocols.

Oligonucleotides for cDNA Synthesis

The following oligonucleotides were used (oligonucleotides 1–3: SMART cDNA Library Construction Kit; CLONTECH Laboratories).

(1) SMART template-switching oligonucleotide:

5′-d(AAGCAGTGGTATCAACGCAGAGTGGCCATTA'TGGCC)-r(GGG)-3′

*Sfi*IA site

r(GGG) = ribonucleotide

(2) cDNA synthesis (CDS) primer:

5′-d(ATTCTAGAGGCCGAGG'CGGCCGACATGT$_{(30)}$VN)-3′

*Sfi*IB site

(N = A, G, C or T; V = A, G or C)

(3) 5′ anchor primer:

5′-d(AAGCAGTGGTATCAACGCAGAGT)-3′

(4) double-stranded adaptor (for conventional library construction)

5′-d(AATTCGCGGCCGCGTCGTCGAC)

d(GCGCCGGCGCAGCAGCTG)-PO$_4$-5′

First-Strand cDNA Synthesis and Template Switching

Human skeletal muscle poly(A)$^+$ RNA was used as starting material to synthesize first-strand cDNA. Briefly, a 5-µL cDNA synthesis mixture containing 1 µL skeletal muscle poly(A)$^+$ RNA (1 µg), 1 µL cDNA synthesis (CDS) primer (10 µM), 1 µL template-switching (TS)-oligonucleotide (10 µM) and 2 µL deionized water were incubated at 72°C for 2 min and then immediately placed in an ice-water bath for 2 min. The volume was then adjusted to 10 µL with the following reagents: 2 µL 5× first-strand synthesis buffer (250 mM Tris-HCl, pH 8.3, 30 mM $MgCl_2$, and 375 mM KCl), 1 µL dithiothreitol (DTT) (20 mM), 1 µL dNTP mixture (10 mM each dATP, dCTP, dGTP, and dTTP), and 1 µL (200 U) MMLV reverse transcriptase (RNase H- point mutant). Reverse transcription and template switching was carried out at 42°C for 60 min in an air incubator. The reaction was terminated by placing the tube on ice.

Double-Stranded cDNA Synthesis by Low-Cycle PCR

After first-strand synthesis, RNA was degraded by adding 1 µL 25 mM NaOH to the first-strand synthesis mixture and incubating at 68°C for 30 min. Double-stranded cDNA was synthesized by low-cycle PCR in a 100-µL reaction containing: 40 mM Tricine-KOH, pH 9.2; 15 mM KOAc; 3.5 mM $Mg(OAc)_2$; 0.2 µM 5′-anchor primer; 0.2 µM CDS primer; 0.2 mM each dATP, dGTP, dCTP, and dTTP; and 2 µL Advantage® cDNA Polymerase Mix (50×; contains KlenTaq-1 and Deep Vent polymerases; CLONTECH Laboratories). Low-cycle PCR was carried out on a DNA Thermal Cycler 480 (Applied Biosystems, Foster City, CA, USA) using the

following cycling conditions: 72°C for 10 min, 95°C for 1 min, and 3 cycles at 95°C for 15 s and 68°C for 8 min. Double-stranded cDNA synthesis was analyzed by visualization on 1.1% agarose/ethidium bromide gels.

cDNA Directional Cloning

Following double-stranded cDNA synthesis, the 50 µL double-stranded cDNA was incubated with proteinase K (Qiagen, Valencia, CA, USA) at final concentration of 0.8 µg/µL to degrade the thermostable DNA polymerases (11). After incubation at 45°C for 20 min, deionized water was added to a final volume of 100 µL. Then, the reaction mixture was extracted one time with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was ethanol-precipitated and digested with *Sfi*I (200 U; New England Biolabs) at 50°C for 2 h. After digestion, the cDNA was purified from a low-melt agarose gel to remove small fragments (<1000 bp) and was directionally cloned into *Sfi*I A&B-digested, dephosphorylated λTriplEx2 arms as previously described (24,26).

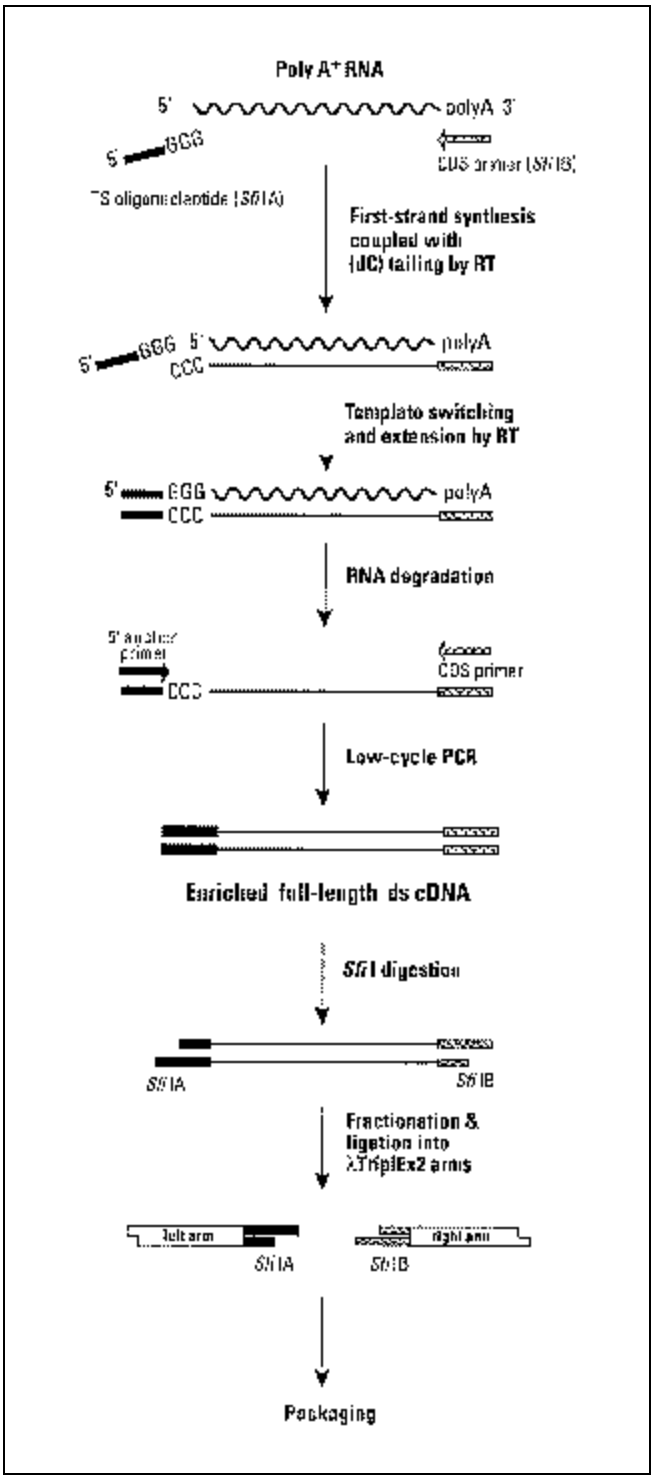

Figure 1. Scheme of the SMART cDNA library construction method.

Conventional cDNA Library Construction

As a control, conventional cDNA synthesis was carried out from 10 µg human skeletal muscle poly(A)$^+$ RNA. First-strand cDNA was synthesized using the CDS primer and MMLV reverse transcriptase. Second-strand synthesis was performed using the modified Gubler-Hoffman method (15,16,24). After polishing by T4 DNA polymerase (New England Biolabs) to generate blunt ends, a double-stranded adaptor with *Eco*RI sticky ends was ligated to both ends using T4 DNA ligase. Following adaptor ligation, the double-stranded cDNA was phosphorylated at the *Eco*RI sites, size-fractionated, and ligated into *Eco*RI-digested, dephosphorylated λTriplEx-1 arms (CLONTECH Laboratories).

PCR Insert Screening

Sixty clones were randomly picked from each of the SMART and conventional libraries, and inserts were screened by PCR using λTriplEx LD-Insert Screening Amplimers (CLONTECH Laboratories) in accordance with the manufacturer's protocol. Five microliters of each PCR product were analyzed on 1.1% agarose gels.

Sequencing and Sequence Analysis

The randomly selected clones from λTriplEx-2 (SMART) and λTriplEx-1 (conventional) cDNA libraries were converted to pTriplEx-2 and pTriplEx-1 in accordance with the manufacturer's protocol (CLONTECH Laboratories). After purification using the NucleoBond® Plasmid Kit (CLONTECH Laboratories), the cDNA clones were sequenced at both the 5′ and 3′ ends using a model 373 automated DNA sequencer (Applied Biosystems). Between 160 and 390 nucleotides were read for each clone. The sequences were compared with the GenBank database using BLAST (2).

RESULTS AND DISCUSSION

Principle of SMART Library Construction

The strategy used in this study for SMART cDNA library construction is illustrated in Figure 1. Briefly, first-strand synthesis is carried out in the presence of two oligonucleotides: a "lock-docking" CDS primer (5) that contains an *Sfi*IB site and primes reverse transcriptase and a TS-oligonucleotide that contains an *Sfi*IA site. When reverse transcriptase reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds additional nucleotides (predominantly dCTP) that are not encoded by the template (10,20). The TS-oligonucleotide

Table 1. Database Search of Random cDNA Clones from Both Human Skeletal Muscle Libraries

Categories	SMART cDNA library clones		Conventional cDNA library clones	
	Number	%	Number	%
Known genes, novel genes, and expressed sequence tags (ESTs)	170	99%	78	81%
Alu-like repetitive sequences	0	0%	2	2%
Mitochondrial DNA sequences	2	1%	12	13%
Cloning oligonucleotide or adaptor dimer contaminants	0	0%	4	4%
Ribosomal RNA sequences	0	0%	0	0%
Total	172	100%	96	100%
mRNA-derived clones:	99% (170/172)		81% (78/96)	

Table 2. Percentage of Clones Containing Intact ORFs by Size Category

Size of gene ORF	<2.0 kb	2.0–4.0 kb	>4.0 kb
SMART method	96% (24/25)	74% (37/50)	44% (4/9)
Conventional method	52% (15/29)	21% (3/14)	0% (0/2)

contains three consecutive rG nucleotides at the 3′ end to serve as a second template for reverse transcriptase. When reverse transcriptase reaches the 5′ end of the mRNA, the complementary interaction of the $r(G)_3$ stretch at the 3′ end of the TS-oligonucleotide and dC-rich extended sequence of the cDNA promotes template switching. Reverse transcriptase then transcribes the oligonucleotide, adding the *Sfi*IA anchor sequence to the end of first-strand cDNA (8,20). The RNA component of the cDNA-RNA duplexes is then degraded by NaOH treatment. Next, double-stranded cDNA is generated with three cycles of PCR catalyzed by a long-distance polymerase mixture (3,18). This reaction uses a 5′ anchor primer, which is complementary to the *Sfi*IA sequence and the CDS primer that contains the *Sfi*IB sequence. Digestion of the double-stranded cDNA with *Sfi*I generates two different sticky ends, *Sfi*I A&B, at the 5′ and 3′ ends, respectively. Because *Sfi*I sites are extremely rare in mammalian DNA, nearly all cDNA fragments remain intact, eliminating the need for methylation of the cDNA. After size fractionation, the *Sfi*I-digested cDNAs are directionally cloned into λTriplEx2, a phagemid vector capable of *Cre-lox*-mediated subcloning.

Quality of the SMART cDNA Library versus a Conventional Library

In a previous study, we showed that SMART cDNA synthesis and the conventional Gubler-Hoffman method yield cDNA samples with comparable banding patterns and size representation when analyzed by agarose gel electrophoresis (8). Furthermore, PCR analysis of 45 different genes, including transcription factors, cytokines, cytokine and other receptors, and housekeeping genes revealed only four genes that did not show the same representation in both cDNA populations (8). Taken together, these data indicate that the SMART and conventional methods yield very similar gene representation.

Based on the strategy shown in Figure 1, we used the SMART method to construct a human skeletal muscle cDNA library in λTriplEx-2 (*Sfi*I A&B) starting with 1 µg poly(A)$^+$ RNA. As a control, a conventional cDNA library was constructed in λTriplEx-1(*Eco*RI/*Eco*RI) using the Gubler-Hoffman method with 10 µg poly(A)$^+$ RNA. The complexity of the libraries were 3×10^6 independent clones/µg poly(A)$^+$ RNA for the SMART library and 0.3×10^6 independent clones/µg poly(A)$^+$ RNA for the conventional library. Although both cDNA libraries contained an equal number (3×10^6) of independent clones, the amount of starting RNA was 10-fold less for the SMART library.

Analysis of 60 randomly picked clones from each library showed that the average insert size for both libraries was approximately 2 kb, with a range of 0.5–7.0 kb. These data indicate that the SMART method avoids the bias in cloning short cDNA products that is a common problem in PCR-based cDNA library construction technologies (1,4,19).

The percentage of recombinant phage clones was determined for each library by PCR insert and blue/white screenings. The two methods showed 95% recombinants for the SMART library and 85% for the conventional library. The high percentage of recombinants seen in the SMART library was likely due to the directional cloning (*Sfi*I A&B) method. In conventional adaptor ligation based cloning methods, the molar ratio of adaptor to cDNA must be at least 100:1 to achieve a high ligation efficiency. This high concentration of adaptors typically results in adaptor self-ligation, producing an abundance of undesirable fragments that cannot be fully removed by size fractionation. Typically, these small adaptor dimers are preferentially ligated into phage vectors. Since adaptor dimer inserts generally do not disrupt *LacZ* α complementation because of their small size, the actual percent recombinants is underrepresented in the analysis of the conventional library (unpublished data).

Sequence Analysis

To compare further the quality of the two libraries, we partially sequenced the 5′ ends of 172 random clones from the SMART library and 96 clones from the conventional library. The clones were analyzed for homology to sequences in the GenBank and EMBL databases. As shown in Table 1, the fraction of clones originating from mRNA was 99% (170/172) in the SMART cDNA library and 81% (78/96) in the conventional library. The analysis revealed that among the clones of non-mRNA origin in the conventional library were significant numbers of mitochondrial DNA clones. The substantial presence of these DNA sequences was likely a byproduct of the adaptor ligation-based cloning process in which adaptors can be ligated to any DNA contaminants in the poly(A)$^+$ RNA population. Additionally, a significant number of adaptor dimer clones were found in the conventional library.

We then performed single-pass 3′ end sequencing on all clones that matched known sequences to compare the percentage of intact open reading frames (ORFs) between the libraries. Of 84 clones from the SMART library, 65 (77%) contained intact ORFs. Of 45 clones from the conventional library, 18 (40%) contained intact ORFs. When clones were grouped by size, the SMART library contained a higher percentage of intact clones in each category (Table 2). Furthermore, of the 65 intact ORF clones in the SMART library, 56 (86%) contained longer 5′ end sequences than the longest entries in the GenBank database. This was the case for only 4 (22%) of the 18 conventional clones with intact ORFs. These results indicate that the SMART cDNA library construction method enriches for full-length cDNA clones and retains more 5′ end sequences.

The observed enrichment for full-length cDNA clones in the SMART library is likely the result of three principal factors. First, MMLV reverse transcriptase terminal transferase and template-switching activities are most efficient when the enzyme has reached the 5′ end of the RNA template (8). Consequently, template switching is unlikely in first-strand synthesis events in which reverse transcriptase prematurely aborts polymerization after encountering strong mRNA secondary structure. Thus, the truncated cDNAs lack *Sfi*IA anchors and are eliminated during the cloning process. Second, previous experiments (12) have shown that conventional cDNA library construction technologies that rely on the combined action of T4 DNA polymerase and RNase H to produce blunt ends for adaptor ligation often remove 1–30 bp from the cDNA ends. The template-switching mechanism avoids the need for these enzymatic steps and the consequent cDNA degradation. Third, the long-distance PCR enzyme mixture (3,18) used for the low-cycle PCR step in the SMART method ensures processive second-strand synthesis and amplification and maintains accurate size representation.

Sequence analysis also revealed that among the SMART library cDNAs that matched known genes, none was a chimerical clone, a single clone with sequences from multiple genes. By contrast, 11% (5/45) of the matched clones in the conventional library were chimerical. Although reverse transcriptase can produce chimerical cDNAs during first strand synthesis (6), the chimerical sequences in the conventional library are most likely artifacts of adaptor ligation.

Comparison with Other Full-Length cDNA Library Construction Methods

Table 3 compares SMART library construction with the Cap Trapper (7) and oligo-capping (26) methods. These data show that among the three methods, SMART library construction yielded the largest average intact ORF size (2.4 kb). Although oligo-capping (26) produced the highest percentage of full-length clones (79%) in the greater than 2 kb size range, this method only yielded complete ORFs up to 3.3 kb in length. In contrast, the SMART library contained complete ORFs that were as long as 5.3 kb. Additionally, the procedural details listed illustrate that the SMART method requires fewer steps, less time, and a smaller quantity of starting RNA than the other technologies.

All three studies listed in Table 3 used thermostable DNA polymerases to synthesize double-stranded cDNAs. The SMART method used three cycles of PCR with a polymerase mixture containing KlenTaq-1 and Deep Vent (3,18); Cap Trapper used 1 cycle of PCR with Ex*Taq* (Takara, Shiga, Japan) (7); oligo-capping used 10 cycles of PCR with the XL PCR kit (Applied Biosystems) (26). Based on our data, the representation of the SMART library is comparable to that of the conventional library. This level of heterogeneity is also consistent with that seen in the Cap Trapper and oligo-capping libraries (7,26), indicating that low-cycle PCR does not lead to loss of representation. An additional concern for PCR-based methods is the rate of error introduction, a problem that is strongly influenced by PCR cycle number. Experiments

Table 3. Comparison of Full-Length cDNA Library Construction Methods

Method	Quantity of Starting Poly(A)$^+$ RNA	Full-Length cDNA Selection		Fraction of Clones Containing Intact ORFs			
		time	reaction step(s)	<2kb[a]	>2kb[a]	average size	(range)
SMART	1 µg	1–2 h	single	96% (24/25)	70% (41/59)	2.4 kb	(0.61–5.3 kb)
CAP Trapper[b]	10–20 µg	24–48 h	multiple	95% (62/65)	67% (6/9)	1.1 kb	(0.15–5.4 kb)
Oligo-capping[c]	5–10 µg	12–24 h	multiple	94% (15/16)	79% (15/19)	1.9 kb	(0.68–3.3 kb)

[a]Published size of intact ORF.
[b]Reference 7.
[c]Reference 26.

have shown that the thermostable polymerase mixture used in this study incorporates on average one error per 42 000 bp after three cycles of PCR (our unpublished data). Although the optimal number of cycles for minimizing PCR-induced mutations has not been rigorously tested for SMART library construction, performing three cycles using a suitable polymerase mixture should be sufficient to limit the number of errors to a tolerable level.

In this paper, we have described a cDNA library construction method that harnesses the template-switching activity of MMLV reverse transcriptase to increase the fraction of full-length clones. Using this method, the proportion of clones with intact ORFs is estimated to be about 77%, 2–3 times higher than that seen in conventional cDNA libraries. Of the clones with intact ORFs, 86% contained more of the 5′ untranslated regions (UTRs) than the longest sequences deposited in the GenBank database. In comparison to other recently reported full-length cDNA library construction methods, the SMART method is less complex, faster, and the 1 µg poly(A)$^+$ RNA required is 5- to 20-fold less.

REFERENCES

1. **Akowitz, A. and L. Manuelidis.** 1989. A novel cDNA/PCR strategy for efficient cloning of small amounts of undefined RNA. Gene *81*:295-360.
2. **Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. *215*:403-410.
3. **Barnes, W.M.** 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc. Natl. Acad. Sci. USA *91*:2216-2220.
4. **Belyavsky, A., T. Vinogradova, and K. Rajewsky.** 1989. PCR-based cDNA library construction: general cDNA libraries at the level of a few cells. Nucleic Acids Res. *17*:2919-2932.
5. **Borson, N.D., W.L. Salo, and L.R. Drewes.** 1992. A lock-docking oligo(dT) primer for 5′ and 3′ RACE PCR. PCR Methods Appl. *2*:144-148.
6. **Brakenhoff, R.H., J.G.G. Schoenmarkers, and N.H. Lubsen.** 1991. Chimeric cDNA clones: a novel PCR artifact. Nucleic Acids Res. *19*:1949.
7. **Carninci, P., C. Kvam, A. Kitamura, T. Ohsumi, Y. Okazaki, M. Tioh, M. Kamiya, K. Shibata et al.** 1996. High-efficiency full-length cDNA cloning by biotinylated CAP trapper. Genomics *37*:327-336.
8. **Chenchik, A., Y.Y. Zhu, L. Diatchenko, R. Li, J. Hill, and P.D. Siebert.** 1998. Generation and use of high quality cDNA from small amounts of total RNA by SMART PCR, p. 305-319. *In* P.D. Siebert and J.W. Larrick (Eds.), RT-PCR Methods for Gene Cloning and Analysis. Eaton Publishing, Natick, MA.
9. **Chomczynski, P. and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*:156-159.
10. **Clark, J.M.** 1988. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucleic Acids Res. *16*:9677-9686.
11. **Crowe, J.S., H.J. Cooper, M.A. Smith, M.J. Sims, D. Parker, and D. Gewert.** 1991. Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion. Nucleic Acids Res. *19*:184.
12. **D'Alessio, J.M. and G.F. Gerard.** 1988. Second-strand cDNA synthesis with *E. coli* DNA polymerase I and RNase H: the fate of information at the mRNA 5′ terminus and the effect of *E. coli* DNA ligase. Nucleic Acids Res. *16*:1999-2014.
13. **Edery, I., L.L. Chu, N. Sonenberg, and J. Pelletier.** 1995. An efficient strategy to isolate full-length cDNAs based on an mRNA retension procedure (CAPture). Mol. Cell. Biol. *2*:161-170.
14. **Furuichi, Y and K. Miura.** 1975. A blocked structure at the 5′ terminus of mRNA from cytoplasmic polyhedrosis virus. Nature *253*:374-375.
15. **Gubler, U. and B.J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene *25*:263-269.
16. **Huynh, T.V., R.A. Yong, and R.W. Davis.** 1985. Procedures for cDNA cloning, p. 88-110. *In* DNA Cloning: A Practical Approach. Vol. I. IRL Press, Oxford.
17. **Kato, S., S. Sekine, S.W. Oh, N.S. Kim, Y. Umezawa, N. Abe, M. Yokoyama-Kobayashi, and T. Aoki.** 1994. Construction of a human full-length cDNA bank. Gene *150*:243-250.
18. **Kellogg, D.E., I. Rybalkin, S. Chen, N. Mukhamedova, T. Vlasik, P. Siebert, and A. Chenchik.** 1994. TaqStart antibody: "hot start" PCR facilitated by a neutralizing monoclonal antibody directed against *Taq* DNA polymerase. BioTechniques *16*:1134-1137.
19. **Korneev, S., S. Blackshaw, and J.A. Davies.** 1994. cDNA libraries from a few neural cells. Prog. Neurobiol. *42*:339-346.
20. **Kulpa, D., R. Topping, and A. Telesnitsky.** 1997. Determination of the site of first strand transfer during Moloney murine leukemia virus reverse transcription and identification of strand transfer-associated reverse transcriptase errors. EMBO J. *16*:856-865.
21. **Lewin, B. (Ed.)** 1980. Structure of messenger RNA. *In* Gene Expression Volume 2: Eucaryotic Chromosomes. 2nd Edition. John Wiley & Sons, NY. p. 687.
22. **Maruyama, K. and S. Sugano.** 1994. Oligo-capping: a simple method to replace the cap structure of eucaryotic mRNAs with oligoribonucleotides. Gene *138*:171-174.
23. **McClelland, M., M. Nelson, and E. Raschke.** 1994. Effect of site-specific modification on endonucleases and DNA modification methyltransferases. Nucleic Acids Res. *22*:3640-3659.
24. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed. CSH Laboratory Press, Cold Spring Harbor, NY.
25. **Sudo, K., K. Chinen, and Y. Nakamura.** 1994. 2058 expressed sequence tags (ESTs) from a human fetal lung cDNA library. Genomics *24*:276-279.
26. **Suzuki, Y., K. Yoshitomo-Nakagawa, K. Maruyama, and A. Suyama.** 1997. Construction and charactrrization of a full length-enriched and a 5′ end-enriched cDNA library. Gene *200*:149-156.

Address correspondence to Paul D. Siebert, Clontech Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303, USA. e-mail: pauls@clontech.com