Takara Bio USA

SMART® cDNA Library Construction Kit User Manual

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I. Introduction

A. Summary

The **SMART cDNA Library Construction Kit** (Cat. No. 634901) provides a method for producing high-quality, full-length cDNA libraries from nanograms of total or poly A^+ RNA. The kit offers a choice of two different methods for synthesizing the cDNA, depending on your starting material—and a single method for constructing libraries from cDNA generated using either synthesis method. The cDNA synthesis methods include:

- **cDNA Synthesis by Long Distance (LD) PCR:** A novel, PCR-based method for researchers limited by their starting material (i.e., 50 ng of total RNA; see Section I.D).
- **cDNA Synthesis by Primer Extension:** A more traditional method for researchers with abundant amounts of starting material (i.e., 1 µg or more poly A^+ RNA; See Section I.E).

Both methods utilize the proprietary SMART IV (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) Oligonucleotide in the first-strand synthesis to generate high yields of full-length, double-stranded (ds) cDNA (Figure 1).

B. SMART Enriches for Full-Length cDNA with Complete 5' Ends

All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded (ss) DNA in the first-strand reaction. In some cases, RT terminates before transcribing the complete mRNA sequence. This is particularly true for long mRNAs, especially if the first-strand synthesis is primed with oligo(dT) primers only or if the mRNA contains abundant secondary structures. In addition, conventional cDNA cloning procedures use T4 DNA polymerase to generate blunt cDNA ends after the second-strand synthesis and adaptor ligation steps. As a result, under-represented 5' ends of genes in cDNA populations tend to be 5–30 nucleotides shorter than the original mRNA (D'Allessio, 1988).

- **The SMART protocols are designed to preferentially enrich for full-length cDNAs**, while eliminating blunt end adaptor ligations.
- **SMART libraries are proven to contain a higher percentage of full-length clones** than libraries constructed by conventional methods or other full-length cDNA synthesis protocols (Okayama & Berg, 1982; Kato *et al.*, 1994). Thus, clones isolated from SMART cDNA libraries contain sequences corresponding to the complete 5' untranslated region of the mRNA (ibid.).

C. SMART Preserves the Complete 5' mRNA Sequence

Libraries made with SMART cDNA can facilitate preliminary mapping of transcription start sites (Fromont-Racine, 1993) due to the high percentage of full-length clones. However, SMART cDNA libraries may not be suitable for immunoscreening for certain proteins; as in some cases, 5' untranslated regions (UTRs) may contain stop codons in frame with the initiating translation start site in the expression vector.

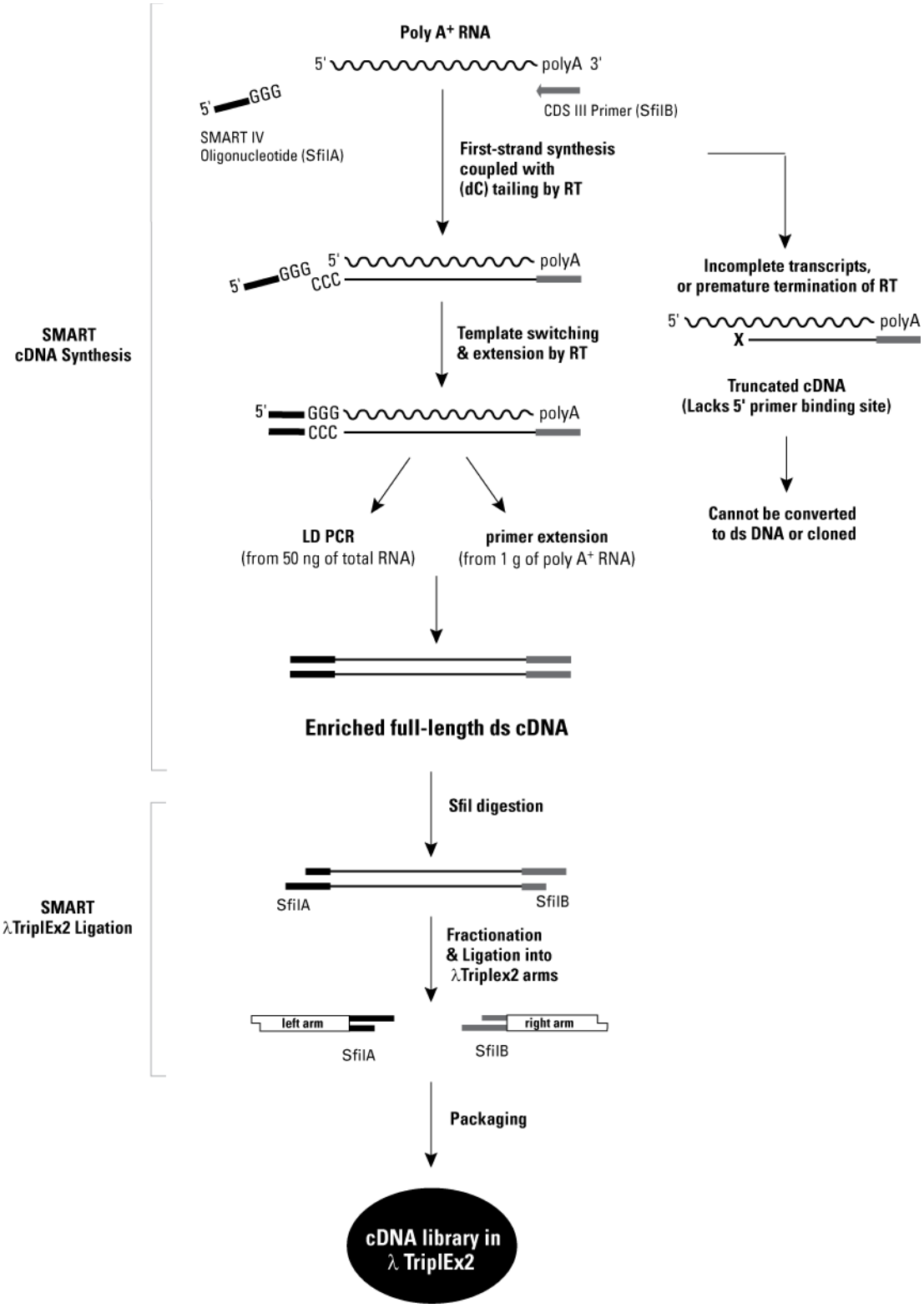

Figure 1. Flow chart of the SMART cDNA Library Construction Kit protocols. The right side of the flow chart shows the fate of incomplete transcripts caused by RNA degradation or premature termination of reverse transcription.

D. cDNA Synthesis by Long-Distance PCR

In Section V.C, long-distance PCR (LD PCR; Barnes *et al.*, 1994, Cheng *et al.*, 1994) is used to generate a full-length ds cDNA after first strand cDNA synthesis in Section V.B (Figure 1). This protocol is ideal for researchers who are limited by the amount of their available RNA starting material (i.e., 50 ng of total RNA).

Protocol Summary

1. A modified oligo(dT) primer (CDS III/3' PCR Primer) primes the first-strand synthesis reaction, and the SMART IV Oligo serves as an acceptor template for template switching.
    - When the RT reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA.
    - The SMART IV Oligo, which has an oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide.
2. The resulting full-length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo, which then serves as a universal priming site (SMART anchor) in the subsequent amplification by LD PCR (Chenchik *et al.*, 1998) to generate a full-length ds cDNA.
    - Only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and can be exponentially amplified. Incomplete cDNAs and cDNA transcribed from poly A^- RNA will lack the SMART anchor and will not be amplified. Thus, contamination by genomic DNA and nonpolyadenylated RNA is eliminated.
    - This selective amplification allows you to construct a cDNA library using nanogram amounts of either total RNA or poly A^+ RNA with a high percentage of full-length clones.

E. cDNA Synthesis by Primer Extension

In Section V.D, primer extension (Green & Sambrook, 2012), is used to generate a full-length ds cDNA after first strand cDNA synthesis in Section V.B (Figure 1). This protocol is ideal for researchers who are not limited by their starting material (i.e., 1 µg or more of poly A^+ RNA).

Protocol Summary

1. Using a procedure similar to the first-strand cDNA synthesis summarized in Section I.D, Step 1, the CDS III/3' Primer is used to prime the first-strand reaction, and the SMART IV Oligo serves as a short, extended template at the 5' end of the mRNA.
2. After first-strand cDNA synthesis, the primer-extension step generates full-length, ds cDNA.

F. Optimized Reagents and Conditions

The SMART kit incorporates carefully optimized reagents and conditions that improve the efficiency of all library construction steps.

- **A specially formulated first-strand buffer and modified lock-docking oligo(dT) primers** (Borson *et al.*, 1992) optimize the efficiency of first-strand synthesis. Lock-docking primers are designed to anneal to the junction of the poly A tail and the encoded transcript. This increases the efficiency of cDNA synthesis by eliminating unnecessary reverse transcription of long stretches of poly A.

- **Advantage® 2 PCR Kits** (Cat. Nos. 639206 & 639207; not supplied—order separately) are strongly recommended for PCR amplification.
- **Incorporation of asymmetrical SfiI (A & B) restriction enzyme sites (Figure 2) at the 5' and 3' cDNA ends**, respectively (Figure 1) allows directional cloning into λTriplEx2 Vector. After digestion with SfiI and size fractionation using CHROMA SPIN™ Columns, SMART cDNA is ready for ligation into the SfiI-digested λTriplEx2 Vector. λTriplEx2, which contains the asymmetrical SfiI sites (A & B) in the MCS, eliminates adaptor ligation, and facilitates directional cloning. Furthermore, SfiI sites are extremely rare in mammalian DNA; therefore, all SMART cDNAs remain intact after SfiI digestion. Methylation steps are eliminated, allowing valuable internal restriction sites to be preserved. Typical results of cDNA synthesis, using the Mouse Liver Poly A^+ RNA provided, are shown for LD-PCR at the end of Section V.C, and for primer extension at the end of Section V.D.

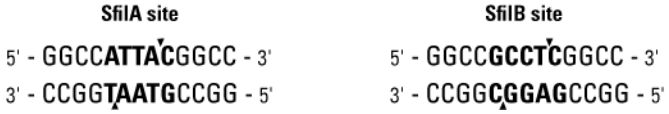

Figure 2. Comparison of the SfiI (A & B) recognition sequences.

G. Benefits of λ TriplEx2 Vector

Clontech's λTriplEx2 phagemid vector offers the following advantages for cDNA cloning:

- High-titer libraries, blue/white screening for recombinants, regulated expression of cloned inserts, and ease of converting clones from phage to a plasmid vector via Cre-lox-mediated subcloning.
- Lambda arms that can accept inserts of up to 13 kb, making this vector more suitable for large-insert libraries.
- Every cDNA inserted into the MCS of λTriplEx2 is expressed in all three reading frames (Figure 3).

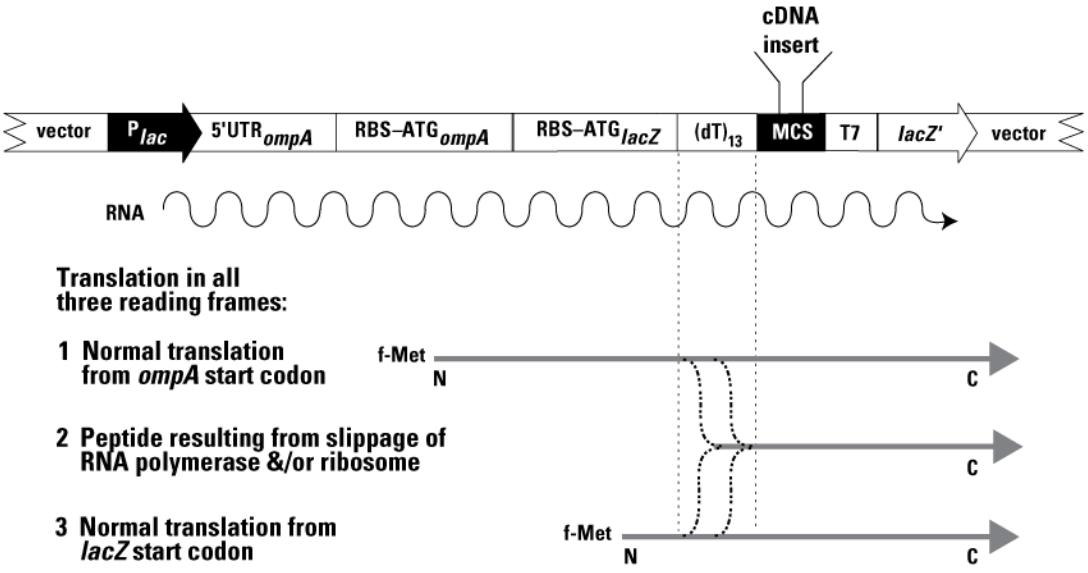

Figure 3. Generation of polypeptides from all three reading frames in a single recombinant λTriplEx2 clone. λTriplEx2 contains two translation start sites (i.e., two sets of ribosome-binding sites and ATG start codons) in different reading frames and a slip site (a stretch of dTs) that can cause ribosomes to shift frames between the regulated lac promoter and the MCS. RNA polymerase may also slip during transcription of the (dT)13 region. By the time the ribosomes begin translating the insert, roughly one-third will be in each of the three reading frames.

II. List of Components

Store CHROMA SPIN Columns, 1X Column Buffer, and Deionized H_2O at room temperature. Store BM25.8 and XL1-Blue *E. coli* host strains, Control RNA, and SMART IV Oligonucleotide at –70°C. Store all other reagents at –20°C.

A. SMART cDNA Library Construction Kit Contents

First-Strand Synthesis

- 10 µl **SMART IV Oligonucleotide** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'
- 25 µl **CDS III/3' PCR Primer** (12 µM)
  5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$N$_{-1}$N-3'
  (N = A, G, C, or T; N$_{-1}$ = A, G, or C)
- 10 µl **SMARTScribe™ MMLV Reverse Transcriptase** (100 units/µl)
- 30 µl **5X First-Strand Buffer**
  - 250 mM Tris (pH 8.3)
  - 30 mM $MgCl_2$
  - 375 mM KCl
- 20 µl **DTT** (dithiothreitol; 20 mM)
- 5 µl **Control Poly A$^+$ RNA** (Mouse Liver; 1.0 µg/µl)

cDNA Amplification

- 20 µl **5' PCR Primer** (12 µM)
  5'-AAGCAGTGGTATCAACGCAGAGT-3'

Digestion of PCR Enzymes

- 20 µl **Proteinase K** (≥600 mAnsonU/ml))

SfiI Digestion

- 80 µl **SfiI Enzyme** (20 units/µl)
- 80 µl **10X SfiI Buffer**
- 10 µl **100X BSA**

cDNA Purification

- 10 **CHROMA SPIN-400 Columns**
- 30 ml **1X Fractionation Column Buffer**

Vector Ligation

- 30 µl **λTriplEx2 (SfiI A & B-digested arms)** (0.5 µg/µl)
- 20 µl **T4 DNA Ligase** (400 units/µl)
- 30 µl **10X DNA Ligation Buffer**
  - 500 mM Tris-HCl (pH 7.8)
  - 100 mM $MgCl_2$
  - 100 mM DTT
  - 0.5 mg/ml BSA
- 20 µl **ATP** (10 mM)
- 5 µl **Control Insert** (SfiI A & B) (50 ng/µl)

Host Cells

- 0.5 ml ***E. coli* BM25.8** (in 25% glycerol, genotype in Table 1)
- 0.5 ml ***E. coli* XL1-Blue** (in 25% glycerol, genotype in Table 1)
- 60 µl **5'-Sequencing Primer** (20 µM)
- 60 µl **3'-Sequencing Primer** (20 µM)

General Reagents

- 20 µl **dNTP mix** (dATP, dCTP, dGTP, dTTP, 10 mM each)
- 200 µl **Sodium Acetate** (3 M; pH 4.8)
- 20 µl **Sodium Hydroxide** (25 mM)
- 60 µl **Glycogen** (20 µg/µl)
- 5 µl **Deionized H_2O** (Milli-Q-filtered, not DEPC-treated)

B. Host Strain Information

Table 1. Bacterial Host Strain Genotypes

Strain	Genotype
XL1-Blue	endA1, gyrA96, hsdR17, lac$^-$, recA1, relA1, supE44, thi-1, [F' lacI ^{q}Z ΔM15, proAB, Tn10] **Note**: Tn10 confers resistance to tetracycline. **Reference:** Wood *et al.*, 1985
BM25.8	supE44, thi Δ(lac–proAB) [F' traD36, proAB$^+$, lacI^{q}Z ΔM15] λimm434 (kan^R)P1 (cam^R) hsdR (rk12$^-$ mk12$^-$) **Note**: BM25.8 is lysogenic for phages λ and P1 and is used for automatic subcloning. **Reference:** Palazzolo *et al.*, 1990

Table 2. Host Strain Applications & Media Additives

Host Strain	Stock Plate	Application(s)
XL1-Blue	LB/tet (15 µg/ml)	Library plating & screening Blue/white (β-galactosidase) screening Regulated expression of cloned genes
BM25.8	LB/kan (50 µg/ml)/ cam (34 µg/ml)	Cre-lox-mediated excision of pTriplEx2 from λTriplEx2 (see Appendix A and Figure 9)

Media Additives Recommended for Lambda Phage Transductions

For λ phage transductions, including plaque titering, library plating, and screening, use the following media additives for optimal adsorption of phage to bacteria:

- 10 mM $MgSO_4$ in LB agar and LB top agar
- 10 mM $MgSO_4$ and 0.2% maltose in LB broth when growing overnight bacterial cultures for λ phage transductions.
- Before using **XL1-Blue** overnight cultures in phage transductions, centrifuge the cells, pour off the supernatant, and resuspend the pellet in 10 mM $MgSO_4$ (in H_2O).
- Before using **BM25.8** overnight cultures in phage transductions, add $MgCl_2$ to a final concentration of 10 mM.
- If the bacterial strains are always maintained on stock plates containing the appropriate antibiotic, there is no need to add antibiotics to the LB broth when growing overnight cultures.

III. Additional Materials Required

The following reagents are required but not supplied.
Store all reagents and solutions at room temperature (20–22°C) unless specified otherwise.

For First-Strand cDNA Synthesis & SMART PCR ds cDNA Synthesis

- Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207)
- Sterile, 0.5 ml microcentrifuge tubes
- Poly A^+ or total RNA
- Mineral oil (We recommend Sigma Cat. No. M-3516.)
- DNA size markers (1 kb DNA Ladder, Takara Bio Cat. No. 3412B)
- 1.1% Agarose/EtBr gel (containing 0.1 µg/ml ethidium bromide)

For Proteinase K Digestion

- Sterile, 0.5 ml microcentrifuge tubes
- 95% ethanol
- 80% ethanol

For cDNA Size Fractionation

- 1.5 ml sterile microcentrifuge tubes
- Ring-stand with small clamp (for holding column)
- 1.1% agarose/EtBr gel (0.1 µg/ml ethidium bromide)
- 95% ethanol (–20°C)
- 1% xylene cyanol

For λ Phage Packaging

- λ phage packaging extract: Several are commercially available. Choose a packaging system that will give you at least 1 x 10^9 pfu/µg of DNA (e.g., MaxPlax Lambda Packaging Extracts, Epicentre Cat. No. MP5120)

For PCR Insert Screening [optional]

- λTriplEx LD-Insert Screening Amplimer Sets

For Routine Plating and Culture of *E. coli*

- Kanamycin stock solution (25 mg/ml in H_2O; 500X); Store at –20°C
- Tetracycline stock solution (15 mg/ml in H_2O; 1,000X); Store at –20°C
- Chloramphenicol stock solution (34 mg/ml in 100% ethanol; 1,000X). Store at –20°C.
- LB broth/LB agar
- LB agar plates
- LB/tet agar plates
- LB/kan/cam agar plates

For transduction and titering of λ phage in *E. coli*

When preparing media for phage transductions or plaque titering, use recipes containing 10 mM $MgSO_4$ for optimal adsorption of phage to bacteria. For the same reason, add 0.2% maltose to the LB broth when growing overnight bacterial cultures for transduction/titering.

- **$MgSO_4$ (1 M stock solution)**

  Dissolve 24.65 g of $MgSO_4 \cdot 7H_2O$ in 100 ml of deionized H_2O. Filter sterilize.

- **20% Maltose stock solution**

  Dissolve 20 g of maltose in 80 ml of deionized H_2O; bring volume up to 100 ml. Filter sterilize and store at 4°C.

- **LB/$MgSO_4$ agar plates**

  To one liter of LB broth, add:

  - 10 ml 1 M $MgSO_4$ (10 mM final concentration)
  - 15 g agar

  Autoclave. Pour plates and store at 4°C.

- **LB/$MgSO_4$ broth**

  To one liter of LB broth, add 10 ml 1 M $MgSO_4$ (10 mM final concentration). Autoclave.

- **LB/$MgSO_4$/maltose broth**

  Prepare 1 L LB/$MgSO_4$ broth as described above. After autoclaving, cool to 50°C before adding maltose to a final concentration of 0.2%. (10 ml of 20% maltose stock solution.)

- **LB/$MgSO_4$ soft top agar**

  To one liter of LB broth, add:

  - 10 ml 1 M $MgSO_4$ (10 mM final concentration)
  - 7.2 g agar

  Autoclave and store at 4°C.

- **10X Lambda dilution buffer**

	Final Conc.	To prepare 1 L of solution
NaCl	1.0 M	58.3 g
$MgSO_4 \cdot 7H_2O$	0.1 M	24.65 g
Tris-HCI (pH 7.5)	0.35 M	350.0 ml of 1 M

  Add H_2O to a final volume of 1 L. Autoclave and store at 4°C.

- **1X Lambda dilution buffer**

  Combine:

  - 100 ml 10X Lambda dilution buffer
  - 5 ml 2% Gelatin (0.01% final concentration)

  Add H_2O to a final volume of 1 L. Autoclave and store at 4°C.

NOTE: The 0.01% gelatin in the 1X lambda dilution buffer stabilizes the library titer for long-term storage. Gelatin addition is optional when diluting the phage for immediate titering.

For amplifying a λ library

- LB/$MgSO_4$ plates
- LB/$MgSO_4$ soft top agar
- 50 ml polypropylene, sterile screw-cap tubes
- Chloroform

Blue/white screening in *E. coli* (i.e., β-galactosidase)

- **IPTG** (100 mM in H_2O)
  Isopropyl β-D-thiogalactopyranoside. Filter sterilize. Store at 4°C.
- **X-Gal** (100 mM)
  Dissolve in dimethyformamide (DMF). Store at –20°C.

Long-term library storage

- 100% Dimethylsulfoxide (DMSO)
- 100% Glycerol

IV. General Considerations

A. Good Laboratory Practices

1. Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.
2. When resuspending pellets or mixing reactions, gently pipet the solution up and down or tap the bottom of the tube. Spin tube briefly to bring contents to the bottom of the tube. Do not vortex samples when resuspending pellets; vortexing may cause shearing of your cDNA.
3. Perform all reactions on ice, unless otherwise indicated.
4. Add enzymes to reaction mixtures last. Make sure that the enzyme is thoroughly blended into the reaction mixture by gently pipetting the mixture up and down.
5. Do not increase the size (volume) of any of the reactions. All components have been optimized for the volumes specified.
6. Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing this reagent. For more information, see Green & Sambrook (2012). Several EtBr disposable cartridges are also available, such as Clontech's BondEx Ethidium Bromide Detoxification Cartridge.
7. Phenol is a corrosive. Always wear gloves and protective clothing when handling solutions containing this reagent. If possible, handle solutions containing phenol and/or chloroform under a chemical fume hood.

B. Preparation and Handling of Total and Poly A$^+$ RNA

1. **Recommended Precautions**

   To avoid contamination and degradation of RNA (and to minimize the presence of RNases:

   - Wear gloves.
   - Use freshly deionized (e.g., Milli-Q-grade) H_2O, untreated with DEPC (diethyl pyrocarbonate).
   - Rinse all glassware with 0.5 N NaOH, followed by deionized H_2O. Then bake the glassware at 160–180°C for 4–9 hr.
   - Use only single-use plastic pipettes and pipette tips with RNA.
   - Avoid using autoclaved H_2O because recycled steam in some autoclaves can introduce contaminants that may interfere with PCR.

2. **RNA isolation**

   Many procedures are available for the isolation of total RNA and poly A$^+$ RNA (Chomczynski & Sacchi, 1987; Farrell, 1993; Green & Sambrook., 2012). Clontech offers several kits for the isolation of total RNA and subsequent isolation of poly A$^+$ RNA.

3. **RNA analysis**

   Methods for Assessing Total RNA Integrity

   a. **Formaldehyde agarose gel visualization with Ethidium Bromide (EtBr):**
   The integrity of total RNA can be visually assessed by the ratio of 28S:18S RNA on a denaturing formaldehyde agarose gel by staining with EtBr. The theoretical 28S:18S ratio for eukaryotic RNA is approximately 2:1. For mammalian total RNA, you should observe two bright bands at approximately 4.5 and 1.9 kb; these bands represent 28S and 18S ribosomal RNA. The ratio of intensities of these bands should be 1.5–2.5:1. For more information, see Green & Sambrook (2012).

   b. **Formaldehyde agarose gel visualization with SYBR® Green or SYBR Gold:**
   One drawback of visualizing RNA with Ethidium Bromide is the amount of sample required. Alternative dyes such as SYBR Green II or SYBR Gold (Invitrogen, CA) allow you to detect as little as 1 or 2 ng of RNA (using SYBR Gold and SYBR Green II, respectively). These dyes are especially useful if you have a limited amount of RNA.

   c. **Detection with the Agilent 2100 BioAnalyzer (Agilent Technologies, CA):**
   This microfluidics-based technology, which provides an alternative to traditional gel-based analysis, requires only 10 ng of RNA per analysis. In addition to assessing RNA quality, this automated system provides a good estimate of RNA concentration.

   Methods for Assessing mRNA Integrity

   All of the above methods can be used to assess mRNA quality. However, since mRNA does not contain strong ribosomal bands, this assessment will be somewhat subjective. Typically, mRNA appears as a smear between 0.5 kb –6 kb, with an area of higher intensity around 1.5 and 2 kb. This size distribution may be tissue or species-specific. An average mRNA size lower than 1.5 kb could indicate degradation.

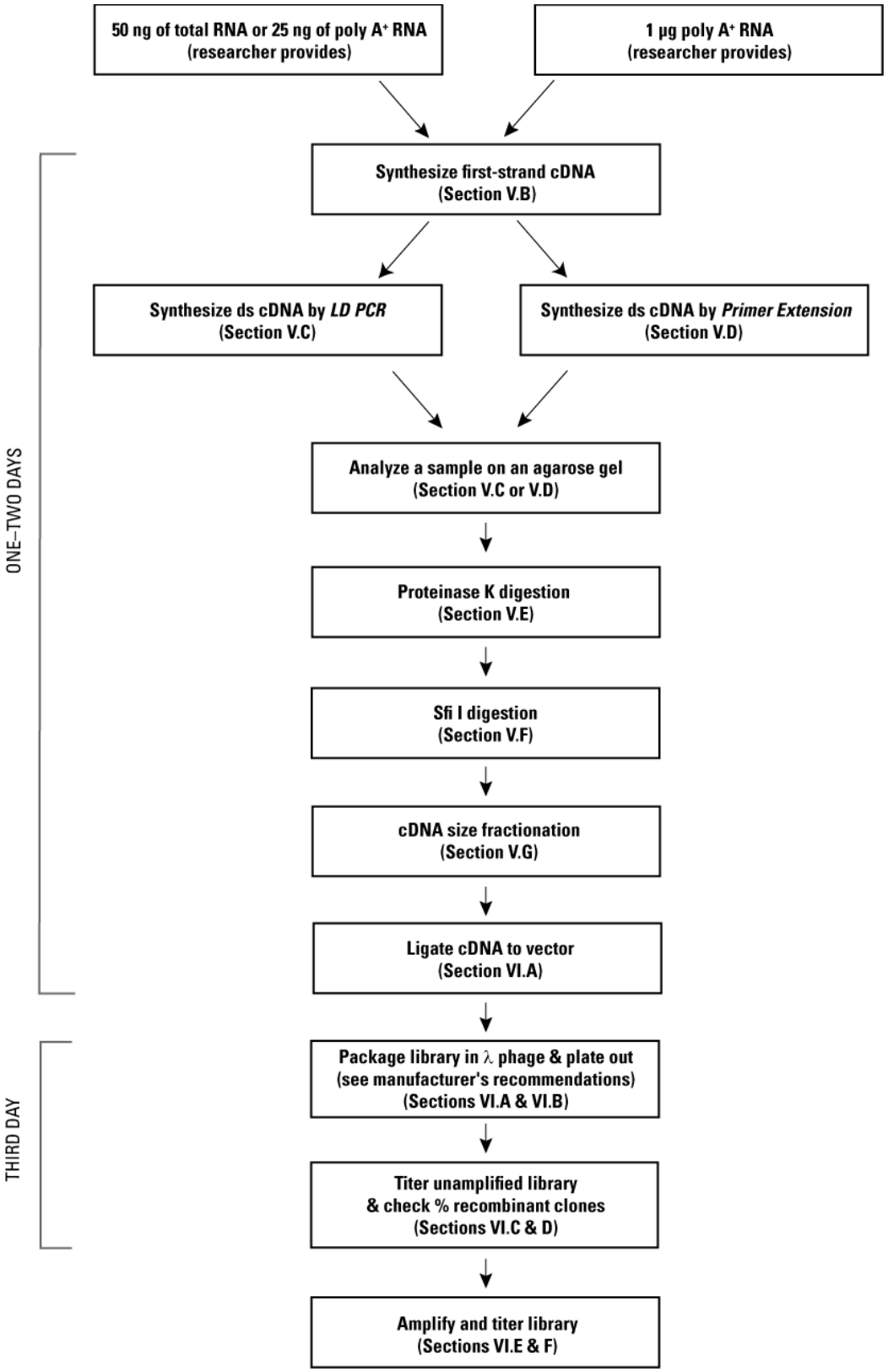

Figure 4. Guide to using the SMART cDNA Library Construction Kit protocols. Be sure to choose the appropriate protocol for your application. Refer to Figure 7 and Section VII for troubleshooting tips.

V. SMART cDNA Synthesis by LD PCR or Primer Extension

This section describes how to synthesize double-stranded SMART cDNA for library construction (see Figure 4) using either LD (long distance) PCR or primer extension:

- **General guidelines** are provided for both methods (Section V.A).
- **First strand cDNA synthesis** is presented in a single protocol for both methods (Section V.B), which differ only as described in Steps 1 & 9.
- **Second strand cDNA synthesis** (to yield a double-stranded cDNA) is presented in separate protocols for **LD PCR** (Section V.C) and **primer extension** (Section V.D).

  NOTE: The differences between the first strand and ds cDNA synthesis protocols used for LD-PCR and primer extension samples are summarized in Table 3 below.

- **Proteinase K digestion** (Section V.E), **SfiI digestion** (Section V.F), and **cDNA size fractionation by CHROMA SPIN-400** (Section V.G) protocols are identical for both methods, as are the SMART cDNA **Library Protocols** in Sections VI.A–F.

Table 3. Differences Between the LD-PCR and Primer Extension Protocols

Protocol Stage	LD-PCR Method	Primer Extension Method
First strand cDNA synthesis	**Section V.B:** • **Step 1.** Starting material: – Total RNA (50 ng–2 µg) or poly A$^+$ RNA (25 ng–1 µg) • **Step 9.** After reaction is complete: – Proceed to ds cDNA synthesis	**Section V.B:** • **Step 1.** Starting material: – Poly A$^+$ RNA (0.5 µg–2 µg) • **Step 9.** After reaction is complete: – Treat with sodium hydroxide – Proceed to ds cDNA synthesis
ds cDNA synthesis	**Section V.C:** • **Step 2.** Use 2 µl of the first strand cDNA synthesized in Section V.B • **Step 5.** LD PCR reaction: – Use a thermal cycling program specific for LD-PCR – Amplify using 18–26 cycles, depending on the amount of starting material	**Section V.D:** • **Step 2.** Use 11 µl of the first strand cDNA synthesized in Section V.B • **Step 5.** Primer extension reaction: – Use a thermal cycling program specific for primer extension – Amplify using 3 cycles
Agarose/EtBr gel analysis of ds cDNA)	**Section V.C:** • **Step 6.** Expect a 0.1–4 kb smear, with some distinct bands from abundant mRNAs	**Section V.D:** • **Step 6.** Expect a 0.1–9 kb smear, with some distinct bands from abundant mRNAs

A. General Considerations for cDNA Synthesis

1. Starting Material Amounts

- **LD PCR protocol (minimum amount):** 50 ng of total RNA or 25 ng of poly A^+ RNA

  In general, the more RNA you start with, the fewer PCR cycles will be required for the second-strand synthesis (see Table 4 in Section V.C). Fewer thermal cycles are less likely to generate nonspecific PCR products, and thus are best for optimal cDNA and library quality. If your RNA sample is not limiting, use the higher starting amounts of RNA shown in the table (up to 2 µg).

- **Primer extension protocol (optimal amount):** 1 µg of poly A^+ RNA (0.5 µg–2.0 µg)

  The optimal amount of starting material for cDNA synthesis is about 1 µg of poly A^+ RNA (0.5 µg–2.0 µg). Reduced cloning efficiency will occur if you use less than 0.5 µg or greater than 2.0 µg of poly A^+ RNA.

2. Positive Control

We strongly recommend that you perform a positive control cDNA synthesis with the mouse liver poly A^+ RNA provided, in parallel with your experimental cDNA synthesis, using either the LD PCR or the primer extension protocol. To identify problems at different stages of the protocol, perform the control:

- **At least once to verify that all the components are working properly.** This is especially important because it may not be possible to visualize your first-strand reaction product on a gel.
- **Through the the ds cDNA synthesis step** to allow you to evaluate the yield and size distribution of the ds cDNA synthesized from your RNA sample.
- **Through the library construction steps** to help you troubleshoot any additional problems.

3. Stopping Places and Storage Temperatures During the Protocol

- If necessary, the procedure (using either method) **can be stopped** after first-strand synthesis by placing the reaction mixture at –20°C, and also at any ethanol precipitation step after the precipitated DNA has been centrifuged or when the supernatant has been removed. The dry or resuspended DNA pellet can be stored at –20°C for up to 3 months.
- For the first-strand synthesis and the PCR reactions, all components and reaction vessels should be prechilled on ice.

B. Protocol: First Strand cDNA Synthesis

1. Combine the following components for your experimental RNA sample and a positive control in separate sterile 0.5 ml microcentrifuge tubes as follows, depending on which dsDNA synthesis protocol you will use (LD-PCR or primer extension):

   - **Samples for LD-PCR protocol:**

Component	Reagent Volume (µl per sample)	
	Experimental RNA Sample	Positive Control RNA Sample
Poly A$^+$ RNA (0.025–0.5 µg) or Total RNA (0.05–1.0 µg)	1–3 µl	—
Control Poly A$^+$ RNA (1.0 µg)	—	1 µl
SMART IV Oligonucleotide	1 µl	1
CDS III/3' PCR Primer	1 µl	1
Deionized H_2O	0–2 µl*	2 µl
Total Volume	5 µl	5 µl

*Depending on how much H_2O is needed to bring the total volume to 5 µl.

   - **Samples for primer extension protocol**:

Component	Reagent Volume (µl per sample)	
	Experimental RNA Sample	Positive Control RNA Sample
Poly A$^+$ RNA (1.0 µg)	1 µl	—
Control Poly A$^+$ RNA (1.0 µg)	—	1 µl
SMART IV Oligonucleotide	1	1
CDS III/3' PCR Primer	1	1
Deionized H_2O	2 µl	2 µl
Total Volume	5 µl	5 µl

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 72°C for 2 min.
4. Cool the tube on ice for 2 min.
5. Spin the tube briefly to collect the contents at the bottom.
6. Add the following reagents to each reaction tube (which already contains 5 µl from Step 1):

2.0 µl	5X First-Strand Buffer
1.0 µl	DTT (20 mM)
1.0 µl	dNTP Mix (10 mM)
1.0 µl	SMARTScribe MMLV Reverse Transcriptase
10.0 µl	Total Volume

7. Mix the contents of the tube by gently pipetting and briefly spinning the tube.
8. Incubate the tube at 42°C for 1 hr in an air incubator or a hot lid thermal cycler.

NOTE: If you use a water bath or thermal cycler for this incubation, cover the reaction mixture with one drop of mineral oil before you close the tube. This will prevent loss of volume due to evaporation.

9. Place the tube on ice to terminate first-strand synthesis and proceed as follows, depending on which dsDNA synthesis protocol you will use (LD-PCR or primer extension):

    - **Samples for LD-PCR protocol:** Proceed to Step 10.
    - **Samples for primer extension protocol**:
        a. Add 1 µl of 25 mM sodium hydroxide (included in kit).
        b. Incubate the tube at 68° for 30 min.
        c. Place tube on ice and proceed to Step 10.

10. If you plan to proceed directly to the ds cDNA synthesis procedure (Section V.C for the LD PCR protocol or Section V.D for the primer extension protocol), first take a 2 µl aliquot from the first-strand synthesis and place this aliquot in a clean, prechilled, 0.5 ml tube on ice. If you used mineral oil in your first-strand reaction tube, be careful to take the 2 µl sample from the bottom of the tube to avoid the oil.

11. Any first-strand reaction mixture that is not used right away should be placed at –20°C. First-strand cDNA can be stored at –20°C for up to three months.

C. Protocol: ds cDNA Synthesis by LD PCR

This protocol is designed for standard library construction of SMART cDNA libraries using LD-PCR.

- See Section V.A for general information on starting material amounts, controls, safe stopping places and storage temperatures during the protocol.
- See Table 4 below for the optimal number of thermal cycles for different amounts of starting material. Use the least number of cycles shown for your amount of RNA when setting up the cycling program in Step 5.

Table 4. Relationship Between Amount of RNA Starting Material and Optimal Number of Thermal Cycles

Total RNA (µg)	Poly A$^+$ RNA (µg)	Number of Cycles
1.0–2.0	0.5–1.0	18–20
0.5–1.0	0.25–0.5	20–22
0.25–0.5	0.125–0.25	22–24
0.05–0.25	0.025–0.125	24–26

NOTES:

- Fewer cycles generally mean fewer nonspecific PCR products.
- Undercycling can be easily rectified by placing the PCR reaction back in the thermal cycler for a few more cycles (see the Troubleshooting Guide, Section VII.C), while overcycling requires repeating the PCR *de novo* using a fresh aliquot of the first-strand reaction product.
- The optimal cycling parameters in Table 4 were determined using the Control Mouse Liver Poly A$^+$ RNA; these parameters may vary with different templates and thermal cyclers.

LD PCR Protocol

1. Preheat the thermal cycler to 95°C.
2. Combine the following components:

2 µl	First-Strand cDNA (from Section V.B, Step 10)
80 µl	Deionized H_2O
10 µl	10X Advantage 2 PCR Buffer
2 µl	50X dNTP Mix
2 µl	5' PCR Primer
2 µl	CDS III/3' PCR Primer
2 µl	50X Advantage 2 Polymerase Mix
100 µl	Total volume

3. Mix contents by gently flicking the tube. Centrifuge briefly to collect the contents at the bottom of the tube.
4. Overlay the reaction mixture with 2 drops of mineral oil if necessary. Cap the tube and place it in a preheated (95°C) thermal cycler.
5. Perform LD-PCR using one of the following programs:

Non-hot-lid thermal cycler:

- 95°C for 1 min
- X cycles*:
  95°C for 15 sec
  68°C for 6 min

Hot-lid thermal cycler:

- 95°C for 20 sec
- X cycles*:
  95°C for 5 sec
  68°C for 6 min

*Refer to Table 4 to determine the optimal number of cycles to use.

6. **Data Analysis**

   - When the LD-PCR reaction is complete, analyze a 5 µl sample on a 1.1% agarose/EtBr gel, alongside a 1 kb ladder DNA size marker (0.1 µg).
   - The ds cDNA should appear as a 0.1–4 kb smear on the gel, with some distinct bands corresponding to the abundant mRNAs for that tissue or cell source. (cDNA prepared from some tissues may not have distinct bands, especially if the mRNA is highly complex.)
   - If your product does not appear as expected, refer to the Troubleshooting Guide (Section VII.A–E). Typical results obtained with Mouse Liver Poly A^+ RNA are shown in Figure 5.

7. Proceed to Section V.E (Proteinase K Digestion), or store ds cDNA at –20°C until use.

Typical Results for LD PCR

Figure 5 shows a typical gel profile of ds cDNA synthesized using the control mouse liver poly A^+ RNA and the SMART protocols via LD PCR, with the following characteristics:

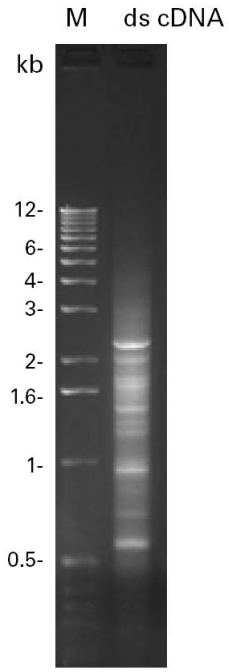

Figure 5. Typical results: ds cDNA synthesized using the SMART control reagents and LD PCR protocol. 1 µl (1.0 µg) of the Control Mouse Liver poly A^+ RNA provided in the kit was used as starting material in a first-strand cDNA synthesis. 2 µl of the ss cDNA served as template for LD-PCR-based, second-strand synthesis using 15 thermal cycles. A 5-µl sample of the ds cDNA product was electrophoresed on a 1.1% agarose/EtBr gel. Lane M: 1 kb ladder DNA size marker (0.1 µg loaded).

1. A moderately strong cDNA smear from 0.1 to 4 kb

Compare the intensity of the banding pattern of your PCR product to the 1 kb ladder DNA size marker (0.1 µg run on the same gel). For cDNA made from all mammalian RNA sources, the overall signal intensity (relative to the marker DNA) should be roughly similar to that shown for the control experiment in Figure 5.

- **If the intensity of the cDNA smear is much stronger than that shown for the control** (relative to 0.1 µg of size markers), especially if no bands are distinguishable, this may indicate that too many thermal cycles were used, i.e., PCR overcycling has occurred (see Section VII.D).
- **If the smear is much fainter and the size distribution is less than 4 kb,** then too few thermal cycles (i.e., PCR undercycling) may be the problem (see Section VII.C).

2. Several distinct bands corresponding to abundant transcripts

The pattern of distinct bands shown in Figure 5 is characteristic of the PCR product made using the control mouse liver poly A^+ RNA.

- **A very strong smear of cDNA in the control reaction without the characteristic distinct bands** may be indicative of PCR overcycling (see Section VII.D).
- **If the characteristic bands are present but weak**, this may be indicative of PCR undercycling (see Section VII.C).

NOTE: The number and position of the bands you obtain with your experimental RNA may differ from those shown for the control reaction. Furthermore, cDNA prepared from some mammalian tissue sources (e.g., human brain, spleen, and thymus) may not display distinct bands due to the very high complexity of the poly A^+ RNA.

3. Some low-molecular-weight material

Most raw PCR reaction products will contain some small cDNA fragments (<0.4 kb) and some very small (i.e., <0.1 kb) non-cDNA contaminants, as seen in Figure 5. The non-cDNA contaminants include unincorporated primers, and SMART oligonucleotides, as well as primer dimers. If most of the material is present in the lower part of the gel (i.e., <0.1 kb), this may indicate that PCR overcycling has occurred (see Section VII.D).

D. Protocol: ds cDNA Synthesis by Primer Extension

This protocol is designed for standard library construction of SMART cDNA libraries using primer extension.

NOTE: See Section V.A for general information on starting material amounts, controls, safe stopping places and storage temperatures during the protocol.

Primer Extension Protocol

1. Preheat the thermal cycler to 95°C.
2. Combine the following components:

11 µl	First-Strand cDNA (from Section V.B, Step 10)
71 µl	Deionized H_2O
10 µl	10X Advantage 2 PCR Buffer
2 µl	50X dNTP Mix
2 µl	5' PCR Primer
2 µl	CDS III/3' PCR Primer
2 µl	50X Advantage 2 Polymerase Mix
100 µl	Total volume

3. Mix contents by gently flicking the tube. Centrifuge briefly to collect the contents at the bottom of the tube.
4. Overlay the reaction mixture with 2 drops of mineral oil if necessary. Cap the tube and place it in a preheated (95°C) thermal cycler.
5. Perform primer extension using one of the following programs:

Non-hot-lid thermal cycler:

- 72°C for 10 min
- 95°C for 1 min
- 3 cycles:
  95°C for 15 sec
  68°C for 8 min

Hot-lid thermal cycler:

- 72°C for 10 min
- 95°C for 20 sec
- 3 cycles:
  95°C for 5 sec
  68°C for 8 min

6. **Data Analysis**

   - When the primer extension reaction is complete, analyze a 5 µl sample on a 1.1% agarose/EtBr gel, alongside a 1 kb ladder DNA size marker (0.1 µg).
   - The ds cDNA should appear as a 0.1–9 kb smear on the gel, with some distinct bands corresponding to the abundant mRNAs for that tissue or cell source. (cDNA prepared from some tissues may not have distinct bands, especially if the mRNA is highly complex.)
   - If your product does not appear as expected, refer to the Troubleshooting Guide (Section VII.A–B, F). Typical results obtained with Mouse Liver Poly A^+ RNA are shown in Figure 6.

7. Proceed to Section V.E (Proteinase K Digestion), or store ds cDNA at –20°C until use.

Typical Results for Primer Extension

Figure 6 shows a typical gel profile of ds cDNA synthesized using the control mouse liver poly A^+ RNA and the SMART protocols via primer extension, with the following characteristics:

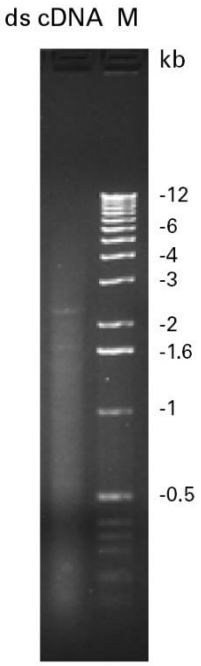

Figure 6. Typical results: ds cDNA synthesized using the SMART control reagents and primer extension protocol. 1 µl (1.0 µg) of the Control Mouse Liver poly A^+ RNA provided in the kit was used as starting material in a first-strand cDNA synthesis. 10 µl of ss cDNA served as a template for primer extension-based, second-strand synthesis using 3 cycles. A 5 µl sample of the ds cDNA product was electrophoresed on a 1.1% agarose/EtBr gel. Lane M: 1 kb ladder DNA size marker (0.1 µg loaded).

1. **A moderately strong cDNA smear from 0.1 to 9 kb (or more)**
   Compare the intensity of the banding pattern of your ds cDNA product to the 1 kb DNA ladder size marker (0.1 µg run on the same gel). Primer extension (Figure 6) generates a longer smear than the ds cDNA synthesized using LD PCR (Figure 5). If your cDNA is less than 3 kb, it is possible that your RNA is degraded (see Section VII.B).

2. **Weak bands correspond to abundant transcripts**
   Primer extension requires more poly A^+ RNA for first-strand synthesis than required by LD PCR; therefore, the bands in Figure 6 appear weaker than the bands in Figure 5. Weaker bands correspond to a higher complexity of the poly A^+ starting material. The number and position of the bands you obtain with your experimental RNA may differ from those shown for the control reaction.

3. **Some low-molecular-weight material**
   The ds cDNA generated using primer extension will contain some degraded RNA fragments (<0.1 kb); a strong signal can be seen at position of about 100 bp in Figure 6. These fragments are removed by the size fractionation columns (see Section V.G & Section VII.G).

E. Protocol: Proteinase K Digestion

1. In a sterile 0.5 ml tube, pipet 50 µl of amplified ds cDNA (2–3 µg), making sure you pipet below the top oil layer of the PCR tube and add 2 µl of Proteinase K (20 µg/µl). Store the remaining ds cDNA at –20°C (up to 3 months).

   NOTE: Proteinase K treatment is necessary to inactivate the DNA polymerase activity. This system was optimized for 2–3 µg (~50 µl/vol) of ds cDNA for subsequent cloning and library construction procedures. Too much ds cDNA (>3–4 µg) will yield a low-titer library. Refer to the Troubleshooting Guide (Section VII.H) for more information.

2. Mix contents and spin the tube briefly.
3. Incubate at 45°C for 20 min. Spin the tube briefly.
4. Add 50 µl of Deionized H_2O to the tube.
5. Add 100 µl of phenol:chloroform:isoamyl alcohol and mix by continuous gentle inversion for 1–2 min.
6. Centrifuge at 14,000 rpm for 5 min to separate the phases.
7. Move the top (aqueous) layer to a clean 0.5 ml tube. Discard the interface and lower layers.
8. Add 100 µl of chloroform:isoamyl alcohol to the aqueous layer. Mix by continuous gentle inversion for 1–2 min.
9. Centrifuge at 14,000 rpm for 5 min to separate the phases.
10. Move the top (aqueous) layer to a clean 0.5 ml tube. Discard the interface and lower layers.
11. Add 10 µl of 3 M Sodium Acetate, 1.3 µl of Glycogen (20 µg/µl) and 260 µl of room temperature 95% ethanol. Immediately centrifuge at 14,000 rpm for 20 min at room temperature.

    NOTE: Do not chill the tube at –20°C or on ice before centrifuging. Chilling the sample will result in coprecipitation of impurities.

12. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
13. Wash pellet with 100 µl of 80% ethanol.
14. Air dry the pellet (~10 min) to evaporate off residual ethanol.
15. Add 79 µl of deionized H_2O to resuspend the pellet.
16. Proceed to Section V.F (SfiI Digestion).

F. Protocol: SfiI digestion

1. Combine the following components in a fresh 0.5 ml tube:

79 µl	cDNA (from Section V.E, Step 15)
10 µl	10X Sfi Buffer
10 µl	SfiI Enzyme
1 µl	100X BSA
100 µl	Total volume

2. Mix well. Incubate the tube at 50°C for 2 hr.
3. Add 2 µl of 1% xylene cyanol dye to the tube above. Mix well.
4. Proceed to Section V.G (cDNA Size Fractionation by CHROMA SPIN-400).

G. Protocol: cDNA Size Fractionation by CHROMA SPIN-400

1. Label sixteen 1.5-ml tubes and arrange them in a rack in order.
2. Prepare the CHROMA SPIN-400 column for drip procedure:
    a. Invert the column several times to completely resuspend the gel matrix.
    b. Remove air bubbles from the column. Use a 1,000-µl pipettor to resuspend the matrix gently; avoid generating air bubbles. Then remove the bottom cap and let the column drip naturally. (If the column does not drain after 3 min, recap the top cap. This pressure should cause the column to drain).
    c. Attach the column to a ring stand.
    d. Let the storage buffer drain through the column by gravity flow until you can see the surface of the gel beads in the column matrix. The top of the column matrix should be at the 1.0 ml mark on the wall of the column. If your column contains significantly less matrix, adjust the volume of the matrix to the 1.0 ml mark using matrix from another column (an extra column is provided for this purpose).
    e. The flow rate should be approximately 1 drop/40–60 sec. The volume of 1 drop should be approximately 40 µl. If the flow rate is too slow (i.e., more than 1 drop/100 sec) and the volume of one drop is too small (i.e., less than 25 µl), you should resuspend the matrix completely and repeat the drip procedure until it reaches the above parameters.
3. When the storage buffer stops dripping out, carefully and gently (along the column inner wall) add 700 µl of column buffer to the top of the column and allow it to drain out.
4. When this buffer stops dripping (~15–20 min), carefully and evenly apply ~100 µl mixture of SfiI-digested cDNA and xylene cyanol dye (from Section V.F, Step 3 above) to the top-center surface of the matrix. An unsmooth matrix surface does not harm the following fractionation process.
5. Before proceeding to the next step, allow the sample to be fully absorbed into the surface of the matrix (i.e., there should be no liquid remaining above the surface).
6. With 100 µl of column buffer, wash the tube that contained the cDNA and gently apply this material to the surface of the matrix.
7. Allow the buffer to drain out of the column until there is no liquid left above the resin. When the dripping has ceased, proceed to the next step. At this point, the dye layer should be several mm into the column.
8. Place the rack containing the collection tubes (Section V.G, Step 1) under the column, so that the first tube is directly under the column outlet.
9. Add 600 µl of column buffer and immediately begin collecting single-drop fractions in Tubes #1–16 (approximately 35 µl per tube). Cap each tube after each fraction is collected. Recap the column after Fraction #16 has been collected.

10. Check the profile of the fractions before proceeding with the experiment:
    a. On a 1.1% agarose/EtBr gel, electrophorese 3 µl of each fraction (separately) in adjacent wells, alongside a 1 kb DNA ladder size marker (0.1 µg). Run the gel at 150 V for 10 min (Running the gel longer will make it difficult to see the cDNA bands).
    b. Determine the peak fractions by visualizing the intensity of the bands under UV.
    c. Collect the first three fractions containing cDNA (in most cases, the fourth fraction containing cDNA is usable. Make sure the fourth fraction matches your desired size distribution).
    d. Pool the above fractions in a clean 1.5 ml tube.
11. Add the following reagents to the tube with 3–4 pooled fractions containing the cDNA (105–140 µl, respectively):

1/10 vol.	Sodium Acetate (3 M; pH 4.8)
1.3 µl	Glycogen (20 mg/ml)
2.5 vol.	95% ethanol (–20°C)

12. Mix by gently rocking the tube back and forth.
13. Place the tube in –20°C or a dry-ice/ethanol bath for 1 hr. (**Optional:** you may incubate at –20°C overnight, which may result in better recovery).
14. Centrifuge the tube at 14,000 rpm for 20 min at room temperature.
15. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
16. Briefly centrifuge the tube to bring all remaining liquid to the bottom.
17. Carefully remove all liquid and allow the pellet to air dry for ~10 min.
18. Resuspend the pellet in 7 µl of Deionized H_2O and mix gently. The SfiI-digested cDNA is now ready to be ligated to the SfiI-digested, dephosphorylated λTriplEx2 Vector provided. **Proceed to Section VI, or store cDNA at –20°C until the ligation step.**

VI. SMART cDNA Library Protocols

A. Protocol: Ligation of cDNA to λTriplEx2 Vector

The ratio of cDNA to vector in the ligation reaction is a critical factor in determining transformation efficiency, and ultimately the number of independent clones in the library. The optimal ratio of cDNA to vector in ligation reactions must be determined empirically for each vector/cDNA combination. To ensure that you obtain the best possible library from your cDNA, set up three parallel ligations using three different ratios of cDNA to vector, as shown in Table 5.

IMPORTANT: Use a λ phage packaging system that will give you at least 1 x 10^9 pfu/µg of DNA. Follow the supplier's recommended protocol and perform a parallel packaging reaction with the control insert provided in the packaging kit.

The following protocol is optimized for ligation of SMART cDNA to the λTriplEx2 DNA provided:

1. Set up a test ligation to determine the efficiency of ligating the vector to the Control Insert.
    a. Use 1 µl of vector, 1 µl of Control Insert, 1.5 µl of Deionized H_2O, and other reagents listed in Table 5. Incubate the test ligation at 16°C overnight.
    b. Perform a λ phage packaging reaction and titer the resulting phage (Section VI.C). You should obtain ≥1 x 10^7 pfu/µg of input vector.
2. Label three 0.5 ml tubes and add the indicated reagents (Table 5). Mix the reagents gently; avoid producing air bubbles. Spin tubes briefly to bring contents to the bottom of the tube.

Table 5. Ligations Using Three Different Ratios of cDNA to Phage Vector.

Component	1st ligation (µl)	2nd ligation (µl)	3rd ligation (µl)
cDNA*	0.5	1.0	1.5
Vector (500 ng/µl)	1.0	1.0	1.0
10X Ligation Buffer	0.5	0.5	0.5
ATP (10 mM)	0.5	0.5	0.5
T4 DNA Ligase	0.5	0.5	0.5
Deionized H_2O*	2.0	1.5	1.0
Total volume (µl)	5.0	5.0	5.0

*To set up a test ligation, use the volumes indicated in Section VI.A, Step 1, above.

3. Incubate tubes at 16°C overnight.
4. Perform a separate, λ phage packaging reaction for each of the ligations.
5. Titer each of the resulting libraries (Section VI.C). From the three ligations combined, you should obtain 1–2 x 10^6 independent clones. The unamplified libraries can be stored at 4°C for 2 weeks.
6. **[Optional]** If you obtained <1–2 x 10^6 clones, you may wish to perform another ligation with the remaining cDNA. Repeat the ligation using the cDNA: vector ratio (of the initial three ligations) that gave the best results. Scale up all reagent volumes according to the amount of cDNA used. Then package and titer this scaled-up ligation. If the titer is still low, see Section VII.H (troubleshooting tips).
7. To increase the stability of your library, first combine the packaging reactions from Section VI.A, Step 5 (you should have >1 x 10^6 independent clones), then amplify the library as described in Section VI.E. The amplified library can be stored at 4°C for 6–7 months or at –70°C (in 7% DMSO) for at least one year.

B. Protocol: Bacterial Culture Plating

E. coli XL1-Blue and BM25.8 are provided as a stock in LB medium with 25% glycerol and can be stored indefinitely at –70°C.

1. To recover the frozen cells, streak a small portion (~5 µl) of the frozen stock onto an LB agar plate containing the appropriate antibiotic. This is the primary streak plate.
   - Use LB/tet for XL1-Blue stock plates.
   - Use LB/kan/cam for BM25.8 stock plates.

   NOTE: When streaking bacterial cultures for use as stock plates, use LB agar medium without $MgSO_4$.
2. Incubate at 37°C overnight. Wrap plate in Parafilm and store at 4°C for up to 2 weeks.
3. To prepare a working stock plate, pick a single isolated colony from the primary streak plate and streak it onto another LB/$MgSO_4$ agar plate (with antibiotics).
4. Incubate at 37°C overnight. Wrap plate in Parafilm and store at 4°C for up to 2 weeks. This plate is used as a source of fresh colonies for inoculating liquid cultures and for preparing the next fresh working stock plate.
5. At 2-week intervals, prepare a fresh working stock plate from the previous working stock plate so you will always have a source of fresh colonies.
6. If you suspect contamination on your current working stock plate, prepare a new primary streak plate from the frozen culture.

C. Protocol: Titering the Unamplified Library

Determining the titer (e.g., pfu/µl) of the unamplified library will give you an estimate of the number of independent phage and independent clones in the library. A library having at least 1 x 10^6 independent clones, in most cases, is representative of the mRNA complexity. The following plating/titering protocol can be used to determine:

1) The efficiency of the ligation of vector to positive control insert.
2) The background titer of the vector alone.

NOTES:

- When plating bacteria + phage mixtures using melted top agar, the melted top agar should be at 45°C; higher temperatures will kill the bacteria.
- Before plating with top agar, the agar plates should be prewarmed to 37°C and the agar surface free of excess moisture droplets. To dry the plates, remove the lids and shake off excess droplets from the inside of the lids. Just prior to use, place the agar plates—inverted and partially uncovered—in a 37°C incubator to warm. (Freshly prepared plates at room temperature will be warmed to 37°C in 10–15 min; plates that have been stored at 4°C will require about 1 hr to warm up. Do not over dry the plates.)
- When preparing media for phage transductions or plaque titering, use recipes containing 10 mM MgSO4 for optimal adsorption of phage to bacteria. For the same reason, add 0.2% maltose to the LB broth when growing overnight bacterial cultures for transduction/titering.

1. Pick a single, isolated colony from the working stock plate (Section VI.B, Step 5) and use it to inoculate 15 ml of LB/$MgSO_4$/maltose broth in a 50 ml test tube or Erlenmeyer flask.
2. Incubate at 37°C overnight—while shaking (at 140 rpm) until the OD_{600} of the culture reaches 2.0. Centrifuge the cells at 5,000 rpm for 5 min. Pour off the supernatant, and resuspend the pellet in 7.5 ml of 10 mM $MgSO_4$.
3. Plan the number of 90 mm LB/$MgSO_4$ plates you will need. Warm and dry them (see note above).
4. Make appropriate dilutions of each of the packaging extracts (from Section VI.A., Step 4) in 1X lambda dilution buffer. As a general guideline, an appropriate dilution for an unamplified λ lysate is 1:5 to 1:20. Perform the following steps for each dilution of each extract you wish to plate.
5. Add 1 µl of the diluted phage to 200 µl of the XL1-Blue overnight culture, and allow the phage to adsorb at 37°C for 10–15 min.
6. Add 2 ml of melted LB/$MgSO_4$ top agar. Mix by quickly inverting and immediately pour onto 90 mm LB/$MgSO_4$ plates prewarmed to 37°C. Swirl the plates quickly after pouring to allow even distribution of the top agar.
7. Cool the plates at room temperature for 10 min to allow the top agar to harden. Invert the plates and incubate them at 37°C for 6–18 hr. Periodically check the plates to ensure that plaques are forming.
8. Count the plaques and calculate the titer of the phage (pfu/ml):

$$\text{pfu/ml} = \frac{\text{number of plaques} \times \text{dilution factor} \times 10^3\ \mu\text{l/ml}}{\mu\text{l of diluted phage plated}}$$

9. If you are titering the three test ligations (Section VI.A), compare the titers to determine the optimal ratio of vector arms to cDNA insert. If you obtained <1–2 x 10^6 plaques (clones) altogether, you may wish to repeat the ligation, using the optimal vector to insert ratio. (Sections VI.A, Step 6 & VII.H.)

D. Protocol: Determining the Percentage of Recombinant Clones

In λTriplEx2, as in all other λ expression vectors, the cloning site is embedded in the coding sequence for the α-polypeptide of β-galactosidase (lacZ '). This makes it possible to use lacZ α-complementation (Green & Sambrook, 2012) to easily identify insert-containing phage by transducing an appropriate host strain (such as *E. coli* XL1-Blue) and screening for blue plaques on medium containing IPTG and X-gal. Do not attempt blue/white screening with strain BM25.8.

- **Performing blue/white screening in *E. coli* XL1-Blue**
  Follow the procedure for titering an unamplified library on LB/$MgSO_4$ plates (Section VI.C), except add IPTG and X-gal to the melted top agar before plating the phage + bacteria mixtures. For every 2 ml of melted top agar, use 50 µl each of the IPTG and X-gal stock solutions (Section III). Aim for 500–1,000 plaques/ 90 mm plate. Incubate plates at 37°C for 6–18 hr, or until plaques and blue color develop.

- **Estimating recombination efficiency**
  The ratio of white (recombinant) to blue (nonrecombinant) plaques will give you a quick estimate of recombination efficiency. A successful ligation using cDNA synthesized from the Control Poly A^+ RNA provided will result in at least 80% recombinants. If your recombination efficiency for the control is lower than this, see Section VII.I for troubleshooting tips.

E. Protocol: Library Amplification

The number of plates required depends on how many independent clones are in the library to be amplified. A general guideline is to aim for 6–7 x 10^4 clones (or plaques) per 150 mm plate when working with λTriplEx2; thus, a library of 1 x 10^6 clones will require 20 plates. (If you are using another vector with a larger or smaller plaque size, the optimal number of clones per plate may be different.)

1. Pick a single, isolated colony from the primary working plate of XL1-Blue (Section VI.B, Step 5 above), and use it to inoculate 15 ml of LB/$MgSO_4$ maltose broth. Incubate at 37°C overnight with shaking (140 rpm) until the OD_{600} of the culture reaches 2.0. Centrifuge the cells for 5 min at 5,000 rpm, pour off the supernatant, and resuspend the pellet in 7.5 ml of 10 mM $MgSO_4$.
2. Plan the number of LB/$MgSO_4$ agar plates you will need. Warm and dry them as explained in the Notes in Section VI.C.
3. Set up the required number of 4 ml tubes with 500 µl of overnight bacterial culture (from Section VI.E, Step 1, above) and enough diluted lysate to yield 6–7 x 10^4 plaques per 150 mm plate.
4. Incubate in a 37°C water bath for 15 min.
5. Add 4.5 ml of melted LB/$MgSO_4$ soft top agar to each tube.
6. Quickly mix and pour the bacteria + phage mixture onto LB/$MgSO_4$ agar plates. Swirl the plate quickly, while pouring, to promote even distribution of the melted agar over the plate.
7. Cool the plates at room temperature for 10 min to allow the top agar to harden. Invert the plates and incubate at 37°C for 6–18 hr, or until the plaques become confluent (i.e., are touching each other).
8. Add 12 ml of 1X lambda dilution buffer to each plate. Store plates at 4°C overnight. The plaques are now ready to be pooled in 1X lambda dilution buffer to form an amplified library lysate.
9. On a platform shaker (~50 rpm), incubate the plates at room temperature for 1 hr.
10. Pour the λ phage lysates into a sterile beaker. This is the pooled, amplified library lysate.
11. To clear the phage lysate of cell debris and to lyse any remaining intact cells:
    a. Mix the phage lysate well and pour it into a sterile, 50 ml polypropylene, screw-cap tube.
    b. Add 10 ml of chloroform to the lysate.
    c. Screw on the cap and vortex for 2 min.
    d. Centrifuge in a Beckman J2-21 centrifuge at 7,000 rpm (5,000 x g) for 10 min. Collect the supernatant into another sterile 50 ml tube, fasten cap tightly and place at 4°C.
12. Determine the titer of the amplified library (Section VI.F below).
13. The amplified library can be stored at 4°C for up to 6 months. For long-term storage (up to one year), make 1 ml aliquots, add DMSO to a final concentration of 7% and place at –70°C. Avoid repeated freeze/thaw cycles.

F. Protocol: Titering the Amplified Library

1. Pick a single, isolated colony from the working stock plate of XL1-Blue (Section VI.B, Step 5), and use it to inoculate 20 ml of LB/$MgSO_4$/maltose broth (without antibiotics). Incubate at 37°C overnight with shaking (140 rpm) until the OD_{600} of the culture reaches 2.0. Centrifuge the cells at 5,000 rpm for 5 min, pour off the supernatant, and resuspend the pellet in 7.5 ml of 10mM $MgSO_4$.
2. Warm and dry four LB/$MgSO_4$ agar plates (90 mm size), as explained in the Notes in Section VI.C.
3. Prepare dilutions of phage lysate (library):
    a. Pipet 10 µl of the library lysate into 1 ml of 1X lambda dilution buffer (Dilution 1 = 1:100).
    b. Transfer 10 µl of Dilution 1 into a second tube containing 1 ml of 1X lambda dilution buffer (Dilution 2 = 1:10,000).
4. Prepare four tubes as described in Table 6 using XL1-Blue overnight culture obtained from Section VI.F, Step 1 and phage Dilution 2 from Section VI.F, Step 3 above.

Table 6. Plating Dilutions for Titering an Amplified Library

1X Lambda Tube	1X Lambda Dilution Buffer	Bacterial Overnight Culture	Phage Dilution 2
1	100 µl	200 µl	5 µl
2	100 µl	200 µl	10 µl
3	100 µl	200 µl	20 µl
4 (Control)	100 µl	200 µl	0 µl

5. Incubate tubes in a 37°C water bath for 15 min.
6. Add 3 ml of melted (45°C) LB/$MgSO_4$ top agar to each of the four tubes.
7. Quickly mix and pour the contents from each tube onto separate LB/$MgSO_4$ agar plates. Swirl the plates quickly after pouring to promote an even distribution of the melted agar.
8. Cool plates at room temperature for 10 min to allow the soft agar to harden.
9. Incubate plates (inverted position) at 37°C for at least 6–7 hr.
10. Count the plaques and calculate the titer (pfu/ml) as follows:

$$\text{pfu/ml} = \frac{\text{number of plaques} \times \text{dilution factor*} \times 10^3\ \mu\text{l/ml}}{\mu\text{l of diluted phage plated}}$$

    * In this case, the dilution factor = 1×10^4

11. A successfully amplified library will have a very high titer (~10^{10} pfu/ml).

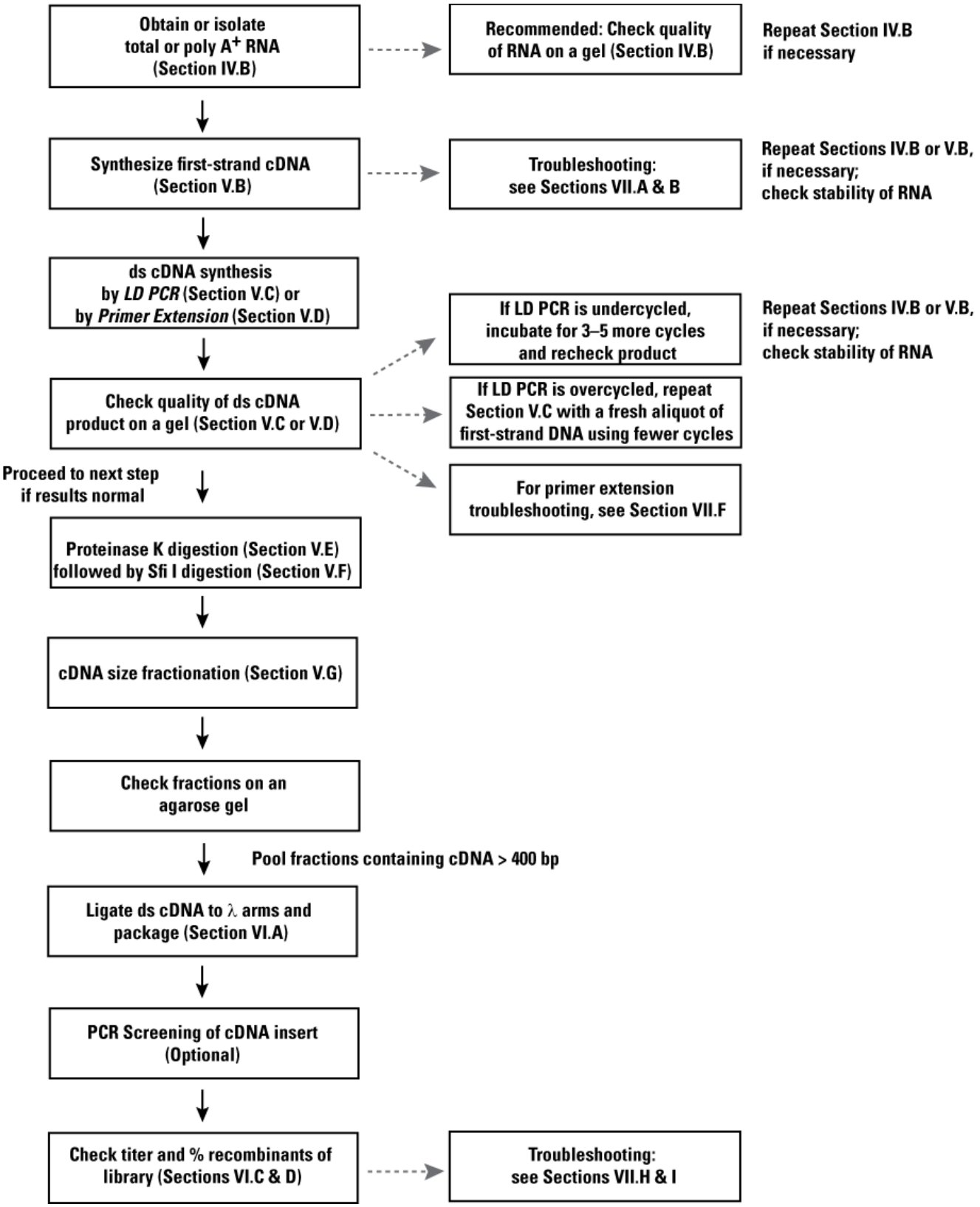

Figure 7. Guide to troubleshooting SMART cDNA synthesis. Please refer to the text for further information on troubleshooting procedures.

VII. Troubleshooting Guide

Table 7. Troubleshooting Guide

Problem	Possible Explanation	Solution
A. No ds cDNA product	Omission of one or more essential reagents from the first-strand synthesis or the ds cDNA synthesis.	Repeat both synthesis reactions, being careful to check off every item as you add it to the reaction.
B. Size distribution of ds cDNA product on an agarose EtBr gel is less than expected. (It should be similar to your mRNA, which typically appears within the range of 0.5–10 kb on an agarose/EtBr gel.)	Your RNA starting material may be degraded, very impure, or too dilute.	1. Check the quality and quantity of your RNA by running a sample on a gel, as recommended in Section IV.B, Step 3. • If the RNA seems too dilute, but otherwise of good quality, restart the experiment using more RNA. • If the RNA seems degraded, restart the experiment using a fresh lot or preparation of RNA. 2. Check the stability of your RNA by incubating a small sample at 37°C for 2 hr. Run it on a gel, parallel to a fresh (unincubated) sample. • If the RNA appears to be unstable, it will yield poor results. • If this is the case, reisolate the RNA using a different method. 3. To diagnose problems with your RNA more easily, perform parallel reactions using the control RNA provided in the kit.
C. Low yield of ds cDNA product using LD-PCR (Section V.C, Step 6)	• PCR undercycling (too few thermal cycles were used in the PCR step)	1. Incubate the PCR reaction mixture for two more cycles and recheck the product. • If you already used the maximum recommended number of cycles indicated in Table 4, increase by three more cycles. • If increasing the number of cycles does not improve the yield of PCR product, repeat the PCR *de novo* using a fresh 2 µl aliquot of the first-strand product. 2. Check to see if the cDNA size distribution <4 kb if the mRNA source was mammalian—another indication of PCR undercycling. (For some sources, such as many insect species, the normal mRNA size distribution may be <2–3 kb.)

Problem	Possible Explanation	Solution
C. Low yield of ds cDNA product using LD-PCR (Section V.C, Step 6; continued)	• Low yield of first-strand cDNA (This may result from using a suboptimal incubation temperature, omitting a component, or insufficient RNA in the reaction. It is also possible that the RNA has been partially degraded by contaminating RNases before or during the first-strand synthesis.)	1. Check the quality of the first-strand cDNA by using a small sample of it as a PCR template with 3' and 5' gene-specific primers. If the first-strand synthesis has been successful, a PCR product of the expected size will be generated. 2. To diagnose problems with your ds cDNA synthesis more easily, perform parallel reactions using the control RNA provided in the kit. If good results were obtained with the control RNA but not with your experimental RNA, then there may be a problem with your RNA. (See Section VII.B above.)
D. No distinct bands distinguishable in the ds cDNA product using LD PCR (Section V.C, Step 6)	• PCR overcycling (too many thermal cycles were used in the PCR step) (As explained at the end of Section V.C, for most mammalian RNA sources, there should be several bands distinguishable against the background smear when a sample of the PCR product is run on a gel. If bands are expected but are not visible, and the background smear is very intense, this is indicative of PCR overcycling.)	Repeat LD PCR (Section V.C) *de novo* with a fresh 2 µl sample of first-strand cDNA, using 2–3 fewer cycles.
	• Gel running conditions which can alter bright band visibility	Be sure to use the following conditions for optimal visibility of your bands: • 1X TAE buffer instead of 1X TBE • Gel concentration of 1.1%–1.2% agarose • Running voltage in the range of 60–90 V.
E. Presence of low-molecular-weight (<0.1 kb) material in the ds cDNA product using LD PCR (Section V.C, Step 6)	PCR overcycling [The raw cDNA (e.g., before size fractionation) is expected to contain some low-molecular-weight DNA contaminants, including unincorporated primers, SMART oligonucleotides and very short PCR products (see Figure 5). However, these small fragments are generally removed from the ds cDNA preparation in the size fractionation step using the columns provided. The presence of mostly low-molecular-weight (<0.1 kb) material in the raw PCR product may be indicative of overcycling.]	Repeat LD PCR (Section V.C) *de novo* with a fresh 2 µl sample of first-strand cDNA, using 2–3 fewer cycles.

Problem	Possible Explanation	Solution
F. No distinct bands distinguishable in the ds cDNA product using Primer Extension (Section V.D, Step 6)	• High complexity of the poly A^+ RNA for some mammalian RNA sources (e.g., human brain, spleen, and thymus) may cause distinct bands to be absent.	If you observe few or no bands, you will need to repeat the primer extension step with the original tube for 2–3 additional cycles.
	• Gel running conditions which can alter distinct band visibility.	Be sure to use the following conditions for optimal visibility of your bands: • 1X TAE buffer instead of 1X TBE • Gel concentration of 1.1%–1.2% agarose • Running voltage in the range of 60–90 V.
G. Presence of low-molecular weight (<0.1 kb) material in the size-fractionated ds cDNA	• Some of your pooled column fractions may contain impurities.	Be sure to check your column fractions on an agarose gel and pool only the first three fractions containing cDNA signal.
	• Failure to follow the protocol exactly and/or improper column handling and storage. [The size fractionation columns are optimized to efficiently remove low-molecular-weight cDNA fragments, small DNA contaminants, and unincorporated nucleotides from the cDNA. Failure to remove these contaminants will result in a library containing mostly very small inserts and/or apparently nonrecombinant clones. (Very small inserts do not necessarily knock out lacZ function.)]	Repeat the ds cDNA synthesis and the size fractionation procedures, noting the following points: 1. The resolving function of the column will be diminished if the protocol is not followed exactly, e.g., using too much or too little column buffer in washing and elution steps, or omitting a step. 2. Do not allow the matrix to dry out between washes or steps. A dry matrix body may shrink away from the inner wall of the column casing. The ds-cDNA mixture can then flow down the sides of the column, allowing small contaminants to enter the body of the matrix too quickly and elute in earlier fractions. 3. The column should be stored and used at room temperature. If it is chilled at 4°C, and then warmed to room temperature for use, bubbles may form, which interfere with the proper functioning of the column. 4. Extreme, uneven deposition of the ds cDNA mixture on the surface of the column can cause inefficient separation of ds cDNA from low molecular weight contaminants. However, a slight uneven surface using the drip method is common and does not affect the column resolution.

Problem	Possible Explanation	Solution
H. Low titer of unamplified library	• Low efficiency of the λ phage packaging reaction.	Check to make sure you are using a λ phage packaging system, according to the manufacturer's specifications, which is expected to yield at least 1 x 10^9 pfu/µg of control λ DNA.
	• Low ligation efficiency of ligation (Section VI.A) due to using a suboptimal amount of the ds cDNA insert [Ligation efficiency is considered low if the unamplified library has a titer <10^6 pfu/µg DNA (all three ligations combined), but the control packaging reaction (using ligated, nonrecombinant λ DNA) yields at least 5 x 10^8 pfu/µg.]	1. Check the concentration of the size-fractionated ds cDNA product using one of the following methods. (The ligation reaction cannot be checked retroactively because the entire ligation mixture is generally used in the packaging reaction): • Electrophorese 1 µl of the resuspended cDNA on an agarose EtBr gel next to a known amount of control DNA. View the gel under UV light. • Alternatively, spot 1 µl of the cDNA on an agarose EtBr plate next to small spots of known amounts (10–1,000 ng) of control DNA. The concentration of the resuspended cDNA should be in the range of 100–200 ng/µl. 2. Confirm that your starting amount of ds cDNA is within 2–3 µg, using one of the following methods. [Too much starting cDNA (>3–4 µg) often yields poor cloning efficiencies or low-titer libraries.]: • Apply 5 µl of ds cDNA product to a DNA fluorometer to determine its concentration. • Obtain a rough estimate of your target cDNA concentration by comparing the intensity of your ds cDNA with cDNA synthesized from the included control RNA, on a 1.1% agarose gel. Typically, the control RNA generates 4–6 µg of ds cDNA by this protocol. 3. Repeat the ligations using the appropriate amount of ds cDNA staring material, as determined above.

Problem	Possible Explanation	Solution
I. Low (<80%) recombination efficiency	Re-ligation of nonrecombinant vectors. (Background due to vector re-ligation should be minimal, since the SfiI (A & B)-digested λTriplEx2 arms provided in the SMART Kit have been dephosphorylated.)	Check for vector re-ligation by performing a control ligation using vector alone (no cDNA) in Section VI.A, Step 1.
	If low recombination efficiency is combined with a high titer, your cDNA population may contain a lot of small DNA fragments, which are preferentially ligated into the vector.	Repeat the ds cDNA synthesis and size fractionation procedures, and check the size distribution of the fractionated cDNA (see Section VII.G).
J. Small insert sizes	Your cDNA preparation was not successfully size-fractionated, if more than 50% of your clones appear to have small insert sizes (i.e., <0.4 kb). (Small cDNA fragments, unincorporated primers, and primer dimers are preferentially ligated to the vector, and should be completely removed before the vector ligation step.)	You will need to repeat the ds cDNA synthesis and the size fractionation procedures, because this problem cannot be retroactively solved. Before you do so, read Section VII.G above for tips on troubleshooting the size fractionation procedure.

VIII. References

Barnes, W. M. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91:**2216–2220.

Birnboim, H. C. & Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7:**1513.

Borson, N. D., Sato, W. L. & Drewes, L. R. (1992) A lock-docking oligo(dT) primer for 5' and 3' RACE PCR. PCR *Methods & Appl.* **2:**144–148.

Chenchik, A., Zhu, Y. Y., Diatchenko, L., Li, R., Hill, J. & Siebert, P. D. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *Gene Cloning and Analysis by RT-PCR* (BioTechniques Books, MA), pp. 305–319.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159.

D'Alessio, J. M. & Gerard, G. F. (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.

Farrell, Jr., R. E. (1993) *RNA Methodologies—A Lab Guide for Isolation and Characterization* (Academic Press, San Diego, CA).

Fromont-Racine, M., Bertrand, E., Pictet, R. & Grange, T. (1993) A highly sensitive method for mapping the 5' termini of mRNAs. *Nucleic Acids Res.* **21:**1683–1684.

Furuichi, Y. & Miura, K. (1975) A blocked structure at the 5' terminus of mRNA from cytoplasmic polyhedrosis virus. *Nature* **253:**374–375.

Green, M. R. & Sambrook, J. (2012) *Molecular Cloning: A Laboratory Manual, Fourth Edition* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Gubler, U. & Hoffman, B. J. (1983) A simple and very efficient method for generating complementary DNA libraries. *Gene* **25:**263–269.

Huynh, T. V., Young, R. A. & Davis, R. W. (1985) *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL Press, Oxford).

Kato, S., Sekine, S., Oh, S.-W., Kim, N.-S., Umezawa, Y., Abe, N., Yokoyama-Kobayashi, M. & Aoki, T. (1994) Construction of a human full-length cDNA bank. *Gene* **150:**243–250.

Okayama, H. & Berg, P. (1982) High efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* **2:**161–170.

NOTE: SMART technology was originally described as Capfinder.

Appendix A. Converting λTriplEx2 to pTriplEx2

A. Background

The conversion of a λTriplEx2 clone to a pTriplEx2 plasmid involves *in vivo* excision and circularization of a complete plasmid from the recombinant phage. The plasmid is released as a result of Cre recombinase-mediated site-specific recombination at the *loxP* sites flanking the embedded plasmid (Figure 8). Release of the plasmid occurs automatically when the recombinant phage is transduced into a bacterial host in which Cre recombinase is being expressed. In this system, *E. coli* BM25.8 (growing at 31°C) provides the necessary Cre recombinase activity. Conversion may be performed on individual positive plaques picked from the secondary or tertiary screening plates. The released version of the plasmid differs from pTriplEx2 by a 100 bp *loxP* insert at the Cla I site (please refer to the Vector Information packet for a vector map and MCS). The excised plasmid is propagated stably in *E. coli*. Do not attempt blue/white screening with strain BM25.8.

B. Conversion Protocol

1. Pick a single, isolated colony from the working stock plate of BM25.8 host cells (Section VI.B, Step 4) and use it to inoculate 10 ml of LB broth in a 50 ml test tube or Erlenmeyer flask. Incubate at 31°C overnight with shaking (at 150 rpm) until the OD_{600} of the culture reaches 1.1–1.4.
2. Add 100 µl of 1 M $MgCl_2$ to the 10 ml overnight culture of BM25.8 (10 mM final concentration of $MgCl_2$).
3. Pick a well-isolated positive plaque from secondary or tertiary screening plates, and place it in 350 µl of 1X lambda dilution buffer. Vortex the plaque and incubate at 37°C for 3–4 hr without shaking (200–250 rpm). (Alternatively, allow phage to elute at 4°C overnight.)
4. In a 20 ml test tube, combine 200 µl of overnight cell culture with 150 µl of the eluted positive plaque. (Reserve the remainder of the eluted plaque in case you need to repeat the conversion.)
5. Incubate at 31°C for 30 min without shaking.
6. Add 400 µl of LB broth.
7. Incubate at 31°C for an additional 1 hr with shaking (225 rpm).
8. Using a sterile glass spreader, spread 1–10 µl of infected cell suspension on an LB/carbenicillin plate to obtain isolated colonies and grow at 31°C.

   NOTE: Ampicillin can be used instead of carbenicillin, but may result in more satellite colonies.
9. Pick several well-isolated colonies from each clone and prepare plasmid DNA separately from each one. For a quick analysis of insert size, the alkaline lysis method of Birnboim & Doly (1979) is recommended for isolation of bacterial plasmids. The isolated plasmid DNA should be pure enough for direct sequencing. The pTriplEx2 sequencing primers provided may be used with standard ds-DNA-sequencing protocols.

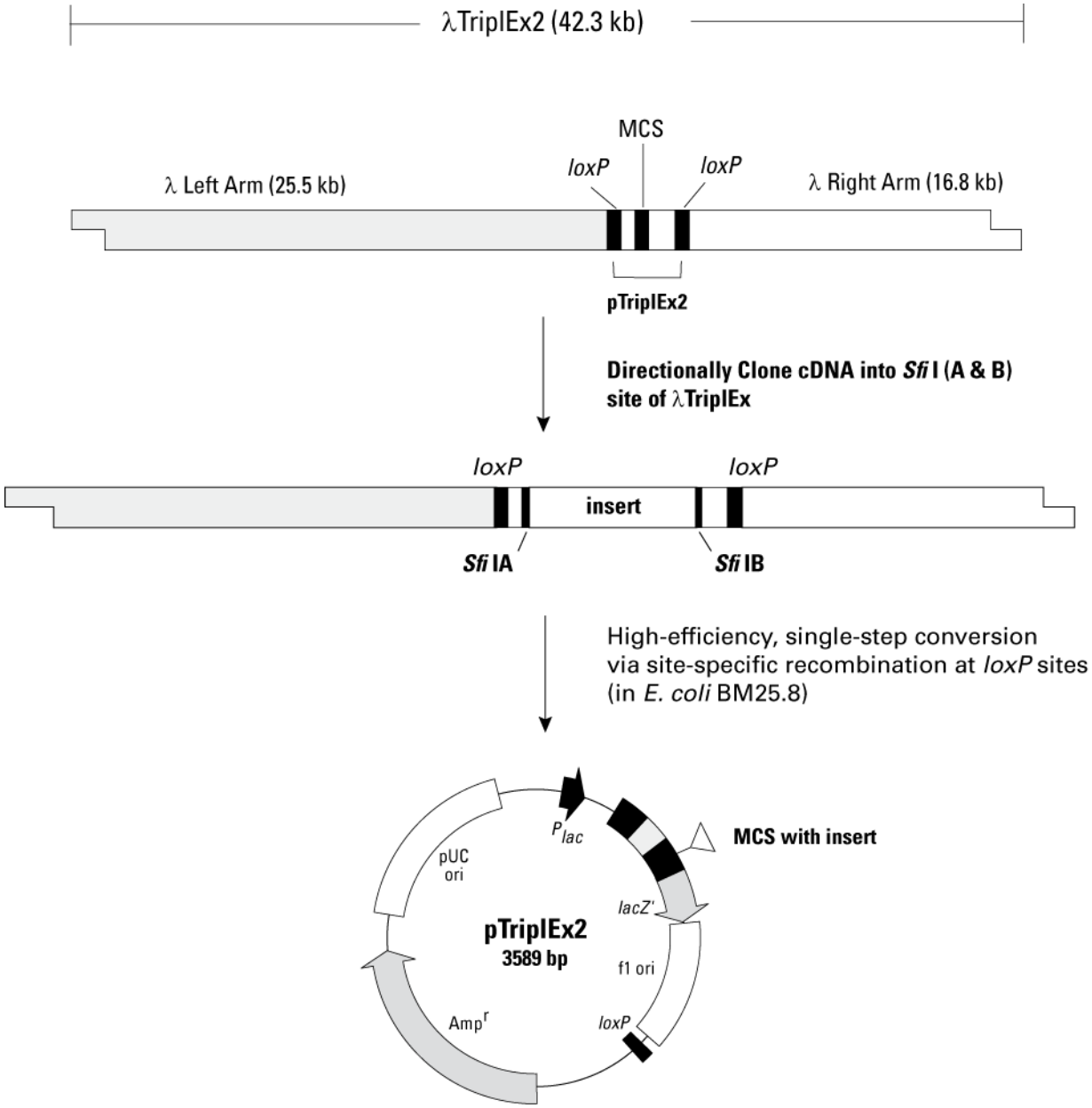

Figure 8. Conversion of a recombinant λTriplEx2 to the corresponding pTriplEx2. The λTriplEx2 MCS is located within an embedded plasmid, which is flanked by loxP sites at the λ junctions. Transduction of a λTriplEx2 lysate into E. coli strain BM25.8 promotes Cre recombinase-mediated release and circularization of pTriplEx2 at the loxP sites. pTriplEx2 carries the bla gene for ampicillin resistance and the pUC ori for autonomous replication in *E. coli*. The MCS provides several unique restriction sites flanking the SfiI A & B sites to facilitate the subcloning and analysis of inserts. See Figure 9 for a restriction map of λTriplEx2.

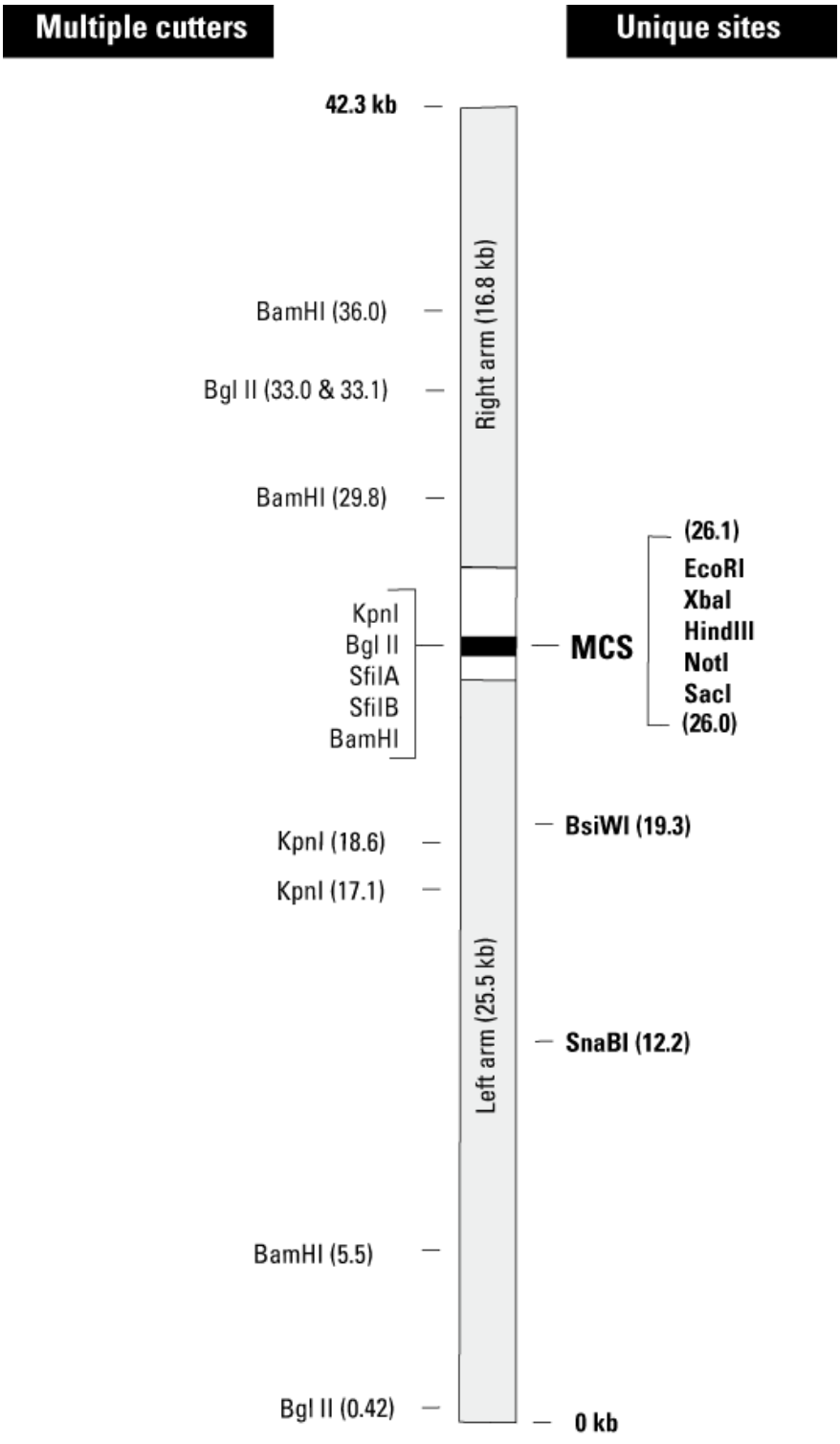

Figure 9. Restriction map of λTriplEx2.

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