Takara Bio USA

In-Fusion® SMARTer® Directional cDNA Library Construction Kit User Manual

Cat. No. 634933
(050421)

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I. List of Components

Store CHROMA SPIN Columns and Deionized H_2O at room temperature. Store Control RNA and SMARTer II A Oligonucleotide at –70°C. Store all other reagents at –20°C.

In-Fusion SMARTer PCR cDNA Synthesis Kit (Cat No. 634934; not sold separately)

- 10 µl **SMARTer V Oligonucleotide** (12 µM)
  5'–AAGCAGTGGTATCAACGCAGAGTAXXXXX–3'
  Rsa I
  (X = undisclosed base in the proprietary SMARTer oligo sequence)
- 10 µl **3' In-Fusion SMARTer CDS Primer** (12 µM)
  5'–CGGGGTACGATGAGACACCATTTTTTTTTTTTTTTTTTVN–3'
  (N = A, C, G, or T; V = A, G, or C)
- 12 µl **SMARTScribe™ Reverse Transcriptase** (100 units/µl)
- 40 µl **5X First-Strand Buffer (RNase-Free)**
  250 mM Tris (pH 8.3)
  30 mM $MgCl_2$
  375 mM KCl
- 50 µl **DTT** (dithiothreitol; 100 mM)
- 100 µl **dNTP mix** (dATP, dCTP, dGTP, dTTP; 10 mM each)
- 10 µl **RNase Inhibitor** (40 U/µl)
- 5 µl **Control Mouse Liver Total RNA** (1.0 µg/µl)
- 200 µl **5' PCR Primer II A** (12 µM)
- 200 µl **3' In-Fusion SMARTer PCR Primer** (12 µM)
  5'– CGGGGTACGATGAGACACCA-3'
- 200 µl **Sodium Acetate** (3 M; pH 4.8)
- 100 µl **Glycogen** (20 µg/µl)
- 1 ml **Deionized H_2O** (Milli-Q-filtered, not DEPC-treated)
- 30 ml **1X Fractionation Column Buffer**
  10 mM Tris (pH 7.4)
  30 mM NaCl
  0.5 mM EDTA (pH 7.4)
- 20 **CHROMA SPIN™ +TE-1000 Columns**

In-Fusion Snap Assembly Master Mix (Cat. No. 638948)

- 100 µl **5X** In-Fusion Snap Assembly Master Mix
- 25 µl **pUC19 Control Vector, linearized** (50 ng/µl)
- 50 µl **2 kb Control Insert** (40 ng/µl)

In-Fusion SMARTer Directional Cloning & Screening Components (Cat. No. 639632; not sold separately)

- 60 µl **pSMART2IFD Linearized Vector** (150 ng/µl)
- 100 µl **Forward Screening Primer** (10 µM)
  TCACACAGGAAACAGCTATGA
- 100 µl **Reverse Screening Primer** (10 µM)
  CCTCTTCGCTATTACGCCAGC

QuickClean Enzyme Removal Resin (Cat. No. 631770)

II. Additional Materials Required

The following reagents are required but not supplied:

- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
  We strongly recommend using the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) for PCR amplification of SMARTer-generated cDNA. This kit includes the Advantage 2 Polymerase Mix, which has been specially formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes 1994). The Advantage 2 Polymerase Mix is comprised of Titanium® *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion mutant of *Taq* DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR (Kellogg et al. 1994)—and a minor amount of a proofreading polymerase. This combination allows you to efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes 1994).

- **Advantage HD Polymerase Mix** (Cat. No. 639241)
  We recommend using Advantage HD Polymerase Mix for inverse PCR amplification of your linearized vector (see Appendix D). If high fidelity is important for your application, we recommend using Advantage HD for PCR amplification of SMARTer cDNA (Appendix E). Advantage HD offers high-fidelity, efficient amplification of long gene segments (>1 kb), and an automatic hot start for increased specificity and reduced background.

- **NucleoSpin RNA kit** (Cat. Nos. 740955.50 & 740955.250)
  We strongly recommend using the NucleoSpin RNA kit for RNA purification. We have found that cells or tissues frozen in RA1 buffer are better preserved, resulting in the isolation of higher quality RNA. The RA1 buffer, which contains guanidinium isothiocyanate, protects cellular RNA by inactivating RNases. The higher quality of RNA reduces the number of cycles required to reach the optimal SMARTer cDNA amplification level, resulting in a more representative cDNA pool. Additional Buffer RA1 (Cat. No. 740961) can be purchased separately.

- **NucleoSpin Gel and PCR Clean-Up** (Cat. Nos. 740609.50 & 740609.250)
  We strongly recommend using NucleoSpin Gel and PCR Clean-Up for PCR product purification.

- **EASY Dilution Solution** (Cat. No. 9160)
  We recommend using EASY Dilution solution for RNA template dilution.

- **Electrocompetent Cells**
  We recommend using electrocompetent cells that have a transformation efficiency >1 x 10^9 cfu/µg.

- **SOC medium**

- **TE buffer** (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA)

- **DNA size markers** (1 kb DNA ladder)

- **50X TAE electrophoresis buffer:**

242.0	g	Tris base
57.1	ml	glacial acetic acid
37.2	g	$Na_2EDTA•2H_2O$
X	ml	Add H_2O to 1 L

- **LB-Amp/IPTG/X-Gal plates** (100 µg/ml ampicillin, 1 mM IPTG, 75 µg/ml X-Gal)

- **1% xylene cyanol in water**

III. Introduction

The **In-Fusion SMARTer Directional cDNA Library Construction Kit** provides a dependable method for producing high-quality, full-length cDNA libraries. The kit utilizes two of Takara Bio's most innovative technologies: SMART® cDNA Synthesis and In-Fusion Cloning. SMART(er) technology enables full-length cDNA synthesis from nanograms of poly A+ or total RNA, and In-Fusion Cloning makes it easy to clone your SMARTer cDNA library into any location within any vector, including the **pSMART2IFD Linearized Vector** included in the kit. Clones isolated from SMARTer libraries can be transferred directly to any linearized expression vector for functional analysis—without the need for compatible restriction sites.

Full-length cDNA clones of mammalian genes are indispensable tools for functional analysis; cDNA libraries remain the main resource for such clones. A common problem with conventionally-generated cDNA libraries is the high percentage of 5'-truncated cDNAs due to truncated starting mRNA and incomplete first strand synthesis (Gubler & Hoffman 1983). To increase the percentage of full-length clones in cDNA libraries, several methods have been developed (Carninci & Hayashizaki 1999; Edery et al. 1995; Kato et al. 1994; Suzuki et al. 1997). All these methods are laborious, involve extensive manipulation of mRNA prior to cDNA synthesis, and require a large amount of starting material (5–100 µg mRNA). In contrast, the In-Fusion SMARTer Directional cDNA Library Construction method is robust and straightforward, and can be performed with much smaller amounts of starting material (0.01–1 µg total RNA or 5–100 ng mRNA).

SMARTer cDNA libraries contain more full-length clones

SMART (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) cDNA synthesis technology is a PCR-based method for cDNA library construction that is designed to preferentially enrich for full-length cDNAs. This unique technology allows the efficient incorporation of synthetic adaptors on both the 5' and 3' ends of cDNA during first-strand synthesis, without adaptor ligation, in one reverse transcription reaction. This enables end-to-end PCR and prevents the generation of chimeric cDNA inserts, resulting in a clean and representative cDNA population.

All commonly used cDNA synthesis methods, including SMART, rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded (ss) DNA. In some cases, reverse transcription terminates before transcribing the complete mRNA sequence, therefore underrepresenting the 5' ends of genes. 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. SMART(er) technology circumvents this problem by selectively amplifying cDNAs that are 5'-tagged via the SMARTer V oligo. This prevents amplification of truncated transcripts because the adaptors are only incorporated if the RT reaches the 5' end of the mRNA. What's more, SMART(er) cDNA cloning does not require T4 DNA polymerase. Conventional cDNA cloning procedures use T4 DNA polymerase to generate blunt cDNA ends after second-strand synthesis. As a result, the 5' ends of the cDNA tend to be 5–30 nucleotides shorter than they were in the original mRNA (D'Alessio 1988). SMART(er) 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). Clones isolated from SMART(er) cDNA libraries are found to contain sequences corresponding to the complete 5' untranslated region of the mRNA (ibid.).

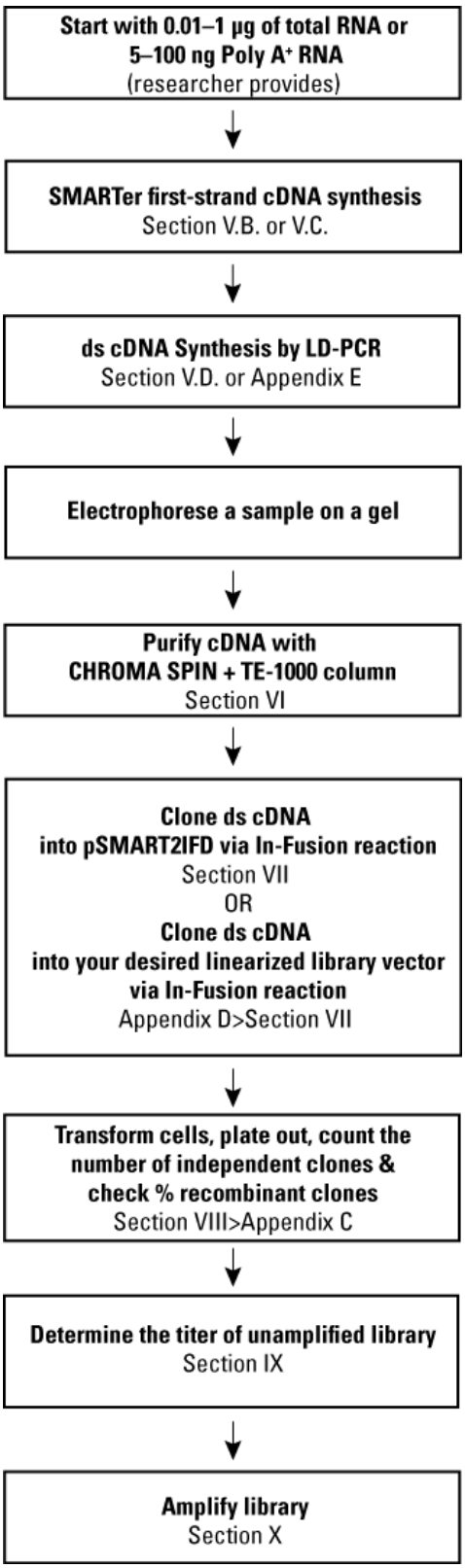

Figure 1. The In-Fusion SMARTer Directional cDNA Library Construction Kit includes a complete protocol for cDNA synthesis, library construction, and library amplification.

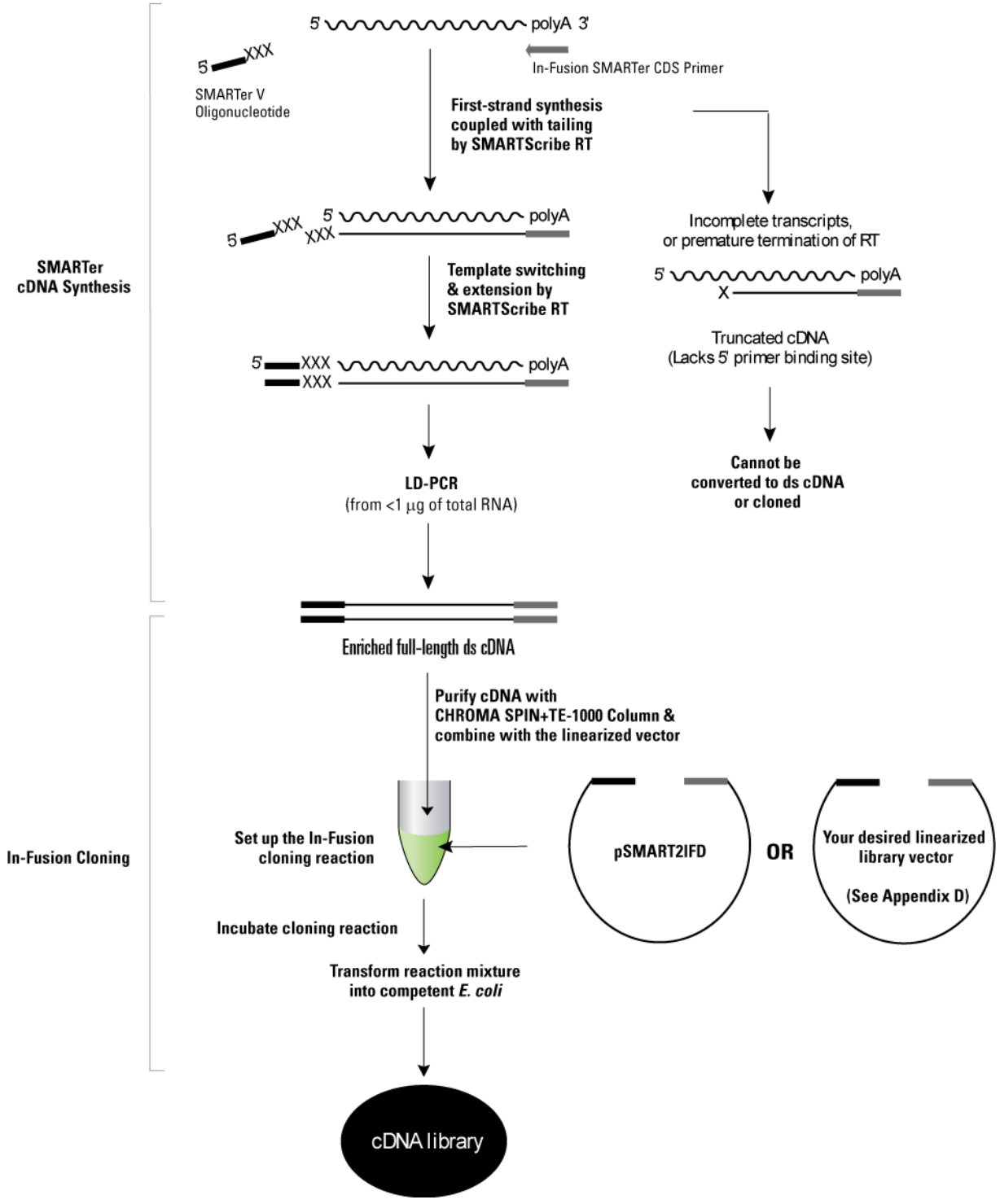

Figure 2. Flow chart of the In-Fusion SMARTer Directional cDNA Library Construction Kit protocol. The right side of the flow chart shows the fate of incomplete transcripts caused by RNA degradation or premature termination of reverse transcription.

ds cDNA Synthesis by Long-Distance PCR—for Limiting Amounts of Starting Material

In the protocol a modified oligo(dT) primer (3' In-Fusion SMARTer CDS Primer) primes the first-strand cDNA synthesis reaction, and the SMARTer V Oligo serves as a short, extended template at the 5' end of the mRNA (Figure 2). When the RT reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the cDNA. The SMARTer V Oligo base-pairs with these additional nucleotides, creating an extended template. The RT then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMARTer V Oligo, which then serves as a universal priming site (SMART anchor) in the subsequent amplification by long-distance PCR (LD-PCR; Barnes et al. 1994, Chenchik et al. 1998, Cheng et al. 1994). Only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and 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 poly A– RNA is eliminated. This selective amplification allows you to construct a cDNA library with a high percentage of full-length clones using nanogram amounts of either total RNA or poly A+ RNA.

Use pSMART2IFD or Any Desired Linearized Vector for Library Cloning

When cDNA is synthesized using SMARTer technology, known sequence is incorporated at each end of the cDNA. As a result, the ends of SMARTer-generated cDNA are complementary to the ends of the pSMART2IFD Linearized Vector. Since In-Fusion Cloning Master Mixes are designed to join pieces of DNA having 15 bp of complementarity at each end, In-Fusion technology can be used to precisely incorporate your SMARTer cDNA into the pSMART2IFD Linearized Vector in just one 30-min reaction. With In-Fusion technology, SMARTer cDNA can be cloned into ANY linearized vector, not just pSMART2IFD. If you would like to clone your library into a vector other than pSMART2IFD, simply linearize your vector of choice by inverse PCR using primers that introduce SMARTer-sequences onto the ends of the vector at the desired cDNA insertion point. See Appendix D for a discussion on primer design and vector preparation for In-Fusion SMARTer cDNA library cloning.

In-Fusion Cloning Allows for Easy Switching Between Expression Systems

In-Fusion Cloning allows you to transfer your gene into multiple expression systems quickly and efficiently (Figure 3). Once you screen your In-Fusion SMARTer library and isolate your clone of interest, you can move the clone to a different vector using In-Fusion, as long as you inverse PCR amplify the destination vector with primers that create linear vector ends containing 15 bp of complementarity to the ends of your clone of interest. The primers must have two characteristics: the 5' end of the primer must contain 15 bases that are complementary to 15 bases at one end of the DNA fragment to which it will be joined (i.e., the insert), and the 3' end of the primer must contain sequence that is specific to the target vector (see Figure 9, Appendix D).

Optimized Reagents and Conditions

The In-Fusion SMARTer Directional cDNA Library Construction 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; Chenchik et al. 1994) 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. Each In-Fusion SMARTer kit is supplied with sequencing/screening forward and reverse primers, which anneal to sites flanking inserts. These primers can be used to determine the percentage of recombinant clones. To obtain the highest yields and the largest SMARTer cDNA, we strongly recommend using our Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) for long-distance PCR amplification. If high-fidelity is important for your application, we recommend using Advantage HD Polymerase Mix (Cat. No. 639241) for PCR amplification of SMARTer cDNA. This User Manual provides a complete protocol for cDNA synthesis, library construction, and library amplification.

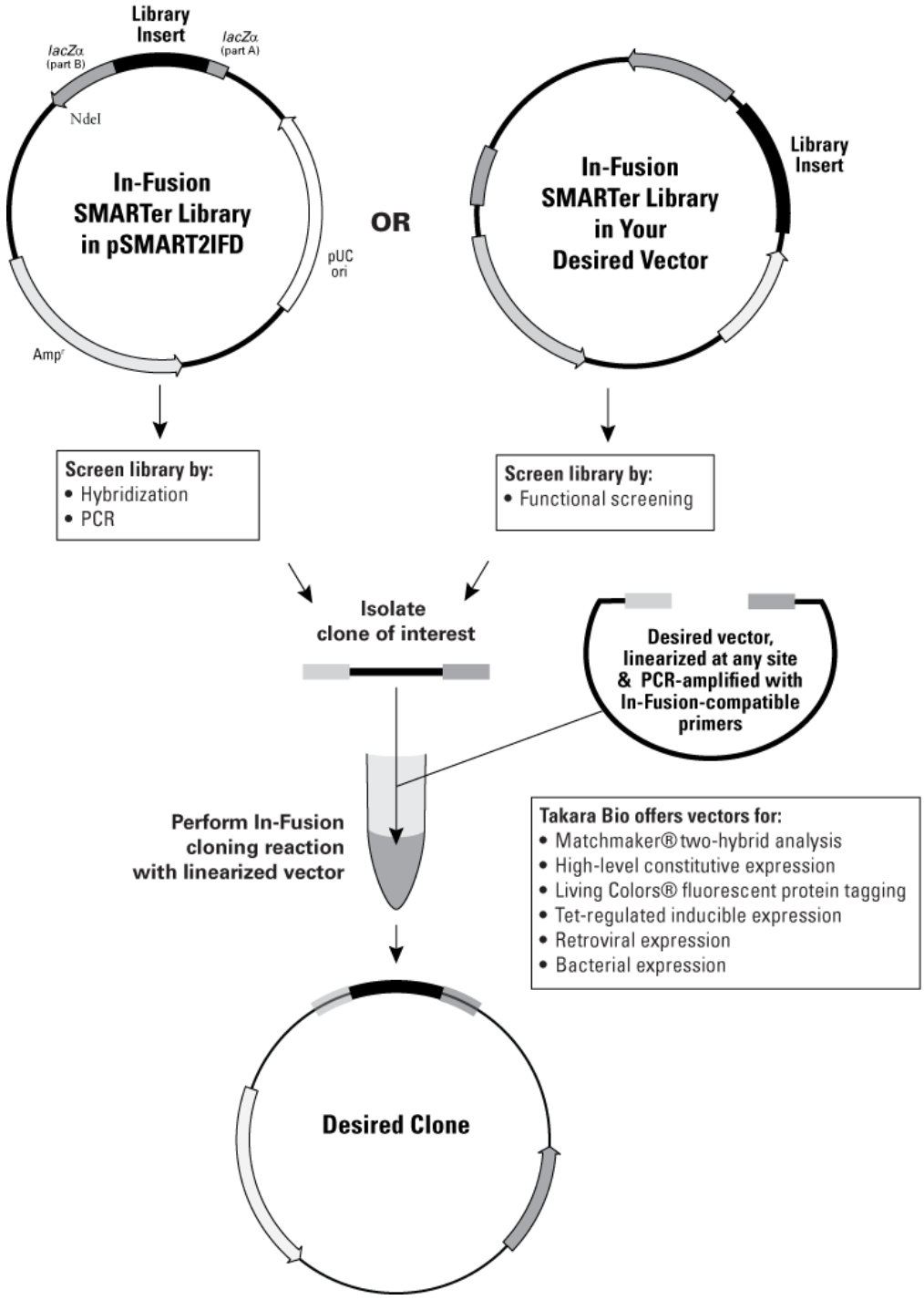

Figure 3. The In-Fusion SMARTer Directional cDNA Library Construction Kit allows you to quickly and efficiently transfer your gene into multiple expression systems via In-Fusion cloning. Simply linearize your vector of choice by inverse PCR, using primers that introduce SMARTer-sequences onto the ends of the vector at the desired cDNA insertion point. Since all SMARTer-generated cDNA is flanked by the same sequence, any cDNA library amplified using SMARTer technology can be In-Fusion cloned into your vector once the SMARTer-sequences have been introduced onto the ends of the vector. See **Appendix D: Preparing Your Vector for In-Fusion SMARTer Directional cDNA Library Cloning** for a discussion on primer design and vector preparation for In-Fusion SMARTer cDNA library cloning. Once you screen your library and isolate your clone of interest, you can move it to a different vector via In-Fusion cloning, as long as the ends of the linearized vector contain 15 bp of complementarity to the ends of your clone of interest. For more information about designing primers for In-Fusion cloning, please see the In-Fusion Snap Assembly User Manual at **takarabio.com/manuals**.

IV. RNA Preparation & Handling

A. General Precautions

The integrity and purity of your total or poly A+ RNA starting material is an important element in high-quality cDNA synthesis. The following precautions will help you avoid contamination and degradation of your RNA:

- Wear gloves throughout the procedure to protect your RNA samples from degradation by nucleases.
- Use freshly deionized (e.g., MilliQ-grade) H_2O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 M 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.
- Ethidium bromide is a carcinogen. Use appropriate precautions when handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manual* by Sambrook & Russell (2001).

B. RNA Isolation

Takara Bio offers several kits for isolating total or poly A+ RNA from a variety of sources. The NucleoBond RNA/DNA Kit (Cat. No. 740650) contains AX-R tips to isolate total RNA from tissue or cells without using phenol or chloroform. With the NucleoSpin RNA kit (Cat. No. 740955.50), you can isolate highly pure total RNA from cells, tissues, or cell-free biological fluids without phenol chloroform extractions. The NucleoTrap mRNA Mini Kit (Cat. No. 740655) combines a spin column filter with oligo(dT)-latex bead technology to isolate high-quality mRNA from total RNA in less than 30 minutes. For more RNA isolation kits, visit our website at www.takarabio.com. Many procedures are available for the isolation of poly A+ RNA (Farrell 1993; Sambrook et al. 1989).

C. RNA Purity

The purity of RNA is the key factor for successful cDNA synthesis and amplification. The presence of residual organics, metal ions, salts or nucleases in your RNA sample could have a large impact on downstream applications by inhibiting enzymatic activity or degrading the RNA. We strongly recommend checking the stability of your RNA to ensure that it is free of contaminants.

To test the stability of your RNA, incubate a small portion of it at 37°C for 2 hours, then compare the sample to a duplicate control stored at –70°C. If the sample incubated at 37°C shows a lower 28S:18S ratio than the control, or the RNA shows a significant downward shift on a formaldehyde agarose gel, the RNA may have nuclease contaminants (see Section IV.D. for methods for assessing RNA quality).

Impurities such as salts or organic contaminants can be removed by repeated ethanol precipitation, subsequent washing with 80% ethanol and the complete removal of all remaining ethanol.

If your RNA template is from a plant or some other species with high pigment levels, please pay special attention to polysaccharide/pigment contamination. Polysaccharides/pigments are hard to remove and can't be detected on an agarose gel. These glycoproteins might interfere with primer binding sites on the RNA during the first-strand cDNA synthesis, leading to reduced cDNA yield.

D. Assessing the Quality of the RNA Template

Methods for Assessing Total RNA Integrity

1. 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 1.9 and 4.5 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 Sambrook & Russell (2001).

2. 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 (Thermo Fisher Scientific) 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.

3. Detection with the Agilent 2100 BioAnalyzer (Agilent Technologies):

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

V. SMARTer cDNA Synthesis by LD-PCR

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING. If you have more than 50 ng of RNA, use the first-strand cDNA synthesis protocol in Section V.B. If you have less than 50 ng of RNA, use the first-strand cDNA synthesis protocol in Section V.C. In general, the more RNA you start with, the fewer PCR cycles will be required for the second-strand synthesis. Using fewer thermal cycles reduces the level of nonspecific PCR products, and therefore is optimal for cDNA and library quality.

IMPORTANT:

- The success of your experiment depends on the quality of your starting sample of total or poly A+ RNA. For best results we strongly recommend that you use the NucleoSpin RNA kit (see Section II for ordering information) to isolate highly pure RNA from cells, tissues or biological fluids (see Section IV.B. RNA Isolation).
- Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants (see Section IV.D. Assessing the Quality of the RNA Template).
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification protocol and reagents.
- Resuspend pellets and mix reactions by gently pipetting the solution up and down or by tapping the bottom of the tube. Then spin the tube briefly to bring all contents to the bottom.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- The first time you use this kit, you should perform cDNA synthesis with the provided Control Mouse Liver Total RNA, in parallel with your experimental sample. Performing this control synthesis at least once will verify that all components (especially the reverse transcriptase) are working properly and will also help you troubleshoot any problems that may arise. This is especially important because it may not be possible to visualize your first-strand reaction product on a gel. Furthermore, performing the control reaction through the PCR and library construction steps in parallel with your experiment will allow you to evaluate the yield and size distribution of the ds cDNA synthesized from your RNA sample, as well as assist in troubleshooting if any problems arise.

A. Recommended Products

- We recommend our **EASY Dilution Solution** (Cat. No. 9160) for RNA template dilution. EASY Dilution Solution can prevent template from sticking to the tube, and allows correct dilution at low concentration.
- We strongly recommend using the **Advantage 2 PCR Kit** (Cat. Nos. 639206 & 639207) for PCR amplification (Section C). This kit includes the Advantage 2 Polymerase Mix, which has been specially formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD-PCR; Barnes 1994).
- For applications that require high-fidelity amplification, we recommend Advantage HD Polymerase (Cat. No. 639241), though the yield will be lower than that obtained using Advantage 2. Please see Appendix E for ds cDNA Synthesis using Advantage HD.

B. Protocol: First-Strand cDNA Synthesis from More than 50 ng of RNA

This protocol is designed for library construction starting from a minimum of 50 ng of total RNA. However, if your RNA sample is not limiting, we recommend that you start from 1 µg of total RNA for cDNA synthesis. If you have less than 50 ng of total RNA, use the first-strand cDNA synthesis protocol in Section V.C. In general, the more RNA you start with, the fewer PCR cycles will be required for the second-strand synthesis (see Table I, Section D). Using fewer thermal cycles reduces the level of nonspecific PCR products, and therefore is optimal for cDNA and library quality. Thus, if your RNA sample is not limiting, use the higher starting amounts of RNA shown in the table.

1. For each sample and Control Mouse Liver Total RNA, combine the following reagents in separate microcentrifuge tubes:

1–3.5 µl	**RNA** (50 ng–1 µg total RNA or 50–100 ng poly A+ RNA)*
1 µl	**3' In-Fusion SMARTer CDS Primer** (12 µM)
X µl	**Deionized H_2O**
4.5 µl	**Total Volume**

   *For the control synthesis, add 1 µl (1 µg/µl) of Control Mouse Liver Total RNA.

2. Mix contents and spin the tube(s) briefly in a microcentrifuge.

3. Incubate the tube(s) at 72°C in a hot-lid thermal cycler for 3 min, then reduce the temperature to 42°C for 2 min.

   NOTE: The initial reaction steps (Step 4-6) are critical for first-strand synthesis and should not be delayed after Step 3. You can prepare your master mix (for Step 4) while your tubes are incubating (Step 3) in order to jump start the cDNA synthesis.

4. Prepare a Master Mix for all reaction tubes at room temperature by combining the following reagents in the order shown:

2.0 µl	**5X First-Strand Buffer**
0.25 µl	**DTT** (100 mM)
1.0 µl	**dNTP Mix** (10 mM)
1.0 µl	**SMARTer V Oligonucleotide** (12 µM)
0.25 µl	**RNase Inhibitor**
1.0 µl	**SMARTScribe Reverse Transcriptase** (100 U)*
5.5 µl	**Total Volume added per reaction**

   *Add the reverse transcriptase to the master mix just prior to use. Mix well by pipetting and spin the tube(s) briefly in a microcentrifuge.

5. Aliquot 5.5 µl of the Master Mix into each reaction tube. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.

6. Incubate the tube(s) at 42°C for 90 min.

7. Heat the tube(s) at 68°C for 10 min to terminate first-strand cDNA synthesis.

8. If you plan to proceed directly to PCR (Section C), take two 2 µl aliquots from the first-strand synthesis and place them in two clean, prechilled PCR tubes. Place the tubes on ice, and proceed to **Section V.D. PROTOCOL: ds cDNA Synthesis by LD-PCR**. 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: First-Strand cDNA Synthesis from Less than 50 ng of RNA

This protocol is designed for library construction starting from a minimum of 10 ng of total RNA. However, if your RNA sample is not limiting, we recommend that you start from 1 µg of total RNA for cDNA synthesis. If you have more than 50 ng of total RNA, use the first-strand cDNA synthesis protocol in Section V.B. In general, the more RNA you start with, the fewer PCR cycles will be required for the second-strand synthesis (see Table I, Section V.D). Using fewer thermal cycles reduces the level of nonspecific PCR products, and therefore is optimal for cDNA and library quality. Thus, if your RNA sample is not limiting, use the higher starting amounts of RNA shown in the table.

1. For each sample and Control Mouse Liver Total RNA, combine the following reagents in separate microcentrifuge tubes:

1–3.75 µl	**RNA** (10–50 ng total RNA or 5–50 ng poly A+ RNA)*
1 µl	**3' In-Fusion SMARTer CDS Primer** (12 µM)
X µl	**Deionized H_2O**
4.75 µl	**Total Volume**

   * For the control synthesis, add 1 µl (50 ng/µl) of Control Mouse Liver Total RNA.

2. Mix contents and spin the tube(s) briefly in a microcentrifuge.

3. Incubate the tube(s) at 72°C in a hot-lid thermal cycler for 3 min, then reduce the temperature to 42°C for 2 min.

   NOTE: The initial reaction steps (Step 4-6) are critical for first-strand synthesis and should not be delayed after Step 3. You can prepare your master mix (for Step 4) while your tubes are incubating (Step 3) in order to jump start the cDNA synthesis.

4. Prepare a Master Mix for all reaction tubes at room temperature by combining the following reagents in the order shown:

2.0 µl	**5X First-Strand Buffer**
0.25 µl	**DTT** (100 mM)
1.0 µl	**dNTP Mix** (10 mM)
1.0 µl	**SMARTer V Oligonucleotide** (12 µM)
0.5 µl	**RNase Inhibitor**
0.5 µl	**SMARTScribe Reverse Transcriptase** (100 U)*
5.25 µl	**Total Volume added per reaction**

   *Add the reverse transcriptase to the master mix just prior to use. Mix well by pipetting and spin the tube(s) briefly in a microcentrifuge.

5. Aliquot 5.25 µl of the Master Mix into each reaction tube. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.

6. Incubate the tube(s) at 42°C for 90 min.

7. Heat the tube(s) at 68°C for 10 min to terminate first-strand cDNA synthesis.

8. If you plan to proceed directly to PCR (Section C), take two 2 µl aliquots from the first-strand synthesis and place them in two clean, prechilled PCR tubes. Place the tubes on ice, and proceed to **Section V.D. PROTOCOL: ds cDNA Synthesis by LD-PCR**. 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.

D. Protocol: ds cDNA Synthesis by LD-PCR using Advantage 2 Polymerase

Table I provides guidelines for optimizing your PCR, depending on the amount of total RNA used in the first-strand synthesis. These guidelines were determined using the Control Mouse Liver Total RNA, Advantage 2 Polymerase and a hot-lid thermal cycler; optimal parameters may vary with different templates, PCR enzymes and thermal cyclers. Additional guidelines, based on the amount of starting material, are also provided in Table II.

For each sample and control, set up an extra reaction tube to determine the optimal number of PCR cycles. In our experience, each 100 µl reaction typically yields 1–3 µg of ds cDNA after the PCR and purification steps (Sections C and D). To ensure that you have sufficient cDNA for your application, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

Table I. Guidelines for Setting Up PCR Reactions

Total RNA (ng)	Poly A RNA (ng)	Typical Optimal Number of PCR Cycles
—	100	14–16
—	50	15–17
1,000	25	16–18
500	10	17–19
250	5	18–20
100	—	19–21
50	—	20–22
10	—	22–24

IMPORTANT: Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles: 15, 18, 21, 24 and 27 cycles.

1. Preheat the thermal cycler to 95°C.
2. Set up two 100 µl PCR reactions. Combine the following reagents in the order shown:

2 µl	**First-strand cDNA (from Step V.B.8 or V.C.8)**
80 µl	**Deionized H_2O**
10 µl	**10X Advantage 2 PCR Buffer**
2 µl	**50X dNTP Mix** (10 mM)
2 µl	**5' PCR Primer II A** (12 µM)
2 µl	**3' In-Fusion SMARTer PCR Primer** (12 µM)
2 µl	**50X Advantage 2 Polymerase Mix**
100 µl	**Total Volume per reaction**

3. Mix well by vortexing and spin the tubes briefly in a microcentrifuge.
4. Cap the tubes, and place in the preheated thermal cycler. If you are NOT using a hot-lid thermal cycler, overlay the reaction mixture with two drops of mineral oil.

IMPORTANT: Typical cycle numbers are provided as a rough guide for those working with extremely small amounts of RNA. We strongly recommend that you perform a range of cycles to determine the optimal number of cycles for your sample and cycling conditions.

Table II. Cycling Guidelines Based on Starting Material

No. of Cells (e.g., HeLa)	Typical Yield of Total RNA (ng)	Typical No. of PCR Cycles
~100	1.5	26
~1,000	15	22
~10,000	150*	20

5. Commence thermal cycling using the following program:

   - 95°C 1 min
   - X cycles*
     95°C 15 sec
     65°C 30 sec
     68°C 6 min

   *Consult Table I for guidelines. Subject all tubes to 15 cycles. Then, divide the PCR reaction mix between the "Experimental" and "Optimization" tubes, using the Optimization tube for each reaction to determine the optimal number of PCR cycles, as described in Step 6. Store the Experimental tubes at 4°C.

6. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30 µl from each tube to a second reaction tube labeled "Optimization". Store the "Experimental" tubes at 4°C. Using the Tester PCR tube, determine the optimal number of PCR cycles (see Figure 4):

   a. Transfer 5 µl from the 15-cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

   b. Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.

   c. Transfer 5 µl from the 18-cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

   d. Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.

   e. Transfer 5 µl from the 21-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

   f. Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.

   g. Transfer 5 µl from the 24-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

   h. Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.

7. Electrophorese each 5-µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 7 (Appendix A) to confirm that your reactions were successful. Determine the optimal number of cycles required for each experimental and control sample.

8. Retrieve the 15-cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until you reach the optimal number.
9. Split your PCR reactions into two tubes (85 µl each), then add 10 µl of 1% xylene cyanol dye to each tube and mix well. Proceed to **Section VI. ds cDNA Purification and Size Fractionation** or store ds cDNA at –20°C until ready to use.

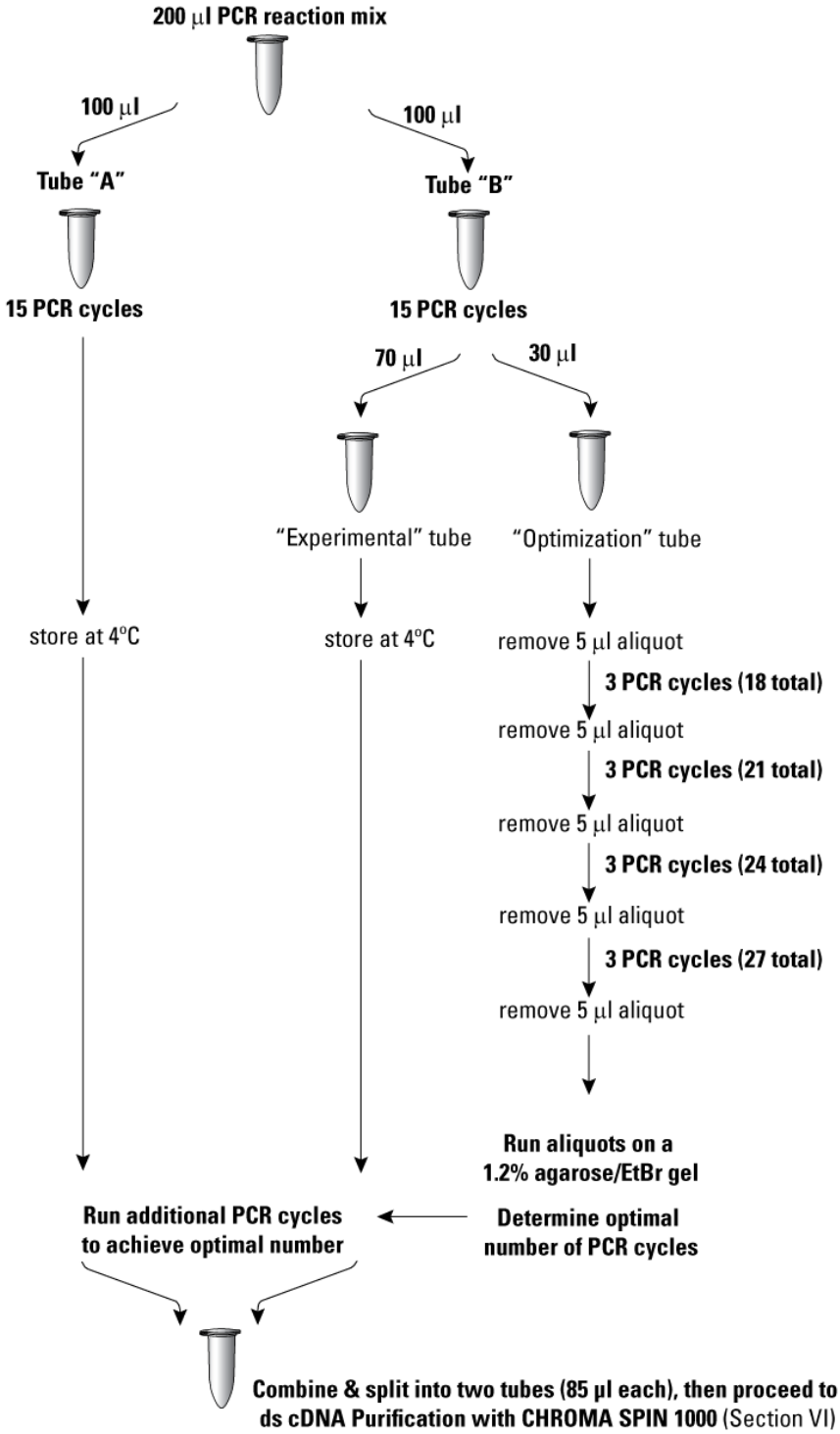

Figure 4. Optimizing PCR parameters for SMARTer cDNA synthesis.

VI. ds cDNA Purification and Size Fractionation

Due to the fact that there is a size bias against large fragments during cloning, clones with very large insert sizes are difficult to find in full-length cDNA libraries (Zhu et al. 2001; Sugahara et al. 2001). To circumvent this size bias, we have included this size fractionation procedure prior to cloning to remove low-molecular-weight cDNA fragments, small DNA contaminants, and unincorporated nucleotides from the cDNA, thereby increasing the proportion of larger inserts in the cDNA library. Such insert sizes are strongly underrepresented in conventionally-generated cDNA libraries (Wellenreuther et al. 2005). Figure 5 shows a typical gel profile of SMARTer ds cDNA before and after size fractionation with the CHROMA SPIN + TE-1000 Column.

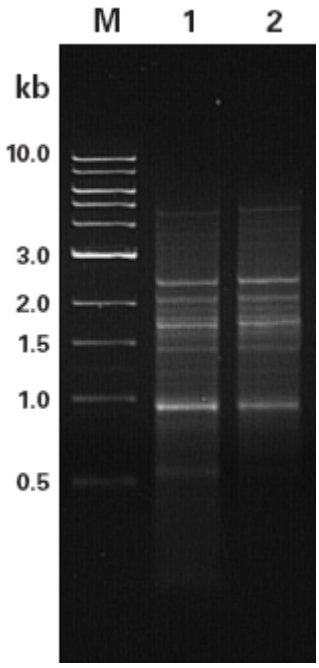

Figure 5. Typical results: ds cDNA PCR product before (Lane 1) & after (Lane 2) size fractionation by the CHROMA SPIN +TE-1000 Column. 1 µl (1 µg) of the Control Mouse Liver Total RNA was used as starting material in a first-strand cDNA synthesis reaction. 2 µl of ss cDNA served as a template for second-strand synthesis using 17 cycles. 2.5 µl samples of the ds cDNA product before (Lane 1) and 1 µl of ds cDNA after (Lane 2) purification with the CHROMA SPIN +TE-1000 column were electrophoresed on a 1.2% agarose/EtBr gel. Lane M: 1-kb DNA size marker (0.1 µg loaded).

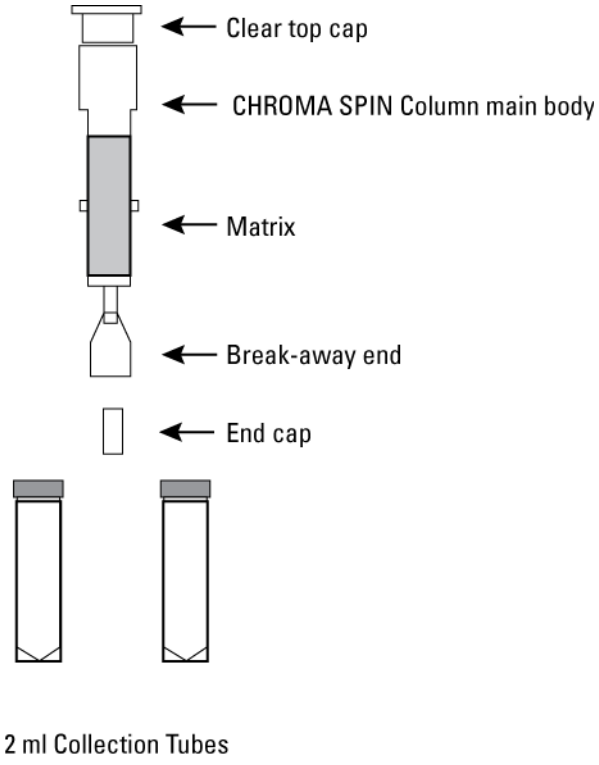

Figure 6. CHROMA SPIN column and collection tubes. Note that a conventional, tapered 1.5-ml microcentrifuge tube can be substituted for the 2-ml collection tube when collecting the purified DNA. This will allow the sample to be confined to a narrower area for easier handling. However, a 2-ml collection tube must be used when purging the equilibration buffer.

A. Protocol: CHROMA SPIN + TE-1000 Column Chromatography

CHROMA SPIN Columns are packed with resin that fractionates molecules based on size. Molecules larger than the pore size are excluded from the resin. These molecules quickly move through the gel bed when the column is centrifuged, while molecules smaller than the pore size are retained and trapped inside the gel matrix. For more information about CHROMA SPIN Columns, please refer to the CHROMA SPIN Columns User Manual, available on our web site at **takarabio.com/manuals**.

1. Label two sets of sixteen 1.5-ml tubes and arrange them in a rack in order.
2. Prepare two CHROMA SPIN +TE-1000 columns for the drip procedure (one for each ~85 µl cDNA sample from Section V.D., Step 9):
    a. Invert the column several times to completely resuspend the gel matrix.
    b. Remove air bubbles from the column by inverting the column several times. 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.
    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 ~85 µl of PCR product and xylene cyanol dye (Section V.D., Step 9) to the top-center surface of the matrix. An unsmooth matrix surface does not hurt 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 (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. On a 1.1% agarose/EtBr gel, electrophorese 3 µl of each fraction (separately) in adjacent wells, alongside a 1-kb DNA size

marker (0.05 µg). Run the gel until the dye front migrates 1–2 cm from the wells (Running the gel longer will make it difficult to see the cDNA bands). Determine the peak fractions by visualizing the intensity of the bands under UV. Collect the first four fractions containing cDNA (in most cases, the fifth fraction containing cDNA is usable. Make sure the fifth fraction matches your desired size distribution). Pool the above fractions in a clean 1.5-ml tube.

11. Repeat steps 4–10 for the second column.
12. Add the following reagents to both tubes with 4–5 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)

13. Mix by gently rocking the tubes back and forth.
14. Place the tubes 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).
15. Centrifuge the tubes at 14,000 rpm for 20 min at room temperature.
16. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
17. Briefly centrifuge the tubes to bring all remaining liquid to the bottom.
18. Carefully remove all liquid and add 600 µl of 80% ethanol.
19. Centrifuge the tubes at 14,000 rpm for 5 min at room temperature.
20. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
21. Carefully remove all liquid and allow the pellets to air dry for ~10 min.
22. Resuspend the pellets in 10 µl of Deionized H_2O and mix gently. Combine the cDNA from both tubes and estimate the yield of cDNA by U.V. spectrophotometry or NanoDrop. Proceed to **Section VII. In-Fusion Cloning of SMARTer ds cDNA into pSMART2IFD**, or store cDNA at –20°C until ready.

VII. In-Fusion Cloning of SMARTer ds cDNA into pSMART2IFD

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING. This protocol is designed for cloning In-Fusion SMARTer cDNA libraries using the pSMART2IFD Linearized Vector. To clone In-Fusion SMARTer cDNA libraries using your own linearized vector, please refer to Appendix D for instructions on how to prepare the vector for cloning. Once you have inverse PCR-amplified your vector with the appropriate primers, you may substitute it for pSMART2IFD in the following protocol. Depending on the antibiotic resistance of your plasmid, you may need different antibiotic plates for the transformation in Section VIII.

A. Protocol: In-Fusion Cloning

The cDNA is now ready to be cloned into the pSMART2IFD Linearized Vector provided with the kit. In the following section three separate cloning reactions are performed (plus one negative control reaction) to ensure an optimal ratio of cDNA to vector. The products of the three cloning reactions will be separately transformed into *E. coli* host cells in Section VIII, and the transformation mixes that produce the highest number of clones will be pooled to form an unamplified library.

Prior to beginning, be sure that you have the competent cells and LB Broth needed for the transformation in Section VIII. You will also need five, 90 mm LB agar plates containing 100 µg/ml ampicillin, 1 mM IPTG, and 75 µg/ml X-Gal.

NOTE: Maximum cloning efficiency is achieved when using a 2:1 molar ratio of insert to vector (~700 ng of SMARTer cDNA plus 300 ng of pSMART2IFD). We recommend using 300 ng of pSMART2IFD (2.7 kb) for a 10-µl In-Fusion Reaction. If using a vector of a different size, the amount used in the reaction should be increased proportionally by size (e.g. 500 ng of a 4.7-kb vector).

1. Set up your In-Fusion cloning reactions as outlined in Table III below:

Table III. Guidelines for Setting Up In-Fusion Cloning Reactions

Reagents	Test Tubes				
	Tube A	Tube B	Tube C	Negative Control	Positive Control
5X In-Fusion Snap Assembly Master Mix	2 µl	2 µl	2 µl	2 µl	2 µl
pSMART2IFD Linearized Vector (150 ng/µl)	2 µl	2 µl	2 µl	2 µl	—
SMARTer cDNA (purified, 150 ng/µl)	4 µl	4.5 µl	5 µl	—	—
ddH_2O	2 µl	1.5 µl	1 µl	6 µl	5 µl
pUC19 Linearized Vector	—	—	—	—	1 µl
Control Insert	—	—	—	—	2 µl
Total Volume	10 µl	10 µl	10 µl	10 µl	10 µl

2. Mix the contents well and spin the tubes briefly in a microcentrifuge.
3. Incubate reactions for **15 min at 50°C**, then transfer the tubes to ice.

4. Add 90 µl of TE and 10 µl of QuickClean Resin to each of the completed reactions from Step 3. Vortex for 1 min, then spin down and transfer the supernatant to fresh tubes. Add 1.2 µl of glycogen to each of the fresh tubes. Mix well and add 280 µl of 100% ethanol. Mix by gently rocking the tubes back and forth. Proceed to Step 5.
5. Place tubes at –70°C or in a dry ice/ethanol bath for 1–2 hr to overnight.

   NOTE: Longer incubation times may increase the yield of DNA after ethanol precipitation.
6. Spin in a microcentrifuge at 15,000 rpm for 20 min and carefully remove the ethanol without disturbing the pellet.
7. Air dry, then resuspend each pellet in 10 µl of sterile H_2O.
8. Proceed to **Section VIII.A. Transformation into Electrocompetent *E. coli*.** If you cannot transform cells immediately, store the completed cloning reactions at –20°C until you are ready.

VIII. Transformation of Recombinant Plasmids into *E. coli*

A. Protocol: Transformation into Electrocompetent *E. coli*

In addition to the experimental transformations described below, we recommend that you perform a positive control transformation with an intact plasmid such as pUC19. Please note that an intact plasmid is not included in the kit, but is typically provided with competent cells.

1. Thaw electrocompetent cells with transformation efficiency >1 x 10^9 cfu/µg on ice. Use the cells promptly after thawing to obtain maximum efficiency in electroporation. Once thawed, cells cannot be refrozen.
2. Add 965 µl of LB broth to 14-ml propylene tubes labeled A, B, and C, and to positive and negative control tubes.
3. Aliquot 25 µl of thawed electrocompetent cells into five pre-chilled 1.5-ml microcentrifuge tubes.
4. Transfer the volumes of reagents listed in the following table to the tubes containing electrocompetent cells mentioned above. Mix DNA and cells gently by pipetting the tubes 4 times.

   Table IV. Guidelines for Setting Up Transformation Mixes

Tube #	Reagents	Volume
1	In-Fusion Reaction A (Tube A, Section VII.A.8)	10 µl
2	In-Fusion Reaction B (Tube B, Section VII.A.8)	10 µl
3	In-Fusion Reaction C (Tube C, Section VII.A.8)	10 µl
4	Negative Control (Section VII.A.8)	10 µl
5	Positive Control (Section VII.A.8)	10 µl

5. Transfer each mixture to a separate, chilled 0.1 cm cuvette.
6. Electroporate and immediately remove the cuvette(s) from the chamber.
7. Transfer the entire volume to the pre-labeled polypropylene tubes containing 965 µl LB broth (prepared in Step 2).
8. Incubate with shaking (225 rpm) for 1 hour at 37°C.

9. During the incubation, label three 1.5-ml polypropylene tubes A, B, and C. Also label tubes for positive and negative controls. Add 50 µl of LB broth to each of these tubes.
10. At the end of the 1-hour incubation, remove 2 µl of each transformation mixture and add it to the appropriate tube containing 50 µl of LB broth. Mix gently by swirling. Store the remaining transformation mixture at 4°C.
11. Spread each 50-µl aliquot on a prewarmed 90-mm LB agar plate containing 100 µg/ml ampicillin, 1 mM IPTG, and 75 µg/ml X-Gal.
12. Allow the plates to dry for 10 min.
13. Invert the plates and incubate at 37°C overnight. The next day, examine your plates and proceed to **Section VIII.B. Pooling Transformation Mixes for Your Library.**

B. Pooling Transformation Mixes for Your Library

1. Some or all of your plates (transformed with 2 µl of transformation mix) should be confluent or nearly confluent (~15,000–30,000 cfu per 150-mm plate). Three such plates should give you a library of approximately 10^6 clones. [The remaining transformation mix (998 µl) contains approximately 500 times more independent clones.]
2. The next day, determine the number of independent clones by plating serial dilutions of the remaining transformation mix.
3. Often one or two of the plates will have considerably fewer colonies than the optimal plate(s). If this is the case (i.e., if you do not have three confluent plates), we recommend that you perform a fourth cloning reaction using all of your remaining ds cDNA. Choose the ratio of cDNA to vector based on which of the original cloning reactions (A, B, or C) gave you the highest number of transformants, and scale up the volumes in Step VII.A.1. Transform and plate this final reaction according to the protocol in Section VIII.
4. If you still do not get three confluent or nearly confluent plates, you may wish to repeat the protocol starting with fresh RNA. However, we do recommend that you pool and amplify the clones that you have at this point. These can be combined with the results of your second construction.
5. [Optional] We recommend that you determine the percentage of recombinant clones in each transformation. To do this, you need to analyze the DNA from 15 independent clones in each transformation. You can screen for inserts by performing PCR directly on colonies (see Appendix C: Protocol for Library Screening by PCR). We recommend that you only add a transformation mix to the pooled library if at least 10 out of 15 clones contain inserts.
6. Based on the above guidelines, pool the desired transformation mixes to generate your original, unamplified cDNA library. You need not perform this or subsequent steps with the positive controls.

IMPORTANT: The original, unamplified library can be stored for up to two weeks at 4°C until you are ready to proceed with **Section X. Amplification of Plasmid Libraries.** Prior to freezing, however, you should determine the titer as outlined in **Section IX. Titrating Plasmid Libraries.**

IX. Titrating Plasmid Libraries

A. Library Titration Precautions

Prior to freezing or amplifying your library, you should determine the titer. To ensure a representative library, the titer should be at least 10-fold higher than the number of independent clones. In general, plasmid libraries should have a titer of at least 10^8 cfu/ml for long-term storage. Keep the following points in mind when titrating the library.

- Diluted libraries are always less stable than undiluted libraries.
- Once 10^{-2} and 10^{-4} dilutions of the library are made, use them within the next hour, before drastic reductions in titer can occur.
- A 2–5-fold range in titer calculations is reasonable, especially if more than one person is doing the titrating.
- Always use the recommended concentration of antibiotic in the medium to ensure plasmid stability.
- Use proper sterile technique when aliquoting and handling libraries.
- Design appropriate controls and include them during plasmid library growth to test for cross-contamination.

B. Protocol: Plasmid Library Titration

1. Prewarm LB/AIX plates at 37°C (or 30°C) for 1–2 hr.
2. Thaw an aliquot of the library and place on ice.
3. Remove 10 µl of the library, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution A (dilution factor = 10^{-2}).
4. Remove 10 µl from Dilution A, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution B (dilution factor = 10^{-4}).

   NOTE: The diluted library is unstable and should be plated within 1 hr.
5. Add 10 µl from Dilution A to 50 µl of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto a prewarmed LB/AIX plate.
6. Remove 50 µl aliquots from Dilution B and spread onto separate LB/AIX plates.
7. Leave plates at room temperature for 15–20 min to allow the inoculum to soak into the agar.
8. Invert the plates and incubate at 37°C (or 30°C) overnight.
9. Count the number of colonies on plates having 30–300 colonies to determine the titer (cfu/ml).

EXAMPLE CALCULATIONS:

1. Calculate the titer (cfu/ml) of your unamplified library:

- titer of your unamplified library =

$$\frac{\text{[\# of colonies on plate]}}{\text{[plating volume (ml)] x [dilution factor]}} = \text{cfu/ml}$$

- titer of your unamplified library =

$$\frac{\text{[50 colonies]}}{\text{[0.05 ml] x [}10^{-4}\text{]}} = 1 \times 10^7 \text{ cfu/ml}$$

X. Amplification of Plasmid Libraries

Use the following protocol to amplify In-Fusion SMARTer cDNA Libraries. You must amplify your cDNA library to obtain enough high-quality plasmid for library screening and for long-term storage of the library.

A. Agar Plate Preparation for Amplification

For initial amplification of the newly constructed unamplified library, all the clones need to be plated and amplified. The exact number of agar plates required depends on the library size. Use the following calculations to determine plating specifications.

EXAMPLE CALCULATIONS:

1. **Calculate the titer (cfu/ml) of your unamplified library:**
   - # of independent clones =
     [titer of pooled unamplified library (cfu/ml)] x [volume of pooled library (ml)]
   - # of independent clones = 1 x 10^6 cfu x [3 ml]) = 3 x 10^6 cfu
2. **Calculate the number of plates to use:**
   - # of plates to use = [# of independent clones] / [20,000 colonies per plate]
   - # of plates to use = [3 x 10^6 cfu] / [20,000 colonies per plate] = 150 plates
3. **Calculate the volume of media needed to plate 150 µl on each plate:**
   - volume of media for plating = [# of plates] x [150 µl per plate]
   - volume of media for plating = [150 plates] x [150 µl per plate] = 2,250 µl = 22.5 ml

1. Dilute the pooled unamplified library into LB/AIX to make up the calculated volume of plating.
   - For example (using the volumes from the example calculations above):

     Dilute 3 ml of pooled unamplified library into 19.5 ml of LB/AIX broth and spread 150 µl onto each of the 150 LB/AIX plates.
2. Allow the agar plates to dry at room temperature for 2–3 days, or at 30°C for 3 hr, prior to plating cells. Moisture droplets on the agar surface can lead to uneven spreading of cells.

B. Protocol: Library Amplification

1. If you have not done so already, titrate the plasmid library (see Section IX).
2. Plate the library directly on selective medium (LB/AIX plates) at a high enough density so that the resulting colonies will be nearly confluent (~20,000–30,000 cfu per 150-mm plate). Plate enough cfu to obtain at least 2–3X the number of independent clones in the library.

   NOTES:
   - The number of independent clones is the number of independent colonies present in the library before amplification.
   - To promote even growth of the colonies, continue spreading the inoculum over the agar surface until all visible liquid has been absorbed, and then allow plates to sit at room temperature for 15–20 min. If using glass beads to spread the colonies, shake the plate back and forth—not round and round.
3. Invert the plates and incubate at 37°C for 18–20 hr.

   NOTE: Growing the transformants on solid medium instead of in liquid culture minimizes uneven amplification of the individual clones.
4. Add 5 ml of LB + 25% glycerol to each plate and scrape the colonies into the liquid. Pool all the resuspended colonies in one flask and mix thoroughly.
   - Set aside five 1-ml aliquots of the library culture in case you wish to re-amplify the library at a later time. Store the aliquots at –80°C.
   - Divide the remainder of the library culture into 50-ml aliquots. Use one of the aliquots for library screening as needed. Store the remainder of the library aliquots at –80°C. For use within one week, aliquots may be stored at 4°C.

XI. Troubleshooting Guide

Problem	Possible Explanation	Solution
Low molecular weight (size distribution < 3 kb, with a majority between 500-200 bp), poor yield, or no PCR product observed for the control mouse liver total RNA	RNAs may have degraded during storage and/or first-strand synthesis. Poor quality RNA starting material will reduce the ability to obtain full-length cDNAs.	RNA must be stored at –70°C. Your working area, equipment, and solutions must be free of contamination by RNase. For best results, freeze cells/tissue immediately following harvest in Buffer RA1 with an RNase inhibitor, then use the NucleoSpin RNA kit to isolate RNA (see Section II. Additional Materials Required for ordering information).
	You may have made an error during the procedure, such as using a suboptimal incubation temperature or omitting an essential component.	Carefully check the protocol and repeat the first-strand synthesis and PCR with your sample and the control RNA.
	The conditions and parameters for PCR may have been suboptimal. The optimal number of PCR cycles may vary with different PCR machines, polymerase mixes, or RNA samples.	Check the protocol and repeat the first-strand synthesis and PCR.
Poor yield or truncated PCR product from your experimental RNA	If your RNA sample was prepared from a nonmammalian species, the apparently truncated PCR product may actually have the normal size distribution for that species. For example, for insects, the normal RNA size distribution may be <2–3 kb.	If you have not already done so, electrophorese a sample of your RNA on a formaldehyde/agarose/EtBr gel to determine its concentration and analyze its quality (see Section IV.D. Assessing the Quality of the RNA Template, for more details).
	The concentration of your experimental RNA is low, but the quality is good.	Repeat the experiment using more RNA and/or more PCR cycles.
	Your experimental RNA has been partially degraded (by contaminating RNases) before or during first-strand synthesis.	Repeat the experiment using a fresh lot or preparation of RNA. Check the stability of your RNA by incubating a small sample in water for 2 hr at 42°C. Then, electrophorese it on a formaldehyde/agarose/EtBr gel alongside an unincubated sample. If the RNA is degraded during incubation, it will not yield good results in the first-strand synthesis. In this case, reisolate the RNA using a different technique, such as the NucleoSpin RNA kit (see Section II. Additional Materials Required, for ordering information).
	Your experimental RNA sample contains impurities that inhibit cDNA synthesis.	In some cases, ethanol precipitation of your existing total RNA, followed by washing twice in 80% EtOH, may remove impurities. If this fails, reisolate the RNA using a different technique, such as the NucleoSpin RNA kit (see Section II. Additional Materials Required, for ordering information).

Problem	Possible Explanation	Solution
Size distribution of ds cDNA product is less than expected	ds cDNA size distribution should be similar to your mRNA, which typically appears within the range of 0.5–10 kb on an agarose/EtBr gel. If the size distributions are not comparable, your RNA starting material may be degraded, very impure, or too dilute.	Check the quality and quantity of your RNA by running a sample on a gel, as recommended in Section IV.D. 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. Also, check the stability of your RNA by incubating a small sample at 37°C for 2 hr. Run it on a gel in parallel with 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. Please note that problems with your RNA are easily diagnosed if you perform parallel reactions using the control RNA provided in this kit.
Low yield of ds cDNA product	Too few thermal cycles used in the PCR step (PCR undercycling). Another indication of PCR undercycling is a cDNA size distribution <4 kb—if the mRNA source was mammalian. (For some sources, such as many insect species, the normal mRNA size distribution may be <2–3 kb.)	If you suspect undercycling, 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 Tables I & II, 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.
	Low yield of first-strand cDNA	Possible problems with the first-strand reaction include a mistake in the procedure (such as using a suboptimal incubation temperature or 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. Reminder: problems with the first-strand cDNA synthesis can be more easily diagnosed if you 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.
No bright bands distinguishable in the ds cDNA product	The conditions and parameters for PCR may have been suboptimal. If bright bands are not visible and the background smear is very intense, this is indicative of PCR overcycling.	If you suspect overcycling, then the PCR step must be repeated *de novo* with a fresh 2-µl sample of first-strand cDNA, using 2–3 fewer cycles.
	The gel running parameters may have altered bright band visibility.	Be sure to use the following conditions for optimal visibility of your bands: a 1X TAE buffer instead of 1X TBE, a gel concentration of 1.1%–1.2% agarose, and a running voltage in the range of 60–90 V.

Problem	Possible Explanation	Solution
Presence of low-molecular-weight material in the ds cDNA product using LD PCR (Section V.C.9)	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. However, these small fragments are generally removed from the ds cDNA preparation in the size fractionation step using the columns provided. Note that a preponderance of low-molecular-weight (<0.1 kb) material in the raw PCR product may be indicative of overcycling.	If you suspect overcycling, then the PCR step must be repeated *de novo* with a fresh 2-µl sample of first-strand cDNA, using 2–3 fewer cycles.
Presence of low-molecular-weight (<0.1 kb) material in the size-fractionated ds cDNA	You may have made an error during the size fractionation procedure (Section VI), such as storing the column at the wrong temperature, or omitting a step. 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 low-molecular-weight contaminants will result in a library having a preponderance of very small inserts and/or apparently nonrecombinant clones.	If you must repeat the ds cDNA synthesis and the size fractionation steps, keep these points in mind: 1. The resolving function of the column will be diminished if the gel matrix becomes dry. 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 elute with the cDNA. 2. 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. **3.** 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.

Problem	Possible Explanation	Solution
No or few colonies obtained from transformation	Plates contained incorrect antibiotic	Check the antibiotic resistance of your fragment. Repeat transformation with appropriate antibiotic plates.
	Low DNA concentration in In-Fusion reaction	It is imperative to obtain the highest DNA concentration possible in your In-Fusion reaction. Either the amount of vector or the amount of PCR fragment was too low. We recommend using 300 ng of pSMART2IFD vector and 600 ng of SMARTer cDNA.
	Bacteria were not competent	Check transformation efficiency. You should obtain >1 x 10^9 cfu/µg for electrocompetent cells.
	Primer sequences are incorrect	If you used your own vector for cloning your In-Fusion SMARTer Library, you may have made a mistake in designing the primers for linearizing the vector by PCR. Check the primer sequences to ensure that they provide 15 bases of homology with the ends of your clone of interest (see Appendix D).
	Bacteria were sensitive to the In-Fusion Enzyme	Use only high transformation efficiency competent cells (>1 x 10^9 cfu/µg) such Stellar™ Electrocompetent Cells. We recommend using QuickClean Enzyme Removal Resin to remove In-Fusion enzyme and any other contaminating proteins from your reaction mixture prior to transformation. In some cases, the QuickClean Enzyme Removal step can double the number of colonies obtained from transformation
Large number of colonies obtained with no insert	Plates too old or contained incorrect antibiotic	Be sure that your antibiotic plates are fresh (<1-month-old). Check the antibiotic resistance of your fragment.
Low titer of unamplified library	Wrong molar ratio of insert:vector	Check the concentration of the size-fractionated cDNA by electrophoresing 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. Check to make sure the total amount of your ds cDNA product is within 2–3 µg, before proceeding to the subsequent cloning procedure. Too much starting cDNA (>3–4 µg) often yields poor cloning efficiencies or low-titer libraries. The control RNA can provide a rough estimate of your target cDNA concentration. On a 1.1% agarose gel, you can compare the intensity of your ds cDNA with the cDNA synthesized from the control RNA provided with this kit. Typically, the control RNA generates 2–4 µg of ds cDNA by this protocol. If the total cDNA amount is higher than recommended, repeat the LD PCR amplification with fewer cycles.

XII. References

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Appendix A: Typical Results of PCR and ds cDNA Synthesis

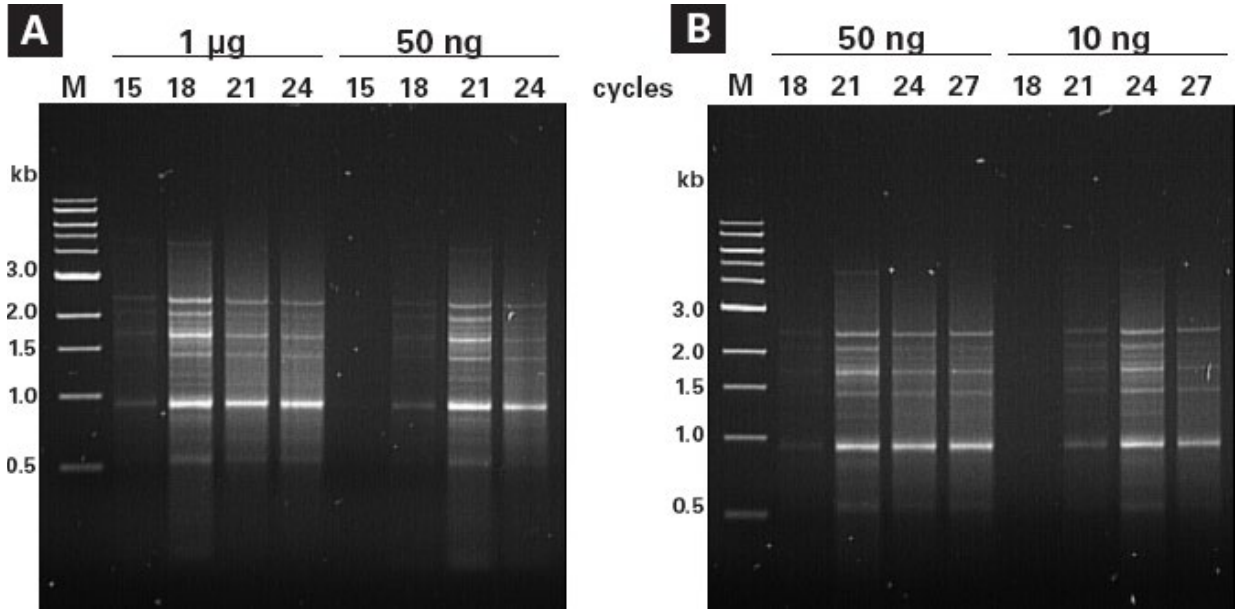
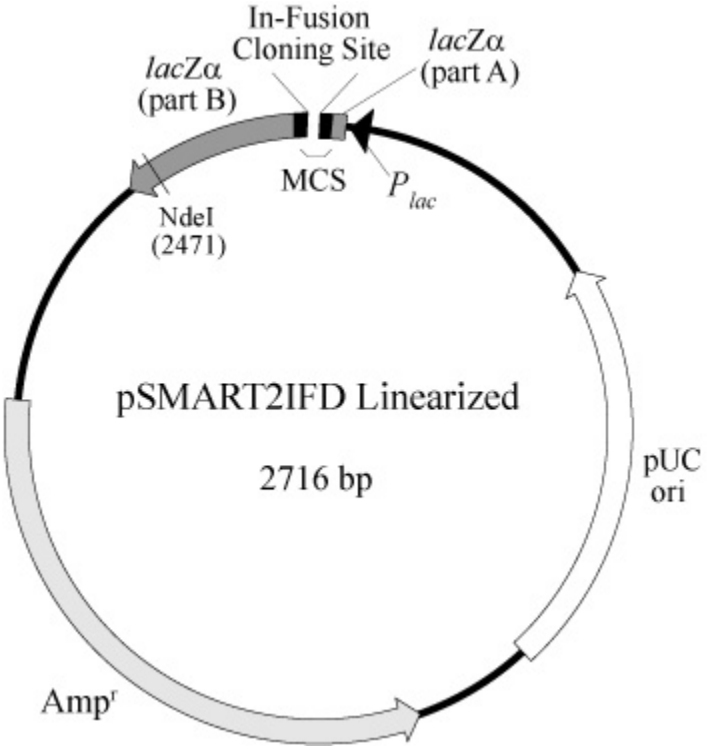


Figure 7. Typical results: ds cDNA synthesized using the SMARTer control reagents and LD PCR protocol. **Panel A**. 1 µg and 50 ng of the Control Mouse Liver Total RNA was subjected to first-strand cDNA synthesis as described in Section V.B, followed by LD-PCR and purification (Sections V.D. & VI). **Panel B.** 50 ng or 10 ng of the Control Mouse Liver Total RNA was subjected to first-strand cDNA synthesis as described in Section V.C, followed by LD-PCR and purification (Sections V.D. & VI). In both experiments, 2 µl of ss cDNA was used for PCR amplification and a range of PCR cycles were performed. 5 µl of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1X TAE buffer following the indicated number of PCR cycles. The optimal number of cycles determined for the experiment in Panel A was 17 for the 1-µg reaction, and 20 for the 50-ng reaction. The optimal number of cycles determined for the experiment in Panel B was 21 for the 50-ng reaction, and 23 for the 10-ng reaction. Lanes M: 1 kb DNA ladder size markers.

Typical results for ds cDNA synthesis using LD-PCR should have the following characteristics:

1. **A moderately strong smear of cDNA from 0.5 to 5 kb (or more):** Compare the intensity of the banding pattern of your PCR product to the 1-kb 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 7. 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 bright bands are distinguishable, this may indicate that too many thermal cycles were used, i.e., PCR overcycling has occurred. 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 XI. Troubleshooting Guide**).
2. **Several bright bands corresponding to abundant transcripts:** The pattern of bright bands shown in Figure 7 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 bright bands may be indicative of PCR overcycling. If the characteristic bands are present but weak, this may be indicative of PCR undercycling. 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 bright 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 7. The non-cDNA contaminants include unincorporated primers, and SMARTer oligonucleotides, as well as primer-dimers. A preponderance of material in the lower part of the gel (i.e., <0.1 kb) may indicate that PCR overcycling has occurred (see **Section XI. Troubleshooting Guide**).

Appendix B: pSMART2IFD Linearized Vector Map and In-Fusion Cloning Site



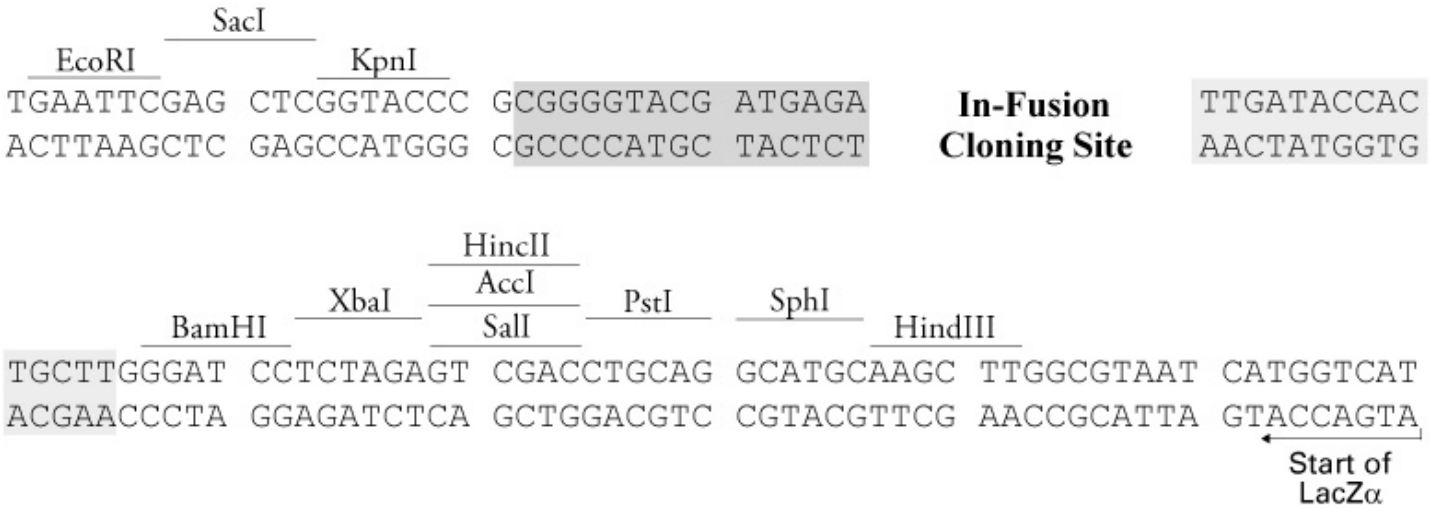


Figure 8. pSMART2IFD linearized vector map and In-Fusion Cloning site. The pSMART2IFD Linearized Vector is a pUC19-based, high copy number, *E. coli* cloning vector. This linearized vector was generated by PCR, and contains the blunt ends shown flanking the In-Fusion Cloning Site in the sequence above. Each end of the vector shares 15 bp of complementarity (shaded above) with a different end of the cDNA generated using the In-Fusion SMARTer PCR cDNA Synthesis Kit (Cat. No. 634934). The ends are different to allow for directional cDNA cloning. The In-Fusion Cloning Site is located within the *lacZα* gene (encoding the N-terminal fragment of β-galactosidase), the expression of which is driven by the *lac* promoter (P_{lac}). The location of the cloning site within *lacZα* allows for blue/white selection (i.e., α-complementation) after In-Fusion cloning. The vector also contains a pUC origin of replication that allows propagation in *E. coli* and temperature-dependent regulation of vector copy number, and an ampicillin resistance gene (Amp^r) for selection in *E. coli*.

Appendix C: Protocol for Library Screening by PCR

In-Fusion SMARTer Libraries can be screened using the primers provided to determine the percentage of recombinant clones. You can also use two gene-specific primers to verify that your target gene is present. We recommend the Advantage 2 PCR Kit (Nos. 639206 & 639207) for efficient long-distance DNA amplification.

1. Pick 15 isolated colonies with sterile toothpicks and inoculate each into 15 µl of H_2O in separate 0.5-ml microcentrifuge tubes.
2. Boil tubes for 5 min, or heat tubes at 95°C in a thermal cycler for 5 min.
3. Set up a 25-µl PCR reaction:

5.0 µl	**Boiled colony lysate**
15.5 µl	**Deionized H_2O**
2.5 µl	**10X Advantage 2 PCR Buffer**
0.5 µl	**50X dNTP Mix** (10 mM each)
0.5 µl	**Forward Screening Primer** (10 µM)
0.5 µl	**Reverse Screening Primer** (10 µM)
0.5 µl	**50X Advantage 2 Polymerase Mix**
25 µl	**Total Volume**

NOTE: If not using a "hot lid" thermal cycler, overlay each tube with 2 drops of mineral oil.

4. Run PCR using the following conditions:

- 3 cycles:
  95°C 30 sec
  65°C 30 sec
  68°C 3 min

- 25 cycles:
  95°C 30 sec
  68°C 3 min

5. Electrophorese 3 µl of PCR product on a 1.2% TAE/agarose gel with DNA size markers. If no band appears, run five additional 2-step cycles. Typically, bands >700 bp in size are seen for colonies containing inserts.

Appendix D: Preparing Your Vector for In-Fusion SMARTer cDNA Library Cloning

In-Fusion SMARTer cDNA Libraries can easily be generated from your desired cloning vector, provided that you prepare your desired vector for In-Fusion cloning as described below.

A. Primer Design

1. Identify the vector you want to use to clone your SMARTer cDNA Library.
2. Choose the location on the vector where you want to insert the library; any location can serve as the insertion point. This will also be the vector linearization site.
3. Design two primers to amplify and linearize the vector by PCR. One primer should include 18–22 bases of sequence from one side of the vector linearization site, plus a 15-base sequence (**TTGATACCACTGCTT**) complementary to the SMARTer V Oligo. The other primer should include 18–22 bases of sequence from the other side of the vector linearization site, plus a 15-base sequence (**TCTCATCGTACCCCG**) complementary to the 3' In-Fusion SMARTer CDS Primer (see Figure 9). The resulting linearized vector will contain the SMARTer V Oligo sequence at one end, and the 3' In-Fusion SMARTer CDS Primer sequence at the other.

B. Protocol: Preparation of a Linearized Vector by PCR

1. In a 200-µl PCR tube mix the following components:

2.0 µl	**Your Vector** (2 ng/µl)
2.0 µl	**Primer 1 (10 µM)** (From Section A. Primer Design)
2.0 µl	**Primer 2 (10 µM)** (From Section A. Primer Design)
10.0 µl	**5X Advantage HD Buffer**
1.0 µl	**10 mM dNTPs**
0.5 µl	**50X Advantage HD Polymerase Mix**
32.5 µl	**RNase-Free H_2O**
50 µl	**Total Volume**

2. Run PCR using the following conditions:

- 23 cycles*:
  96°C 20 sec
  68°C 1 min/kb vector
- 1 cycle:
  72°C 3 min

*** NOTE:** We recommend that you use a range of PCR cycles (20, 22, 24, 26) to determine the optimal number of PCR cycles for your vector preparation.

3. After the PCR reaction has been completed, add 2 µl of Dpn I enzyme (5 U/µl) to the 50-µl PCR reaction and incubate at 37°C for 1 hr.
4. Run a gel with 1 µl of PCR product to make sure there is a single PCR band.
5. Purify the rest of the PCR reaction using a spin-column purification kit such as NucleoSpin Gel and PCR Clean-Up (Cat. Nos. 740609.50 & 740609.250).
6. Once you have amplified and purified your vector, combine the linearized vector with your cDNA library in an In-Fusion cloning reaction, as described in **Section VII.A. In-Fusion Cloning.**

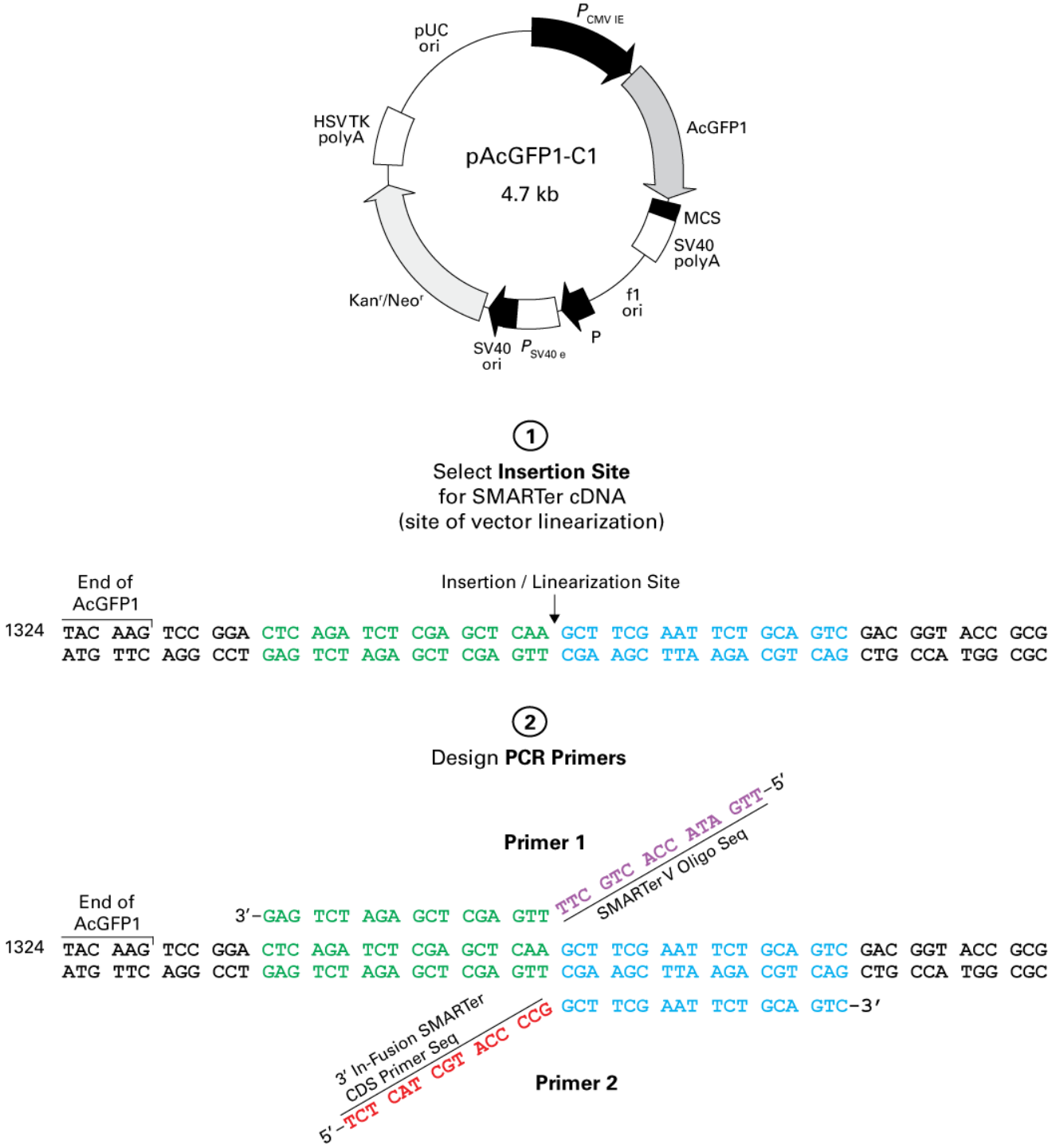


Figure 9. How to simultaneously linearize your vector and introduce In-Fusion SMARTer sequences. Any vector can be used for In-Fusion SMARTer Library cloning—here we use Takara Bio's pAcGFP1-C1 Vector (Cat. No. 632470) to demonstrate. First, select an insertion site/vector linearization site--we chose a site just downstream of the pAcGFP1-C1 coding sequence (identified by the arrow, above). Next, design PCR primers that will introduce SMARTer sequences onto the ends of the vector at the site of linearization. To do this, design one primer so that it contains 18–22 bases of vector sequence from one side of the insertion/linearization site (green, above) and 15 bases of sequence (**TTGATACCACTGCTT**) complementary to the SMARTer V Oligo; design the other primer so that it contains 18–22 bases of vector sequence from the other side of the insertion/linearization site (blue, above) and 15 bases of sequence (**TCTCATCGTACCCCG**) complementary to the 3' In-Fusion SMARTer CDS Primer. The following primers would be required to linearize pAcGFP1-C1 and introduce In-Fusion SMARTer sequences at the site indicated above:

Primer 1: 5'-TTGATACCACTGCTTTTGAGCTCGAGATCTGAG-3'
Primer 2: 5'-TCTCATCGTACCCCGGCTTCGAATTCTGCAGTC-3'

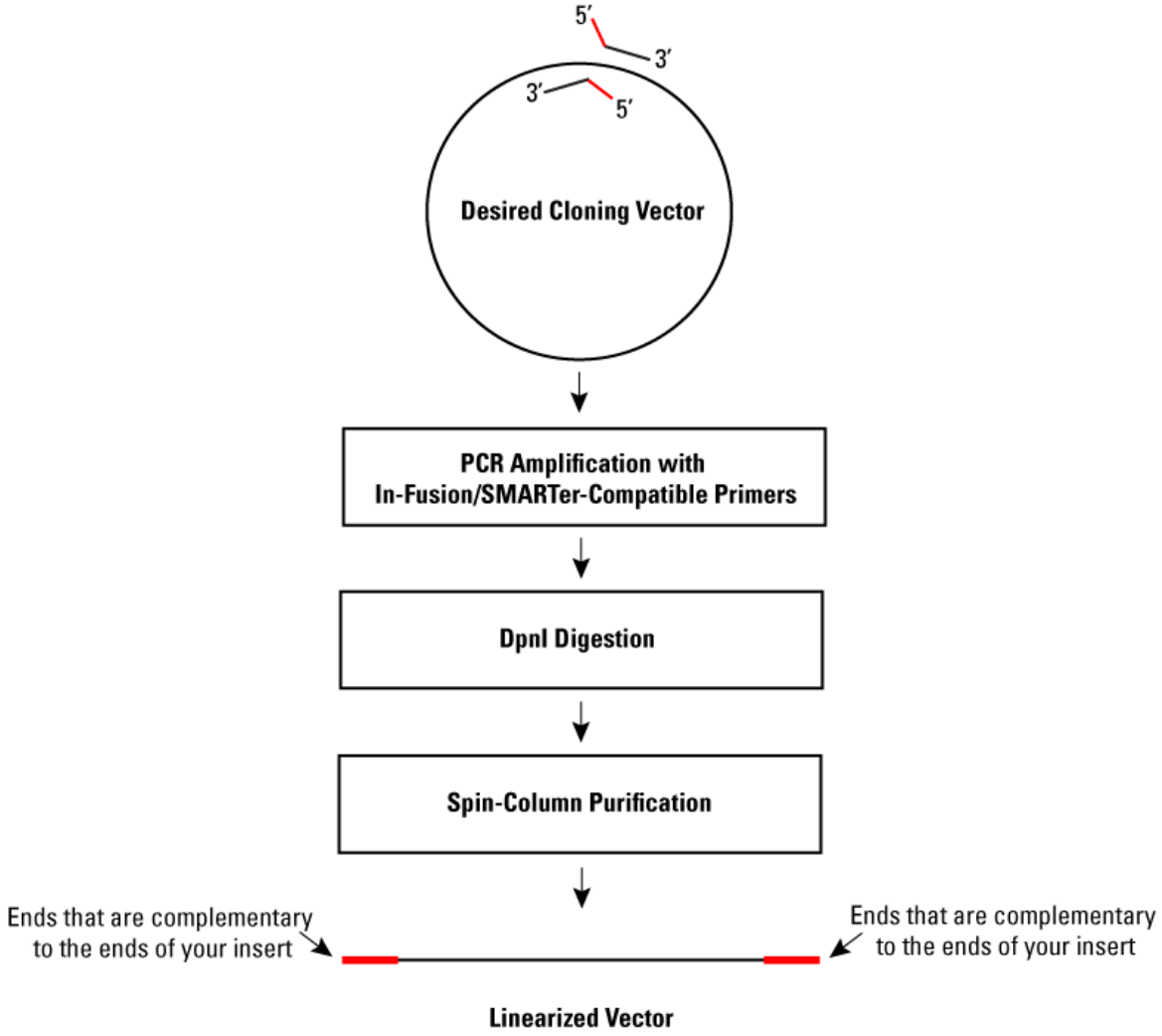


Figure 10. Preparation of a linearized vector by PCR.

Appendix E: SMARTer ds cDNA Synthesis by LD-PCR using Advantage HD

Table I provides guidelines for optimizing your PCR, depending on the amount of total RNA used in the first-strand synthesis. These guidelines were determined using the Control Mouse Liver Total RNA, Advantage HD Polymerase and a hot-lid thermal cycler; optimal parameters may vary with different templates, PCR enzymes and thermal cyclers. Do not use less than 50 ng of Total RNA.

For each sample and control, set up an extra reaction tube to determine the optimal number of PCR cycles. In our experience, each 100 µl reaction typically yields 1–3 µg of ds cDNA after the PCR and purification steps. To ensure that you have sufficient cDNA for your application, you should estimate the yield of SMARTer cDNA by UV spectrophotometry.

Table V. Guidelines for Setting Up PCR Reactions

Total RNA (ng)	Typical Optimal No. of PCR Cycles*
1,000	15–17
250	17–19
50	19–21

***IMPORTANT:** Optimal parameters may vary with different templates and thermal cyclers. Typical cycle numbers are provided as a rough guide for those working with extremely small amounts of RNA. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles to determine the optimal number of cycles for your sample and cycling conditions: 15, 18, 21, 24, and 27 cycles.

1. Preheat the thermal cycler to 95°C.
2. Set up two 100-µl PCR reactions. Combine the following reagents in the order shown:

2 µl	**First-strand cDNA (from Step V.B.8 or V.C.8)**
71 µl	**Deionized H_2O**
20 µl	**5X Advantage HD Buffer**
2 µl	**50X dNTP Mix** (10 mM)
2 µl	**5' PCR Primer II A** (12 µM)
2 µl	**3' In-Fusion SMARTer PCR Primer** (12 µM)
1 µl	**Advantage HD Polymerase Mix**
100 µl	**Total Volume per reaction**

3. Mix well by vortexing and spin the tubes briefly in a microcentrifuge.
4. Cap the tube, and place it in the preheated thermal cycler. If you are NOT using a hot-lid thermal cycler, overlay the reaction mixture with two drops of mineral oil.
5. Commence thermal cycling using the following program:
   - X cycles*:

   98°C 10 sec
   55°C 15 sec
   68°C 6 min

   *Consult Table I for guidelines. **Subject all tubes to 15 cycles.** Then, divide the PCR reaction mix between the "Experimental" and "Optimization" tubes, using the Optimization tube for each reaction to determine the optimal number of PCR cycles, as described in Step 6. Store the Experimental tubes at 4°C.

6. Subject each reaction tube to 15 cycles, then pause the program. Transfer 30 µl from each tube to a second reaction tube labeled "Optimization". Store the "Experimental" tubes at 4°C. Using the Tester PCR tube, determine the optimal number of PCR cycles (see Figure 4):

    a. Transfer 5 µl from the 15-cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

    b. Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.

    c. Transfer 5 µl from the 18-cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/ EtBr gel analysis).

    d. Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.

    e. Transfer 5 µl from the 21-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

    f. Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.

    g. Transfer 5 µl from the 24-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

    h. Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.

7. Electrophorese each 5 µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Determine the optimal number of cycles required for each experimental and control sample.

8. Retrieve the 15-cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until you reach the optimal number.

9. When the cycling is completed, analyze a 5 µl sample of each PCR product alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 7 (Appendix A) to confirm that your reactions were successful.

10. Proceed to **Section VI. ds cDNA Purification and Size Fractionation** or store ds cDNA at –20°C until ready to use.

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