Protocol-at-a-Glance

SMART® cDNA Library Construction Kit
Protocol-at-a-Glance
(PT3000-2)

Please read the *User Manual* (PT3000-1) before using this abbreviated protocol. The Protocol-at-a-Glance is provided for your convenience, but is not intended for first-time users. Some steps are numbered differently here than in the *User Manual*.

A. First-Strand Synthesis

1. Combine the following reagents in a sterile 0.5-ml microcentrifuge tube:
   - 1–3 µl RNA sample
     (For the control reaction, use 1 µl [1 µg] of the control RNA.)
   - 1 µl SMART IV Oligonucleotide
   - 1 µl CDS III/3' PCR Primer

   If total volume is <5 µl, add Deionized H_2O to bring volume up to 5 µl.
2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 72°C for 2 min. Cool on ice for 2 min.
4. Spin the tube briefly to collect the contents at the bottom.
5. Add the following to each reaction tube:
   - 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
6. Mix the contents of the tube by gently pipetting and briefly spinning the tube.
7. Incubate the tube at 42°C for 1 hr. Place the tube on ice. For cDNA amplification by LD PCR, proceed to Step B.
8. *For ds cDNA Synthesis by Primer Extension*:
   Add 1 µl of Sodium Hydroxide to the tube and incubate at 68°C for 30 min.
   Proceed to Step C.

B. cDNA Amplification by *LD PCR*

1. Combine the following components in a fresh 0.5-ml tube:
   - 2 µl First-Strand cDNA (from Step A.7)
   - 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
2. Mix contents by gently flicking the tube. Centrifuge briefly to collect the contents at the bottom of the tube.
3. Add two drops of mineral oil if necessary. Cap the tube, and place it in a preheated (95°C) thermal cycler.

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4. Commence thermal cycling using one of the following programs:

GeneAmp 480	GeneAmp 2400/9600
• 95°C 1 min	• 95°C 20 sec
• x cycles*:	• x cycles*:
95°C 15 sec	95°C 5 sec
68°C 6 min	68°C 6 min

*Refer to Table I for the optimal number of cycles to use.

TABLE I: 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

5. Analyze a 5-µl sample of the PCR product on a 1.1% agarose/EtBr gel, alongside 0.1 µg of 1-kb DNA size marker. If the results are not as expected (Appendix A of the *User Manual*), refer to the Troubleshooting Guide (Section VIII of the *User Manual*).
6. Proceed to Step D, below.

C. ds cDNA Synthesis by *Primer Extension*

1. Combine the following components:

11	µl	First-Strand cDNA (from Step A.8)
71	µl	Deionized H_2O
10	µl	10X Advantage 2 PCR Buffer
2	µl	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

2. Mix contents by gently flicking the tube. Centrifuge briefly to collect the contents at the bottom of the tube.
3. Add 2 drops of mineral oil if necessary. Cap the tube and place it in a preheated (95°C) thermal cycler.
4. Commence thermal cycling using the one of the following programs:

GeneAmp 480	GeneAmp 2400/9600
• 72°C 10 min	• 72°C 10 min
• 95°C 1 min	• 95°C 20 sec
• 3 cycles:	• 3 cycles:
95°C 15 sec	95°C 5 sec
68°C 8 min	68°C 8 min

5. Analyze a 5-µl sample of the PCR product on a 1.1% agarose/EtBr gel, alongside 0.1 µg of 1-kb DNA size marker. If the results are not as expected (Appendix A of the *User Manual*), refer to the Troubleshooting Guide (Section VIII of the *User Manual*).

D. Proteinase K Digestion

1. Combine 50 µl of the amplified ds cDNA (2–3 µg) with 2 µl of Proteinase K (20 µg/µl) in a sterile 0.5-ml tube. Store remaining ds cDNA at –20°C.
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.
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. Transfer 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. Transfer 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.
12. Carefully remove the supernatant.
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.

E. SfiI Digestion

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

79	µl	cDNA (Step D.15)
10	µl	10X *Sfi* Buffer
10	µl	SfiI Enzyme
1	µl	100X BSA
100	µl	Total volume

2. Mix well. Incubate at 50°C for 2 hr.
3. Add 2 µl of 1% xylene cyanol dye to the tube above. Mix well.

F. 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. Remove and warm the CHROMA SPIN Column at room temperature for about 1 hr. Invert the column to completely resuspend the gel matrix.
   b. Remove air bubbles from the column. Use a 1000-µl pipettor to resuspend the matrix gently, and remove the bottom cap to let the column drip.
   c. Attach the column to a ring stand.
   d. Drain the storage buffer by gravity flow. (The top of the column matrix should be at the 1.0-ml mark on the wall of the column.)
   e. The flow rate should be ~1 drop/40–60 sec, and the volume of 1 drop should be ~40 µl.
3. When the storage buffer stops dripping out, add 700 µl of column buffer and allow it to drain out.
4. When this buffer stops dripping, apply ~100 µl mixture of SfiI-digested cDNA and xylene cyanol dye (Step E.3 above) to the top-center surface of the matrix.
5. Allow the sample to be fully absorbed into the surface of the matrix before proceeding to the next step.
6. Wash the tube that contained the cDNA with 100 µl of column buffer, 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.
8. Place the rack containing the collection tubes 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 (~35 µl per tube) in tubes #1–16. Cap each tube after each fraction is collected. Recap the column after fraction #16 has been collected.
10. Electrophorese 3 µl of each fraction on a 1.1% agarose/EtBr gel, alongside 0.1 µg of 1-kb DNA size marker. Run the gel for 10 min at 150 V. Determine the peak fractions by visualizing the intensity of the bands under UV. Collect the first three fractions containing cDNA. 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.
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.

G. Ligation of cDNA to Vector

1. Label three 0.5-ml tubes and add the indicated reagents (Table II). Mix the reagents gently. Spin tubes briefly to bring contents to the bottom of the tube.

TABLE II: LIGATIONS USING THREE DIFFERENT RATIOS OF cDNA TO VECTOR

Component	1st ligation (µl)	2nd ligation (µl)	3rd ligation (µl)
cDNA (from Step F.18)	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 (10mM)	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

2. Incubate tubes at 16°C overnight.
3. Perform a separate λ-phage packaging reaction for each of the ligations.
4. Titer each of the resulting libraries (Section H). 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.
5. [Optional] If you obtained <1–2 x 10^6 clones, you may wish to perform another ligation with the remaining cDNA. Repeat ligation using the ratio of cDNA to vector (of the initial three ligations) that gave the best results. Scale up the volumes of all reagents according to the amount of cDNA used. Then package and titer this scaled-up ligation. If the titer is still low, see Section VIII of the User Manual for troubleshooting tips.
6. To increase the stability of your library, first combine the packaging reactions from Step G.4 above, then amplify the library as described in Section J below. 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.

H. Titering the Unamplified Library

1. Pick a single, isolated colony from the working stock plate and use it to inoculate 15 ml of LB/maltose/$MgSO_4$ broth in a 50-ml test tube. Incubate at 37°C overnight with 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 supernatant, and resuspend the pellet in 7.5 ml of 10 mM $MgSO_4$.

2. Plan and prepare the number of 90-mm LB/$MgSO_4$ agar plates you will need. Prewarm plates at 37°C.
3. Dilute the unamplified lysates in 1X lambda dilution buffer (dilution range = **1:5 to 1:20)**.
4. Add 1 µl of the diluted phage to 200 µl of the XL1-Blue overnight culture, and allow the phage to adsorb for 10–15 min at 37°C.
5. Add 2 ml of melted LB/$MgSO_4$ top agar. Mix by quickly inverting and immediately pour onto 90-mm LB agar plates prewarmed to 37°C.
6. Cool the plates at room temperature for 10 min. Invert the plates and incubate at 37°C for 6–18 hr.
7. Count the plaques and calculate the titer of the phage (pfu/ml):

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

I. Determining the Percentage of Recombinant Clones

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.

To perform blue/white screening, follow the procedure for titering an unamplified library on LB/$MgSO_4$ plates (Step.H, above), 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. 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.

The ratio of white (recombinant) to blue (nonrecombinant) plaques will give you a quick estimate of recombination efficiency. A successful ligation will result in at least 80% recombinants. If your recombination efficiency is lower than this, see Section VIII of the *User Manual* for troubleshooting tips.

[Optional] PCR Insert Screening of SMART PCR cDNA Library

To test ligation efficiency, we recommend screening your cDNA insert using Clontech's Advantage 2 cDNA PCR Kit and λTriplEx LD-Insert Screening Amplimers (639206 and #9107-1, respectively). Efficient ligation of the cDNA to the λTriplEx2 Vector should result in more than 80% recombinants.

J. Library Amplification

The number of plates required depends on how many independent clones are in the library to be amplified. Aim for 6–7 x 10^4 clones (or plaques) per 150-mm plate; thus, a library of 1 x 10^6 clones will require 20 plates.

1. Pick a single, isolated colony from the primary working plate and use it to inoculate 15 ml of LB/maltose/$MgSO_4$ broth. 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 10 mM $MgSO_4$.
2. Plan and prepare the number of LB/$MgSO_4$ agar plates you will need.
3. Set up the required number of 4-ml tubes with 500 µl of bacterial culture and enough diluted lysate to yield 1 x 10^5 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.
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. Incubate plates at room temperature for 1 hr on the platform shaker (~50 rpm).
10. Pour the lambda phage lysates into a sterile beaker. Mix the pooled lysate well and transfer it into a sterile, 50-ml polypropylene screw-cap tube.
11. Add 10 ml of chloroform to the lysate and vortex for 2 min.
12. 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.
13. Determine the titer of the amplified library (Section K below).
14. The amplified library can be stored at 4°C for up to 6 months. For long-term storage (up to at least one year), make 1-ml aliquots, add DMSO to a final concentration of 7%, and place at –70°C. Avoid repeated freeze/thaw cycles.

K. Titering the Amplified Library

1. Pick a single, isolated colony from the working stock plate and use it to inoculate 20 ml of LB/maltose/$MgSO_4$ 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 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. Prepare four LB/$MgSO_4$ agar plates (90-mm size).
3. Pipet 10 µl of the library lysate into 1 ml of 1X lambda dilution buffer (Dilution 1 = 1/100).
4. Transfer 10 µl of Dilution 1 into a second tube containing 1 ml of 1X lambda dilution buffer (Dilution 2 = 1/10,000).
5. Prepare four tubes as described in Table III using the bacterial overnight culture obtained from Step K.1 and phage Dilution 2 from Step K.4 above.

TABLE III: PLATING DILUTIONS FOR TITERING AN AMPLIFIED LIBRARY

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

6. Incubate tubes in a 37°C water bath for 15 min.
7. Add 3 ml of melted (45°C) LB/$MgSO_4$ top agar to each of the four tubes.
8. Quickly mix and pour the contents from each tube onto separate LB/$MgSO_4$ agar plates.
9. Cool plates at room temperature for 10 min to allow the soft agar to harden.
10. Incubate plates (inverted position) at 37°C for at least 6–7 hr.
11. 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

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