

Creator™ SMART™ cDNA Library Construction Kit (Catalog #: K1053)

Full user manual PT3577-1 (PR12792)

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Shortened User Manual (Kasia Hammar)

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

This protocol is for library construction of Creator SMART cDNA libraries using a PCR-based protocol. The minimum amount of starting material for cDNA synthesis is 50 ng of total RNA or 25 ng of poly A+ RNA. In general, the more RNA you start with, the fewer PCR cycles will be required for the second strand synthesis. Using fewer thermal cycles reduces the level of nonspecific PCR products, and therefore is optimal for cDNA and library quality.

Important:

1. Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.
2. When resuspending pellets or mixing reactions, gently pipet the solution up and down or tap the bottom of the tube. Spin tube briefly to bring contents to the bottom of the tube. Do not vortex samples when resuspending pellets; vortexing may cause shearing of your cDNA.
3. Perform all reactions on ice, unless otherwise indicated.
4. Add enzymes to reaction mixtures last. Make sure that the enzyme is thoroughly blended into the reaction mixture by gently pipetting the mixture up and down.
5. Do not increase the size (volume) of any of the reactions. All components have been optimized for the volumes specified.
6. Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing of this reagent.
7. Phenol is a corrosive. If possible, handle solutions containing phenol and/or chloroform under a chemical fume hood or be extra careful.
8. Use only provided Milli-Q-grade Rnase, Dnase free H₂O
9. Use only single-use plastic pipettes and pipette tips.
10. Isolated RNA sample is provided. The integrity of the RNA is estimated by gel electrophoresis and spectrophotometric analysis.

Day 1 10/6 8:30-1pm 3:30-5pm

A. First-Strand cDNA Synthesis

1. Combine the following reagents in a sterile 0.5-ml microcentrifuge tube:
 - 1–3 ul RNA sample
 - 1 ul SMART IV Oligonucleotide
 - 1 ul CDS III/3' PCR PrimerAdd deionized H₂O to a total volume of 5 ul, if necessary.
2. Mix gently and spin the tube briefly in a microcentrifuge
3. Incubate the tube at 72°C for 2 min.
4. Cool the tube on ice for 2 min.
5. Spin the tube briefly to collect the contents at the bottom.

6. Add the following to each reaction tube:
 - 2.0 ul 5X First-Strand Buffer
 - 1.0ul DTT (20 mM)
 - 1.0ul dNTP Mix (10 mM)
 - 1.0ul PowerScript Reverse Transcriptase
 - 10.0 ul total volume
7. Mix the contents of the tube by gently pipetting and briefly spinning the tube and cover the reaction mixture with one drop of mineral oil before you close the tube (prevents loss of volume due to evaporation)
8. Incubate the tube at 42°C for 1 hr (water bath or heating block)
9. Place the tube on ice to terminate first-strand synthesis.
10. If you plan to proceed directly to the PCR step, take a 2-ml aliquot from the first-strand synthesis and place it in a clean, prechilled, 0.5-ml tube. (If you used mineral oil in your first-strand reaction tube, be careful to take the 2-ml sample from the bottom of the tube to avoid the oil.) Place the tube on ice.
11. Any first-strand reaction mixture that is not used right away should be placed at –20°C. First-strand cDNA can be stored at –20°C for up to three months.

B. cDNA amplification by LD PCR (Double Strand cDNA Synthesis)

Use the fewest number of cycles shown for that amount of RNA. Fewer cycles generally mean fewer nonspecific PCR products. Undercycling can be rectified by placing the PCR reaction back in the thermal cycler for a few more cycles.

1. Preheat the PCR thermal cycler to 95°C.

2. Combine the following components in the reaction tube:
 - 2 ul First-Strand cDNA
 - 80 ul Deionized H₂O
 - 10 ul 10X Advantage 2 PCR Buffer
 - 2 ul 50X dNTP Mix
 - 2 ul 5' PCR Primer
 - 2 ul CDS III/3' PCR Primer
 - 2 ul 50X Advantage 2 Polymerase Mix
 - 100 ul total volume
3. Mix contents by gently flicking the tube. Centrifuge briefly to collect the contents at the bottom of the tube.
4. Cap the tube and place it in a preheated (95°C) thermal cycler.
5. Commence thermal cycling using one of the following programs:
 - 95°C 20 sec
 - 2 Temp 20 cycles
 - 95°C 5sec. 68°C 6min.

Gel run

6. When the cycling is complete, analyze a 5-ml sample of the PCR product, alongside 0.1mg of 1-kb DNA size markers, on a 1.1% agarose/EtBr gel.

The ds cDNA should appear as a 0.1–4 kb smear on the gel, with some bright bands corresponding to the abundant mRNAs for that tissue or cell source. cDNA prepared from some tissues may not have distinct bright bands, especially if the mRNA is highly complex.

7. Store ds cDNA at –20°C until use.

Day 2 10/6 9:30-12pm

C. Proteinase K Digestion

1. In a sterile 0.5-ml tube, pipet 50 ul of amplified ds cDNA (2–3 ug), making sure you pipet below the top oil layer of the PCR tube and add 2 ul of proteinase K (20 ug/ul). Store the remaining ds cDNA at –20°C (up to 3 months).
2. Mix contents and spin the tube briefly.
3. Incubate at 45°C for 20 min. Spin the tube briefly.
4. Add 50 ul of H₂O to the tube.
5. Add 100 ul of phenol:chloroform:isoamyl alcohol and mix by continuous gentle inversion for 1–2 min.
6. Centrifuge at 14,000 rpm for 5 min to separate the phases.
7. Move the top (aqueous) layer to a clean 0.5-ml tube. Discard the interface and lower layers.

8. Add 100 ul of chloroform:isoamyl alcohol to the aqueous layer. Mix by continuous gentle inversion for 1–2 min.
9. Centrifuge at 14,000 rpm for 5 min to separate the phases.
10. Move the top (aqueous) layer to a clean 0.5-ml tube. Discard the interface and lower layers.
11. Add 10 ul of 3 M sodium acetate, 1.3 ul of glycogen (20 mg/ml) and 260 ml of room-temperature 95% ethanol. Immediately centrifuge at 14,000 rpm for 20 min at room temperature.

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

12. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
13. Wash pellet with 100 ul of 80% ethanol.
14. Air dry the pellet (~10 min) to evaporate off residual ethanol.
15. Add 79 ul of H_2O to resuspend the pellet.

D. Sfi I Digestion

1. Combine the following components in a fresh 0.5-ml tube:
 - 79 ul cDNA
 - 10 ul 10X Sfi Buffer
 - 10 ul Sfi I Enzyme
 - 1 ul 100X BSA
 - 100 ul total volume
2. Mix well. Incubate the tube at 50°C for 2 hr. (**go to E.**)
3. Add 2 ul of 1% xylene cyanol dye to the tube above. Mix well.

Continue Day 2 1-5pm 7-9pm (if needed)

E. cDNA Size Fractionation

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:
3. Invert the column several times to completely resuspend the gel matrix.
4. Remove air bubbles from the column. Use a 1000-ul pipettor to resuspend the matrix gently; avoid generating air bubbles. Then remove the bottom cap and let the column drip naturally. (If the column does not drain after 3 min, recap the top cap. This pressure should cause the column to drain).
5. Attach the column to a ring stand.

6. Let the storage buffer drain through the column by gravity flow until you can see the surface of the gel beads in the column matrix. The top of the column matrix should be at the 1.0-ml mark on the wall of the column. If your column contains significantly less matrix, adjust the volume of the matrix to the 1.0-ml mark using matrix from another column (an extra column is provided)
7. The flow rate should be approximately 1 drop/40–60 sec. The volume of 1 drop should be approximately 40 ul. 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 ul), you should resuspend the matrix completely and repeat the drip procedure until it reaches the above parameters.
8. When the storage buffer stops dripping out, carefully and gently (along the column inner wall) add 700 ul of column buffer to the top of the column and allow it to drain out.
9. When this buffer stops dripping (~15–20 min), carefully and evenly apply ~100 ml mixture of Sfi I-digested cDNA and xylene cyanol dye to the top-center surface of the matrix. An irregular matrix surface does not hurt the following fractionation process.
10. Before proceeding to the next step, allow the sample to be fully absorbed into the surface of the matrix (there should be no liquid remaining above the surface).
11. With 100 ul of column buffer, wash the tube that contained the cDNA, and gently apply this material to the surface of the matrix.
12. 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.
13. Place the rack containing the collection tubes under the column, so that the first tube is directly under the column outlet.
14. Add 600 ml of column buffer and immediately begin collecting single drop fractions (approximately 35 ul per tube) in tubes #1–16. Cap each tube after each fraction is collected. Recap the column after fraction #16 has been collected.
15. Check the profile of the fractions before proceeding with the experiment.
16. On a 1.1% agarose/EtBr gel, electrophorese 3 ul of each fraction (separately) in adjacent wells, alongside 0.1 mg of a 1-kb DNA size marker. Run the gel at 150 V for 10 min. (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 three fractions containing cDNA (in most cases, the fourth fraction containing cDNA is usable. Make sure the fourth fraction matches your desired size distribution).
17. Pool the above fractions in a clean 1.5-ml tube.
18. Add the following reagents to the tube with 3–4 pooled fractions containing the cDNA: (105–140 ul, respectively)
 - 1/10 vol. sodium acetate (3 M; pH 4.8)
 - 1.3 ul glycogen (20 mg/ml)
 - 2.5 vol. 95% ethanol (–20°C)
19. Mix by gently rocking the tube back and forth.
20. Place the tube in –20°C overnight

Day 3 10/8 10-12pm

E. cDNA Size Fractionation, continued

21. Centrifuge the tube at 14,000 rpm for 20 min at room temperature.
22. Carefully remove the supernatant with a pipette. Do not disturb the pellet.
23. Briefly centrifuge the tube to bring all remaining liquid to the bottom.
24. Carefully remove all liquid and allow the pellet to air dry for ~10 min.
25. Resuspend the pellet in 7 ul of Deionized H₂O and mix gently.
26. Store cDNA at -20°C until the ligation step.

F. Ligation of cDNA to vector

1. Combine ligation components in labeled 0.5 ml tube
 - 1.0ul cDNA
 - 1.0ul pDNR-LIB (0.1 mg/ml)
 - 0.5ul 10X Ligation Buffer
 - 0.5ul ATP (10 mM)
 - 0.5ul T4 DNA Ligase
 - 1.5ul Deionized H₂O
 - 5.0ul total volume
2. Mix the reagents gently; avoid producing air bubbles. Spin tubes briefly to bring contents to the bottom of the tube. Store the unused cDNA at -20 °C for later use.
3. Incubate ligation mix at 16°C 12-16hrs.
4. Add 15 ul H₂O and store at -20C to each ligation mixture

Day 4 Oct. 10/10 (5-6pm)

G. Transformation

1. Prepare ice bucket with cuvette, ligation mix (spun down), SOC, electrocompetent cells (not to be defrosted for more than 10min prior to usage, in -80 freezer), and clean tube.

Note: Thaw electrocompetent cells on ice Use the cells promptly after thawing to obtain maximum efficiency in electroporation. Once thawed, cells cannot be refrozen.

2. Add 970 ul of SOC broth to labeled 14-ml polypropylene tubes
3. Set electroporator 2510 to 1800v and "time constant"
4. Add 25 ul of thawed cells to the 5ul of the ligation reaction.
5. Insert cold sample in the cuvette holder and place in electroporator
6. Pulse 2x, record reading (acceptable values range from 2-6)
7. Place back on ice and add 250ul of SOC broth
8. Pool samples into 970ul of SOC and incubate 37°C . 1hr.
9. Plate 10ul, 20ul on LB agar plates and incubate 37°C . overnight (250 colonies/plate is optimal)
10. Store un plated cells in refrigerator 4°C

Day 5 10/11

- H. Pick colonies to grow overnight (18 hr).

Day 6 10/12

- I. Spin down cultures, template preparation (RevPrep)
- J. Set up sequencing reactions
- K. Precipitation and sequencing run

Day 7 10/13

- L. sequencing finished / analysis