Please read the User Manual for the SMARTer® RACE 5'/3' Kit (Cat. Nos. 634858, 634859) before using this Protocol-At-A-Glance. **This abbreviated protocol is provided for your convenience, but is not intended for first-time users.**

I. Primer Design (Section IV of the User Manual)

Gene-Specific Primers (GSPs) should:

- be 23–28 nt to ensure specific annealing
- be 50–70% GC
- have a T_m ≥65°C; best results are obtained if T_m >70°C, which enables the use of touchdown PCR. (T_m should be calculated based upon the 3' (gene-specific) end of the primer, NOT the entire primer.)
- **not** be complementary to the 3'-end of the Universal Primer Mix
  Long primer = 5'–TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3'
  Short primer = 5'–CTAATACGACTCACTATAGGGC–3'
- be specific to your gene of interest
- both have 15 bp overlaps with the vector at their 5' ends to facilitate In-Fusion® cloning (i.e., add the sequence **GATTACGCCAAGCTT** to the 5' ends of both GSPs' sequences)

II. Generating RACE-Ready cDNA (Section V of the User Manual)

1. Prepare enough of the following Buffer Mix for all of the 5'- and 3'-RACE-Ready cDNA synthesis reactions plus 1 extra reaction to ensure sufficient volume. Mix the following reagents and spin briefly in a microcentrifuge, then set aside at room temperature until Step 6:

4.0 µl	5X First-Strand Buffer
0.5 µl	DTT (100 mM)
1.0 µl	dNTPs (20 mM)
5.5 µl	**Total Volume**

2. Combine the following reagents in separate microcentrifuge tubes:

For preparation of 5'-RACE-Ready cDNA	
1.0–10 µl	RNA*
1.0 µl	5'-CDS Primer A
0–9 µl	Sterile H_2O
11 µl	**Total Volume**

For preparation of 3'-RACE-Ready cDNA	
1.0–11 µl	RNA*
1.0 µl	3'-CDS Primer A
0–10 µl	Sterile H_2O
12 µl	**Total Volume**

*For the control reactions, use 1 µl of Control Mouse Heart Total RNA (1 µg/µl).

3. Mix contents and spin the tubes briefly in a microcentrifuge.

4. Incubate tubes at 72°C for 3 minutes, then cool the tubes to 42°C for 2 minutes. After cooling, spin the tubes briefly for 10 seconds at 14,000 x g to collect the contents at the bottom.
   NOTE: This step can be performed in a thermal cycler. While the tubes are incubating, you can prepare the Master Mix in Step 6.

5. **To just the 5'-RACE cDNA synthesis reaction(s)**, add 1 µl of the SMARTer II A Oligonucleotide per reaction.

6. Prepare enough of the following Master Mix for all 5'- and 3'-RACE-Ready cDNA synthesis reactions. Mix these reagents at room temperatures in the following order:

5.5 µl	Buffer Mix from Step 1
0.5 µl	RNase Inhibitor (40 U/µl)
2.0 µl	SMARTScribe™ Reverse Transcriptase (100 U)
8.0 µl	**Total Volume**

7. Add 8 µl of the Master Mix from Step 6 to the denatured RNA from Step 4 (3'-RACE cDNA) and Step 5 (5'-RACE cDNA), for a total volume of 20 µl per cDNA synthesis reaction.

8. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.

9. Incubate the tubes at 42°C for 90 minutes in an air incubator or a hot-lid thermal cycler.

10. Heat tubes at 70°C for 10 minutes.

11. Dilute the first-strand cDNA synthesis reaction product with Tricine-EDTA Buffer:
    - Add 10 µl if you started with <200 ng of total RNA.
    - Add 90 µl if you started with >200 ng of total RNA.
    - Add 240 µl if you started with poly A^+ RNA.

12. You now have 3'- and 5'-RACE-Ready cDNA samples. Samples can be stored at –20°C for up to three months.

III. Rapid Amplification of cDNA Ends (RACE) (Section VI of the User Manual)

This procedure describes the 5'-RACE and 3'-RACE PCR reactions that generate the 5' and 3' cDNA fragments. We recommend that you also perform positive control 5'- and 3'-RACE using the TFR primers and UPM. Although the Universal Primer Short (UPM short) is provided, nested PCR is generally not necessary in SMARTer RACE reactions.

1. Prepare enough PCR Master Mix for all of the PCR reactions plus one extra reaction to ensure sufficient volume. The same Master Mix can be used for both 5'- and 3'-RACE reactions. For each 50 µl PCR reaction, mix the following reagents:

15.5 µl	PCR-Grade H_2O
25.0 µl	2X SeqAmp™ Buffer
1.0 µl	SeqAmp DNA Polymerase
41.5 µl	**Total Volume**

2. Prepare PCR reactions as shown below in Table 1. Add the components to 0.5 ml PCR tubes in the order shown and mix gently.

Table 1. Setting up 5'- and 3'-RACE PCR Reactions

Component	5’- or 3’-RACE Sample	UPM only (– control)	GSP only (– control)
5’- or 3’-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl
10X UPM	5 µl	5 µl	—
5’ or 3’ GSP (10 µM)	1 µl	—	1 µl
H_2O	—	1 µl	5 µl
Master Mix (Step 1)	41.5 µl	41.5 µl	41.5 µl
Total Volume	**50 µl**	**50 µl**	**50 µl**

3. Commence thermal cycling using one of the following PCR programs (both programs 1 and 2 work with the positive control 5’- and 3’-RACE TFR and UPM Primers). Be sure to choose the correct number of cycles (as noted) based on whether you started with poly A^+ or total RNA.

NOTES ON CYCLING: You may need to determine the optimal cycling parameters for your gene empirically, because the number of cycles necessary depends on the abundance of the target transcript. The optimal extension time depends on the length of the desired amplicon. For 0.2–2 kb amplicons, we typically extend for 2 minutes; for 2–4 kb amplicons, we extend for 3 minutes; and for 5–10 kb amplicons, we extend for up to 10 minutes.

NOTE: The T_m should be calculated based upon the 3’ (gene-specific) end of the primer, and NOT the entire primer.

Program 1 (touchdown PCR—preferred; use if GSP T_m >70°C)

- 5 cycles:
  94°C 30 sec
  72°C 3 min*
- 5 cycles:
  94°C 30 sec
  70°C 30 sec
  72°C 3 min*
- 20 cycles (Poly A^+ RNA) OR 25 cycles (Total RNA):
  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

*If fragments >3 kb are expected, add 1 minute for each additional 1 kb.

Program 2 (use if GSP T_m = 60–70°C)

- 20 cycles (Poly A^+ RNA) OR 25 cycles (Total RNA):
  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

*If fragments >3 kb are expected, add 1 minute for each additional 1 kb.

IV. Characterization of RACE Products (Section VII of the User Manual)

A. Gel Extraction with the NucleoSpin Gel and PCR Clean-Up Kit

For more details on the included NucleoSpin Gel and PCR Clean-Up Kit, please download its User Manual from our website at www.takarabio.com/manuals.

Before you start: Add 24 ml of 96–100% ethanol to Wash Buffer NT3. Mark the label of the bottle to indicate that ethanol was added. Wash Buffer NT3 is stable at room temperature (18–25°C) for at least one year.

1. Electrophorese your RACE DNA sample on an agarose/EtBr gel. We recommend using a buffer system containing either TAE (40 mM Tris-acetate [pH 8], 1 mM EDTA) or TBE (45 mM Tris-borate [pH 8], 1 mM EDTA).

2. Locate the position of your fragment under UV light. Use a clean scalpel or razor blade to excise the DNA fragment of interest. Cut close to the fragment to minimize the surrounding agarose. Estimate the amount of DNA present in the gel slice.
   NOTE: Minimize UV exposure time to avoid damaging the DNA.

3. Measure the weight of the gel slice and transfer it to a clean 1.5 ml microcentrifuge tube.

4. For each 100 mg of agarose, add 200 µl Buffer NTI.

5. Incubate the sample for 5–10 minutes at 50°C. Vortex every 2–3 minutes until the gel slice is completely dissolved.

6. Place a NucleoSpin Gel and PCR Clean-Up Column into a Collection Tube (2 ml) and load up to 700 µl of sample. Centrifuge for 30 seconds at 11,000 x g. Discard flow-through and place the column back into the collection tube. Load remaining sample if necessary and repeat the centrifugation step.

7. Add 700 µl Buffer NT3 to the column. Centrifuge for 30 seconds at 11,000 x g. Discard flow-through and place the column back into the collection tube.

8. Centrifuge for 1 minute at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and collection tube.
   NOTE: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 minutes at 70°C prior to elution (Step 9).

9. Place the column into a new 1.5 ml microcentrifuge tube (not provided). Add 15–30 µl Buffer NE and incubate at room temperature (18–25°C) for 1 minute. Centrifuge for 1 minute at 11,000 x g to elute DNA.

   NOTE: DNA recovery of larger fragments (>1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70°C, and incubation for 5 minutes.

B. In-Fusion Cloning of RACE Products

For more details on the included In-Fusion Snap Assembly Master Mix, please download its User Manual from our website at www.takarabio.com/manuals.

1. Combine:

1 µl	Lineareized pRACE vector (provided with SMARTer RACE 5'/3' Kit Components)
7 µl	Gel-purified RACE product
2 µl	In-Fusion Snap Assembly Master Mix
10 µl	**Total Volume**

2. Incubate for 15 minutes at 50°C and transfer to ice.

3. Follow the protocol provided with your Stellar™ Competent Cells to transform the cells with 2.5 µl of the In-Fusion reaction mixture.

   IMPORTANT: DO NOT add more than 5 µl of the reaction to 50 µl of competent cells. More is not better. Using too much of the reaction mixture inhibits the transformation.

4. Place 1/100–1/5 of each transformation reaction into separate tubes and bring the volume to 100 µl with SOC medium. Spread each diluted transformation on a separate LB plate containing 100 µg/ml of ampicillin.

5. Centrifuge the remainder of each transformation at 6,000 rpm for 5 minutes. Discard the supernatant and resuspend each pellet in 100 µl fresh SOC medium. Spread each sample on a separate LB plate containing the appropriate antibiotic. Incubate all of the plates overnight at 37°C.

6. The next day, pick individual isolated colonies from each experimental plate. Isolate plasmid DNA using a standard method of your choice (e.g. miniprep). To determine the presence of your RACE insert, analyze the DNA by PCR screening (with your GSPs) or restriction digest (with EcoRI and HindIII, which flank the cloning site).

   NOTE: For 5'-RACE products, we recommend picking at least 8–10 different independent clones in order to obtain the maximum amount of sequence at the 5'end.

C. Sequencing RACE Products

Once you have identified the clones containing the largest gene-specific inserts, obtain as much sequence data as you can. Ideally, you will be able to sequence the entire open reading frame, as well as the 5' and 3' untranslated regions.

NOTE: The provided pRACE vector is a pUC19-based vector, and is compatible with M13 sequencing primers for characterization of your cloned insert(s). Because In-Fusion cloning is directional, you can preferentially use the M13F primer to sequence into the UPM end, and the M13R primer to sequence into the gene-specific end.

The UPM contains a T7 priming site which can be used for Sanger sequencing, but we recommend using M13 primers to get full clean reads of your experimental sequence. The T7 priming sites are too close to the 5’- and 3’-cloning sites to ensure complete coverage in the sequencing trace.

Options for generating full-length cDNA

After the RACE products have been characterized by partial or complete sequencing, you can generate the full-length cDNA by one of two methods:

- By long distance PCR (LD PCR) using primers designed from the extreme 5’ and 3’ ends of your cDNA and the 5’-RACE-Ready cDNA as a template.
- By cloning overlapping 5’- and 3’-RACE fragments using a restriction site in the overlapping region (if available). If no suitable restriction sites are available, you can alternately design new GSPs suitable for multi-fragment In-Fusion cloning.

NOTE: Details on multi-fragment In-Fusion cloning can be found in our tech note In-Fusion Multiple Fragment Cloning.

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