Rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends (**RACE**) is a technique used in molecular biology to obtain the full length sequence of an RNA transcript found within a cell. RACE results in the production of a cDNA copy of the RNA sequence of interest, produced through reverse transcription, followed by PCR amplification of the cDNA copies (RT-PCR). The amplified cDNA copies are then sequenced and should map to a unique genomic region. RACE is commonly followed up by cloning before sequencing of what was originally individual RNA molecules. RACE can provide the sequence of an RNA transcript from a small known sequence within the transcript all the way to the 5' end (5' RACE-PCR) or 3' end (3' RACE-PCR) of the RNA.

Material Required

Please refer to provided protocol for list of reagents. (Clontech: Cat#634858)

Storage conditions

Store SMARTer II A Oligonucleotide and Control Mouse Heart Total RNA at –70°C.
Store all other components at –20°C.

General Principal of RACE Experiment

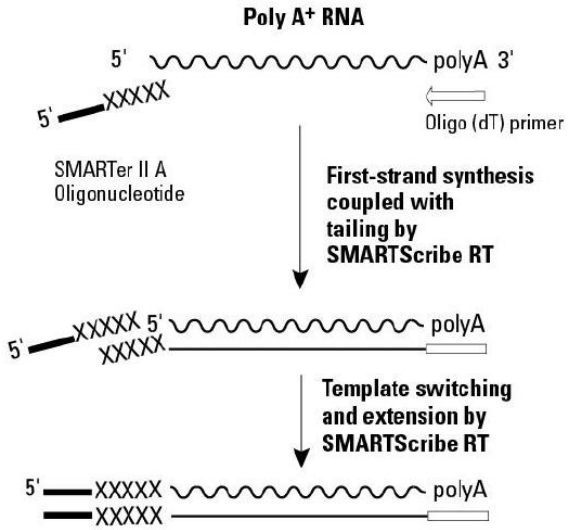


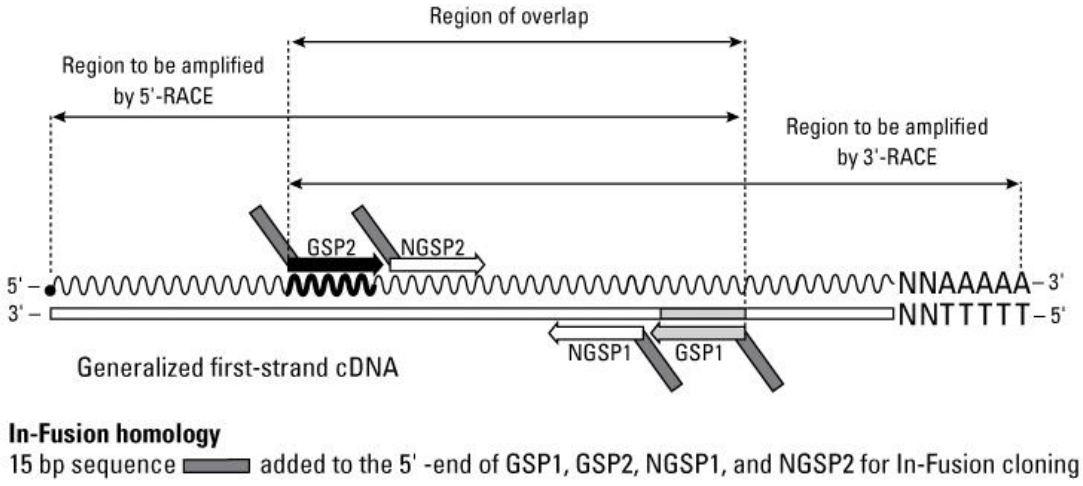
Figure 1. Mechanism of SMARTer cDNA synthesis. First-strand cDNA synthesis is primed using a modified oligo (dT) primer. After SMARTScribe Reverse Transcriptase (RT) reaches the end of the mRNA template, it adds several nontemplated residues. The SMARTer II A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for SMARTScribe RT.

Primer Design(Adapted from ClonTech User Manual)

Gene-Specific Primers (GSPs) Requirements:

1. 23–28 nt to ensure specific annealing
2. 50–70% GC
3. $T_m \geq 65°C$; 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.)
4. **NOT** be complementary to the 3’-end of the Universal Primer Mix Long primer = 5’–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3' Short primer = 5’–CTAATACGACTCACTATAGGGC–3’
5. Ensure specificity to your gene of interest. Use PRIMER BLAST or IN SILICO PCR (UCSC)
6. Add 15 bp overlaps with the vector at their 5’ ends (i.e., add the sequence **GATTACGCCAAGCTT** to the 5’ ends of both GSPs’ sequences; see details below)

The relationship of the primers used in the SMARTer RACE reactions to the template and resulting RACE products are shown in detail in Figure below.



RACE: First Strand cDNA Synthesis

- All reactions occur in 1.5mL Eppendorf Tubes, unless otherwise state, All reaction are made on ICE, unless otherwise stated)
- Please prepare the following master Mix. If you use the Positive control, please adjust to 2x.
- This reagent is made first and is left to incubate at Room Temperature until the Synthesis Reaction.

Volume (1x)	**Reagent**
4.0uL	5X First Strand Buffer
0.5uL	DTT (100mM)
1.0uL	dNTPs (20mM)
5.5uL	**Total**

- Per Reaction (Sample and Control), please prepare the following in separate tubes:

Sample	**Control**	**Reagent**
7.5uL	1.0uL	RNA (10ng Lower Limit)
1.0uL	1.0uL	5’ CDS PRIMER A
2.5uL	9.0uL	Sterile Water
11uL	**11uL**	**Total**

- Mix and spin very briefly in microcentrifuge. (Just the 11uL Samples)

- Incubate Samples: (Use ThermalCycler)

  72^{o}C for 3minutes

  42^{o}C for 2 minutes

- Spin Samples: 14,000 RPM for 10 Seconds
- After spin, add 1uL of “SMARTer II A Oligonucleotide” per reaction (from -80^{o}C)
- Prepare the Synthesis Reaction Master Mix (Can be done during ThermalCycler Step):

Be sure to prepare these at Room Temperature!!

Volume (1x)	**Reagent**
5.5uL	Buffer Mix from first Step!
0.5uL	RNase Inhibitor (40U/uL)
2.0uL	SmartScribe Reverse Transcriptase (100U)
8.0uL	**Total**

- Add the 8.0uL of Synthesis Reaction mixture to the 12uL of Samples.
- Mix by gentle pipetting.
- Spin tubes briefly.
- Incubate Samples: (Use ThermalCycler with HOT LID setting!)

  42^{o}C for 90 minutes

  70^{o}C for 10 minutes

- Add 10uL of Tricine-EDTA Buffer (Total now 30uL)

cDNA Synthesis is now complete.

Samples can either be stored in -20^{o}C or used immediately.
According to the protocol, there is enough for 12 reactions.

RACE: Rapid Amplification of cDNA Ends

- Prepare Master Mix:

Volume (1x)	Reagent
15.5uL	PCR GRADE WATER
25.0uL	2X SeqAmp Buffer
1.0uL	SeqAmp DNA Polymerase
41.5uL	**Total**

- Prepare PCR Reaction to a 0.5mL PCR tube: (In this exact order!)

For Human Oocyte	For Mouse Heart	Reagent
2.5uL	2.5uL	cDNA
5.0uL	5.0uL	10x Universal Primer (UPM Short)
1.0uL	1.0uL	5’ Gene Specific Primer (GSP)
41.5uL	41.5uL	Master Mix (above)
50uL	**50uL**	**Total**

- Mix briefly, spin down.
- Run PCR Reaction in ThermalCycler:

Temp	Time	Cycles
94ºC	30 seconds	5x
72ºC	3 minutes	
94ºC	30 seconds	5x
70ºC	30 seconds	
72ºC	2 minutes	
94ºC	30 seconds	25x
68ºC	30 seconds	
72ºC	2 minutes	

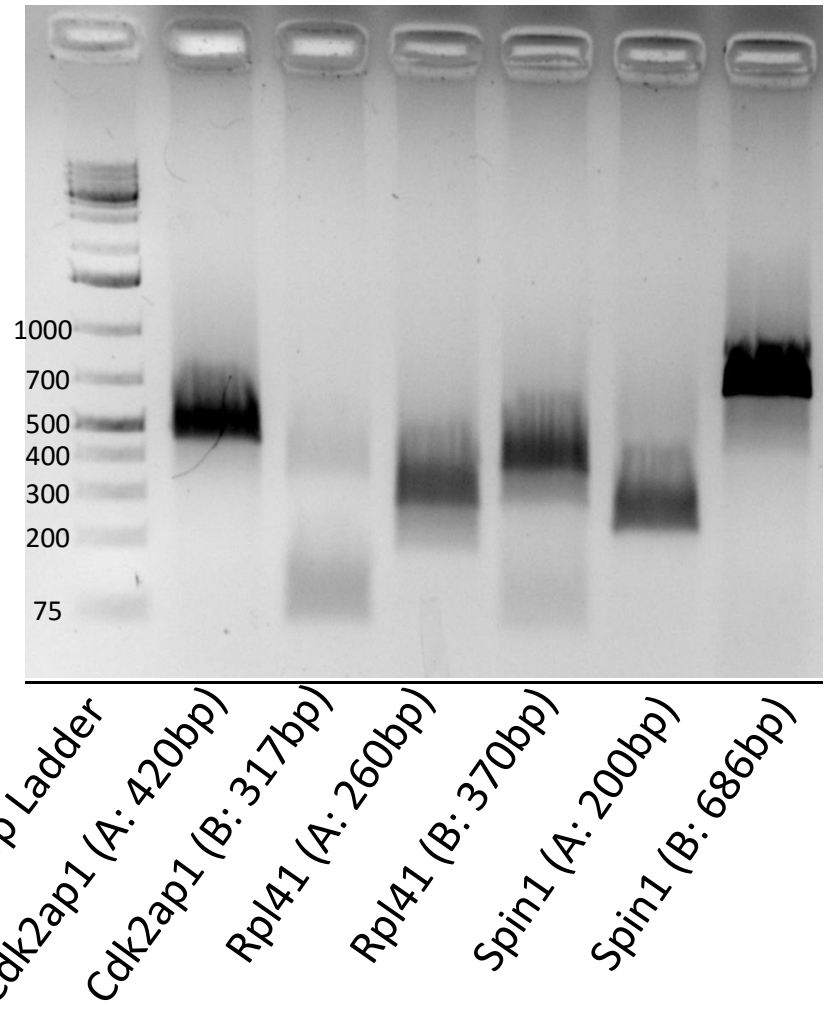
- **TEST**: Prepare 1.2% Agarose Gel and run 5uL of product. If no band is seen, return to thermal cycler for additional PCR rounds:

Temp	Time	Cycles
94ºC	30 seconds	5x
68ºC	30 seconds	
72ºC	2 minutes	

- If this still fails to yield any bands, proceed with Nested PCR strategy (Next Page).

If this is successful, please proceed to GEL EXTRACTION section.

TYPICAL EXAMPLE OF RESULTS AT THIS POINT



NESTED PCR

(Optional if initial PCR fails or produces a smear)

- Dilute 5uL of above PCR product with 245uL of Tricine EDTA buffer.

- Prepare Master Mix:

Volume (1x)	**Reagent**
15.5uL	PCR GRADE WATER
25.0uL	2X SeqAmp Buffer
1.0uL	SeqAmp DNA Polymerase
41.5uL	**Total**

- Prepare PCR Reaction to a 0.5mL PCR tube: (In this exact order!)
- The nR2 primer will work with both R1 and nR1 from the previous section.

For Human Oocyte	**For Mouse Heart**	**Reagent**
5.0uL	5.0uL	DILUTED PCR PRODUCT
1.0uL	1.0uL	10x Universal Primer (UPM Short)
1.0uL	1.0uL	5’ NESTED Gene Specific Primer
41.5uL	41.5uL	Master Mix (above)
48.5uL	**48.5uL**	**Total**

- Mix briefly, spin down.
- Run PCR Reaction in ThermalCycler:

Temp	**Time**	**Cycles**
94ºC	30 seconds	20x
68ºC	30 seconds	
72ºC	2 minutes	

- **TEST**: Prepare 1.2% Agarose Gel and run 5uL of product. If no band is seen, return to thermal cycler for additional PCR rounds, attempt this exact strategy one final time with the final Nested PCR Primer.

If this is successful, please proceed to GEL EXTRACTION section.

GEL EXTRACTION: (Nucleospin Kit Provided for 10 Reactions)

Before you start: Add 24 ml of 96–100% ethanol to Wash Buffer NT3.

- Cut appropriate sized bands from 1.2% Agarose Gel.
- Place slice into 1.5mL Eppendorf tube.
- Measure weight of Gel Slice.
- Add 200uL for every 100mg of Gel removed with Buffer NTI.
- Incubate at 50ºC for 10 minute or until gel is dissolved. (Vortex every 2 minutes).
- Place Nucleospin Column into 2mL Collection Tube.
- Load up to 700uL of sample.
- Centrifuge 30seconds at 11,000xG
- Discard flowthrough and place back into collection tube.
- (Add more samples and spin, if needed).
- Add 700uL of Buffer NT3.
- Centrifuge 30seconds at 11,000xG
- Discard Flowthrough.
- Additional centrifugation for 1 min at 11,000xG to completely remove NT3.
- Incubate tubes at 70ºC for 2 minutes to remove EtOH.
- Place in clean and labeled 1.5mL Eppendorf tube.
- Add 20uL Buffer NE to column.
- Incubate at Room Temperature for 1 minute.
- Centrifugation for 1 min at 11,000xG to elute DNA.

In-Fusion Cloning of RACE Products

(In-Fusion HD Cloning Kit Provided)

- For each sample, combine:

Volume (1x)	**Reagent**
1.0uL	Linearized pRACE Vector
7.0uL	Gel Purified RACE Product
2.0uL	In-Fusion HD Master Mix
10.0uL	**Total**

- Incubate at 50ºC for 15minutes
- Place on ICE.

Transformation of RACE Products: (STELLAR Competent Cells Provided)

- Thaw Stellar Competent Cells in an ice bath just before use.
- After thawing, mix gently to ensure even distribution.
- Move 50µl of competent cells into a 14-ml round-bottom tube (falcon tube).
- (1.5mL Eppendorf tubes can be used, but the protocol states this will reduce efficiency. I had hundreds of colonies but maybe listen to them).
- Do not vortex.
- Add no more than 5ul of RACE Product for transformation.
- Place tubes on ice for 30 min.
- Heat shock the cells for exactly 45 sec at 42°C.
- Place tubes on ice for 1–2 min.
- Add SOC medium to bring the final volume to 500 µl. SOC medium should be warmed to 37°C before using.
- Incubate by shaking (160–225 rpm) for 1 hr at 37°C.
- Pre-Warm 100ug/mL Amplicillin supplemented plates to 37ºC.
- The protocol recommends that you make dilutions before plating.
- For example:
    - Dilute 1uL transformed cells in 100uL SOC
    - Dilute 20uL transformed cells in 100uL SOC
    - Spin remaining Transformed cells down (479uL) for 6000rpm for 5 min, resuspend in 100uL
- Plate all 100uL of each dilution onto Amp Plates overnight at 37ºC.
- Pick 10 different colonies.
- Miniprep samples.
- Sequence samples with M13F.
- CELEBRATE!!