艾科瑞生物
Accurate Biology

Instruction for Use

5’/3’ RACE Kit

AG11618
Version.V2E1

Research Use Only
Not For Diagnosis Procedures

1. Description

RACE (Rapid Amplification of cDNA Ends) is a technique used for the rapid amplification of unknown cDNA end sequences based on a known partial sequence region. This product is a kit designed for reverse transcription using Total RNA or Poly(A)+ RNA as a template, enabling the rapid amplification of both the 5′ and 3′ ends of cDNA. The kit includes all necessary components for both 5′ RACE and 3′ RACE amplification, allowing users to choose according to their experimental needs. During 5′ RACE, the template-switching activity of reverse transcriptase is utilized to add an adaptor sequence to the 5′ end of the RNA. This allows direct RACE PCR amplification without the need for adaptor ligation, making the entire process faster and easier to perform. The PCR amplification system employs L-Exp Taq HS DNA Polymerase, a high-performance enzyme developed by our company with strong amplification capability for long or GC-rich fragments. The carefully optimized buffer system ensures high specificity and minimizes non-specific amplification.

This product contains all reagents optimized for 5′ RACE and 3′ RACE reactions. Please use the reagents provided in the kit for reverse transcription and PCR amplification. If substitution is necessary, validation should be performed in advance.

2. Kit Information

Kit Name	Cat. No	Specification
5′/3′ RACE Kit	AG 11618	10 rxns

3. Transportation and Storage

Storage	Package 2-1 Store at −80°C ; Package 2-2 Store at −20°C
Transportation	Transport at −20°C Dry Ice or Blue Ice Condition

4. Kit Components

Package 2-1

Kit Components	Volume
Template Switching Oligo (TSO) (24 μM)	10 μl
Control Mouse Liver Total RNA (1 μg /μl)	10 μl

Package 2-2

Kit Components	Volume
3′ RACE RT Primer (12 μM)	10 μl
5′ RACE RT Primer (12 μM)	10 μl
Random Primer (20 μM)	10 μl
RNase Inhibitor (40U/μl)	10 μl
5X RACE RT Buffer	40 μl
Evo M-MLV RTase for RACE (100U/μl)	20 μl
dNTP Mix (10mM each)	120 μl
10X Universal Primer Mix	400 μl
Tricine EDTA Buffer	1 ml x 2 Pcs
Universal Short Primer (10 μM)	50 μl
Control 5′-Gene-Specific Primer (10 μM)	25 μl
Control 3′-Gene-Specific Primer (10 μM)	25 μl
L-Exp Taq HS DNA Polymerase (5U/μl)	25 μl
5X L-Exp Taq PCR Buffer (Mg2+ plus)	500 μl
Nuclease Free Water	1 ml

* When using the Control 5′-Gene-Specific Primer, the amplified DNA fragment length is 1.6 kb;
when using the Control 3′-Gene-Specific Primer, the amplified DNA fragment length is 0.6 kb.

5. Experimental Precautions

1) When designing Gene-Specific Primers (GSP) or Nested Gene-Specific Primers (NGSP), please follow the "GSP Primer Design Requirements" described in the Preparation Before Experiment section. Failure to do so may result in non-specific amplification. It is also recommended to design multiple primers targeting the same transcript to increase amplification success rate.
2) It is recommended to use intact and contamination-free RNA. Degraded or impure RNA will affect the completeness and length of synthesized cDNA, leading to RACE amplification failure. It is advisable to check RNA integrity before the experiment using agarose gel electrophoresis or the Agilent RNA 6000 Pico Kit (Agilent, Code 5067-1513).
3) If using RNA without a Poly(A) tail to study the 5′ end sequence, the Random Primer can be used instead of the 5′ RACE RT Primer, following the same usage and concentration. To study the 3′ end sequence, Poly(A) polymerase should be used to add a Poly(A) tail before subsequent experiments.
4) It is recommended that the total amount of RNA for reverse transcription does not exceed 1 μg. However, if the target gene has low abundance, the RNA input may be increased appropriately.
5) During reverse transcription, take care to avoid RNase contamination. Use sterile, RNase-free consumables; avoid speaking during operation; and wear lab coats and disposable gloves to prevent RNA contamination or degradation.
6) Because this product has high detection sensitivity, avoid cross-contamination with other experiments. It is recommended to perform reverse transcription in a dedicated, clean PCR area (or biosafety cabinet).
7) If the PCR amplification shows no clear target band or poor specificity, perform a nested PCR as a second-round amplification.
8) It is recommended to keep all procedures on ice throughout the experiment.
9) When preparing the reaction mixture, it is recommended to add the enzyme as the last step.

6. Experimental Workflow

Figure 1. Workflow of 3′ RACE amplification

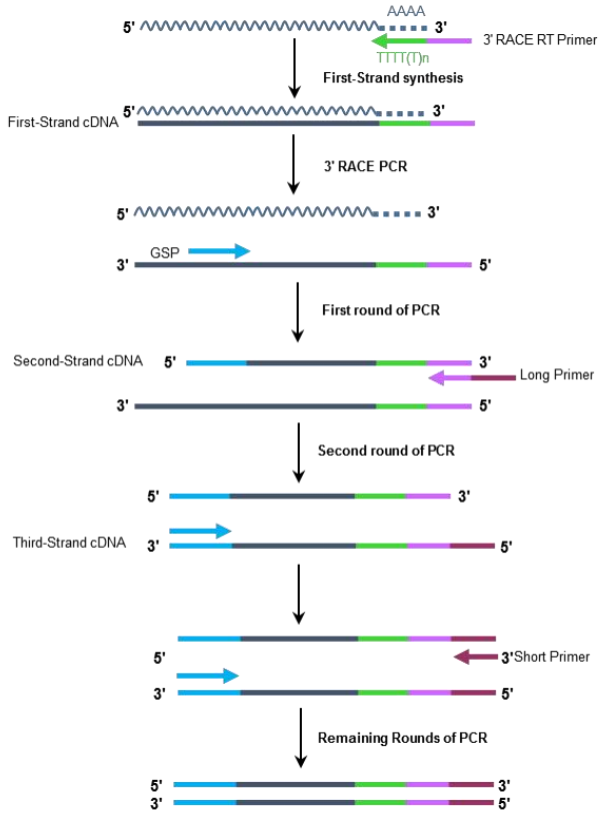
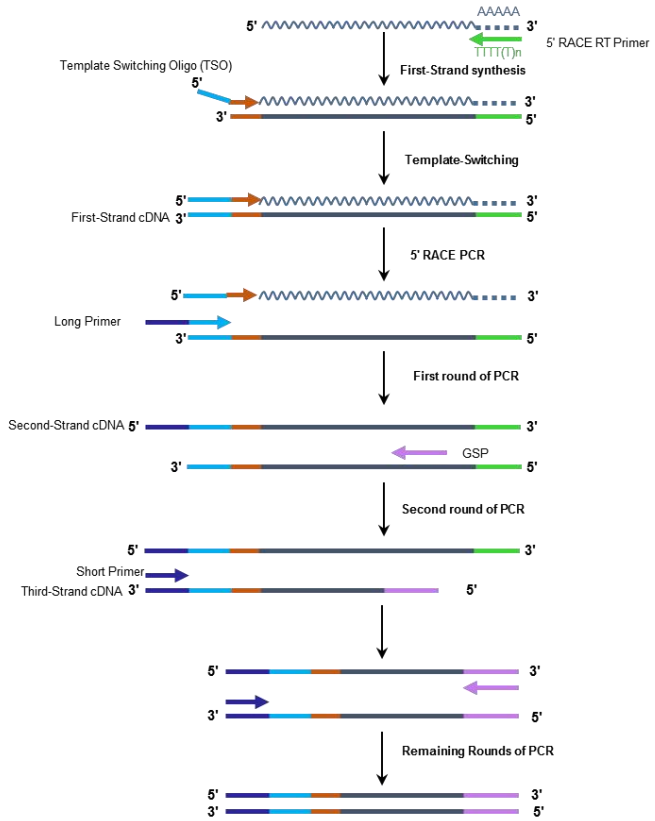


Figure 2. Workflow of 5′ RACE Amplification



3′ RACE Principle

3′ RACE (Rapid Amplification of cDNA 3′ Ends) is a technique used to amplify the DNA fragment between a known gene sequence and the 3′ end of the transcript.

Using the Poly(A) tail of mRNA as the priming site, an Oligo(dT) primer containing an adaptor sequence is used for reverse transcription. Under the action of reverse transcriptase, a cDNA strand with an adaptor sequence at its 5′ end is synthesized.
A gene-specific primer (GSP) is then used as the upstream primer, and a universal primer containing part of the adaptor sequence is used as the downstream primer.
PCR amplification is performed using the first-strand cDNA as the template to obtain the cDNA 3′ end sequence (from the gene-specific primer → to the 3′ end).

5′ RACE Principle

5′ RACE (Rapid Amplification of cDNA 5′ Ends) is a technique used to amplify the DNA fragment between a known gene sequence and the 5′ end of the transcript. Using mRNA containing a Poly(A) tail as a template, Oligo(dT) is used as the primer for cDNA synthesis. Relying on the template-switching activity of reverse transcriptase, several (3–5) dC residues are automatically added to the 3′ end of the newly synthesized cDNA when reverse transcription reaches the mRNA's 5′ end.
These dC residues pair with the Oligo(dG) sequence at the 3′ end of a universal adaptor primer, allowing reverse transcriptase to switch templates and extend along the adaptor sequence. As a result, a universal adaptor sequence is added to the 3′ end of the cDNA.
Using the first-strand cDNA as a template, a universal primer containing part of the adaptor sequence serves as the upstream primer, and a gene-specific primer (GSP) serves as the downstream primer for PCR amplification, thereby obtaining the cDNA 5′ end sequence (from the gene-specific primer → to the 5′ end).

7. Experimental Preparation

1) GSP Primer Design Requirements

- It is recommended that the primer length be 23–28 nucleotides with a GC content of 50–70% to ensure specific annealing. Adjustments can be made as needed.
- The Tm value is critical for product specificity. It should be ≥65°C; if Tm > 70°C, the Touchdown PCR method can be used to improve specificity.
- It is recommended to use the Tm value calculated by IDT: sg.idtdna.com/calc/analyzer
- For long transcripts (>10 kb), primers should be designed as close as possible to the end of the cDNA, ideally within 3 kb of the end.
- GSP primers should not be complementary to the 3′ end of the 10X Universal Primer Mix (UPM).
- Long primer: 5′- CATATACCATTACGTCAAGGGCATGCGATGCTTAGCCAGCAATACG -3′
- Short primer: 5′- CATATACCATTACGTCAAGGGC –3′
- Design principles for Nested Gene-Specific Primers (NGSP): The length, GC content, and Tm value follow the same rules as GSP design.
- Position: NGSP should be designed near the 3′ end of the GSP binding region, and the 5′ end of NGSP should not overlap with the GSP primer.

2) RNA Preparation

- The integrity and purity of the RNA starting material are crucial for synthesizing high-quality cDNA.
- When extracting RNA, observe the following points:

Use dedicated pipettes in a separate workspace to avoid RNase contamination.
Always wear gloves and a mask during extraction to prevent RNA degradation.
Use RNase-free, filter tips to avoid contamination.

- RNA Purity Check:

Organic contaminants, metal ions, salts, or nucleases in RNA samples may inhibit enzyme activity or degrade RNA.
Check RNA purity to ensure no contamination.
Impurities can be removed by multiple ethanol precipitations, washing with 80% ethanol, and completely removing residual ethanol.

- When extracting RNA from plants or pigment-rich samples, be cautious of polysaccharide or pigment contamination, which

cannot be detected on agarose gels and are difficult to remove.

- These substances can interfere with primer binding during first-strand cDNA synthesis and reduce cDNA yield.
- RNA Integrity Check:

Use an Agilent 2100 Bioanalyzer (Agilent, Code 5067-1513) or equivalent product. Each run requires 2–7 ng RNA.

It is recommended to use RNA with RIN ≥ 7.

- If an Agilent 2100 Bioanalyzer is not available, use agarose gel electrophoresis to assess RNA integrity.
- For eukaryotic RNA, the 28S:18S ratio should be approximately 2:1.
- If the 28S:18S ratio < 1, the RNA sample is not suitable for RACE experiments.

3) Reagents and Consumables (Not Provided)

Cloning and transformation products: e.g., DNA Ligation Kit (Code: AG11801) or equivalent; E. coli HST08 competent cells (Code: AG11804) or equivalent.

Other materials: RNase-free PCR tubes, primers, RNA templates, filter tips, ice bath or ice box.

4) Instruments

PCR machine, Pipettes, Vortex mixer, Mini centrifuge, Electrophoresis system, Gel imaging system, Agilent 2100 Bioanalyzer

8. Procedure

I. First-Strand cDNA Synthesis

This step converts 10 ng – 1 µg of total RNA or Poly(A)+ RNA into the first-strand cDNA for RACE.

Before proceeding, ensure that the RNA is intact and free of contamination.

It is recommended to use mouse liver RNA (included in this kit) as a positive control for cDNA synthesis.

To distinguish non-specific amplification, a negative control (without Evo M-MLV RTase for RACE) can also be set up.

For RNA without a Poly(A) tail:

In 5′ RACE, replace the 5′ RACE RT Primer with a Random Primer (use the same method and dosage).

In 3′ RACE, perform Poly(A) tailing with Poly(A) polymerase before reverse transcription.

1) RNA Denaturation and Annealing

a) Since the total reaction volume is small, use a 200 µL tube, and add reagents according to the recommended setup.

b) Preheat the PCR instrument and set the appropriate program in advance.

c) Thaw all reagents on ice, mix gently, and prepare the reaction mixture as shown below.

All steps should be carried out on ice.

Components	3′ RACE Reaction	5′ RACE Reaction
RNA	10 ng – 1 µg	10 ng – 1 µg
3′ RACE RT Primer (12 µM)	1.0 µL	-
5′ RACE RT Primer (12 µM)	-	1.0 µL
Nuclease-Free Water	Up to 11.5 µL	Up to 10.5 µL

d) Mix the solution gently using a pipette, spin briefly to collect liquid at the tube bottom, and run the following PCR program:

Reaction Program:

Temperature	Time
72°C	3 min
4°C	-

e) After annealing, spin briefly and immediately place the tubes on ice.

2) cDNA Synthesis

a) On ice, prepare the reverse transcription reaction mixture as shown below.

(It is recommended to prepare enough for one extra reaction to ensure adequate volume.)

Mix gently with a pipette and briefly centrifuge to collect liquid at the tube bottom.

Components	Input Volume
5× RACE RT Buffer	4.0 µL
dNTP Mix (10 mM each)	2.0 µL
RNase Inhibitor (40 U/µL)	0.5 µL
Evo M-MLV RTase for RACE (100 U/µL) *²	2.0 µL
Total Volume	8.5 µL

2*: It is recommended to add Evo M-MLV RTase for RACE last.

b) Add 8.5 µL of the above reverse transcription reaction mix to the annealed RNA mixture prepared in the previous step.

For 5′ RACE, add an additional 1.0 µL of Template Switching Oligo (TSO).

Components	3′ RACE Reaction	5′ RACE Reaction
Denatured and annealed RNA mix	11.5 µL	10.5 µL
Reverse transcription mix	8.5 µL	8.5 µL
Template Switching Oligo (24 µM)	-	1.0 µL
Total Volume	20 µL	20 µL

c) Mix the solution gently with a pipette, spin briefly to collect the liquid at the bottom of the tube.

d) Immediately place the tubes in a pre-set PCR instrument or water bath for the following reaction:

Reaction Program:

Temperature	Time
42°C	90 min
70°C	15 min
4°C	Hold

e) After the reaction, place the synthesized first-strand cDNA on ice. Because the expression levels of target transcripts vary among RNA samples, excessive template may cause non-specific amplification during subsequent PCR.

It is recommended to dilute cDNA with Tricine-EDTA Buffer.

For example:

If the starting RNA amount is 200 ng, add 20 µL Tricine-EDTA Buffer to the 20 µL cDNA reaction. The dilution ratio can be adjusted depending on RNA input and target gene abundance.

f) The obtained 5′ RACE cDNA and 3′ RACE cDNA can be stored at –20°C for up to 3 months.

For long-term storage, keep at –80°C.

II. Rapid Amplification of cDNA Ends (RACE PCR Amplification)

The efficiency of RACE PCR amplification depends on the abundance of the target gene in the RNA sample.

Since different primers may require different annealing temperatures, refer to <Appendix 2: Common Problems and Solutions> for PCR optimization guidelines. For positive control, use the Mouse Liver RNA provided in this kit to synthesize cDNA and perform amplification using the 5′ and 3′ RACE control GSP primers together with the 10× Universal Primer Mix (UPM).

1) PCR Reaction Preparation

Prepare the PCR reaction mixture on ice as shown below. Mix gently and spin briefly before loading into the PCR machine.

(It is recommended to prepare enough for one extra reaction to ensure sufficient volume.)

5′ RACE*[3]

Components	Final Concentratio	Volume
5′ RACE cDNA	-	2.5 μL
10× Universal Primer Mix	1X	5 μL
5′ Gene-Specific Primer (10 μM)	0.2 μM	1 μL
5× L-Exp Taq PCR Buffer (Mg^{2+} plus)	1X	10 μL
L-Exp Taq HS DNA Polymerase (5 U/μL)	2.5 U	0.5 μL
dNTP Mix (10 mM each)	0.4 mM	2 μL
Nuclease-free water	-	Up to 50 μL

3′ RACE*[3]

Components	Final Concentratio	Volume
3′ RACE cDNA	-	2.5 μL
10× Universal Primer Mix	1X	5 μL
3′ Gene-Specific Primer (10 μM)	0.2 μM	1 μL
5× L-Exp Taq PCR Buffer (Mg^{2+} plus)	1X	10 μL
L-Exp Taq HS DNA Polymerase (5 U/μL)	2.5 U	0.5 μL
dNTP Mix (10 mM each)	0.4 mM	2 μL
Nuclease-free water	-	Up to 50 μL

3*: To distinguish non-specific background products, it is recommended to prepare single-primer control reactions using only UPM or GSP primers. Detailed setup can be found in <Appendix 1: Single-Primer Control Preparation Method>.

2)PCR Programs

Choose the appropriate PCR program based on the Tm value of the gene-specific primer (GSP).

Program I (for primers with Tm = 60–70°C)

Step	Temperature	Time	Cycles
Initial denaturation	94°C	1 min	1
Denaturation	94°C	30 sec	
Annealing	55–68°C	30 sec	1
Extension	72°C	3 min	
Final extension	72°C	5 min	1

Program II (for primers with Tm > 70°C, use Touchdown PCR)

Step	Temperature	Time	Cycles
Initial denaturation	94°C	1 min	1
Denaturation	94°C	30 sec	
Extension	72°C	3 min*[4]	5
Denaturation	94°C	30 sec	
Annealing	70°C	30 sec	5
Extension	72°C	3 min*[4]	
Denaturation	94°C	30 sec	
Annealing	68°C	30 sec	20-25*[5]
Extension	72°C	3 min*[4]	
Final extension	72°C	5 min	1

4*: The optimal extension time depends on the target amplicon length: ≤3 kb: 3 min; 3 kb: add 1 min per extra kb

5*: Recommended cycles: 25 cycles for Poly(A)+ RNA; 30 cycles for Total RNA

Adjust according to the target gene abundance — for low-expression genes, increase cycle number as needed.

Recommended PCR Program for Kit Positive Control

Step	Temperature	Time	Cycles
Initial denaturation	94°C	1 min	1
Denaturation	94°C	30 sec	
Annealing	56°C	30 sec	30
Extension	72°C	3 min	
Final extension	72°C	5 min	1

3) Analysis

After PCR, analyze an appropriate volume of the reaction product by agarose gel electrophoresis.

If no clear target band or diffuse bands appear, perform Nested PCR amplification.Refer to the section <Optional Step: Nested PCR Amplification> for detailed procedure.

Optional Step: Nested PCR Amplification

If the previous PCR round did not yield a clear target band or produced diffuse/non-specific bands, you can perform a Nested PCR using the following procedure:

1) Template Preparation

Take 5 µL of the previous PCR product and add it to 245 µL of Tricine-EDTA Buffer.

Vortex gently to mix thoroughly.

2) Nested PCR Reaction Setup

Prepare the Nested PCR mixture on ice as shown below. Mix gently using a pipette, spin briefly, and proceed with PCR amplification.

Components	Final Concentration	Volume
Diluted PCR product (from Step 1)	-	5 µL
Universal Short Primer (10 µM)	0.2 µM	1 µL
Nested Gene-Specific Primer (NGSP)	0.2 µM	-
5× L-Exp Taq PCR Buffer (Mg^{2+} plus)	1X	10 µL
L-Exp Taq HS DNA Polymerase (5 U/µL)	2.5 U	0.5 µL
dNTP Mix (10 mM each)	0.4 mM	2 µL
Nuclease-free water	-	Up to 50 µL

3) PCR Program

Run the PCR program following Program I described in the section <RACE PCR Amplification>.

III. Purification and Detection of RACE Products

To verify amplification results, it is recommended to confirm products by cloning and sequencing:

If the amplified band is single and clear, use SteadyPure PCR Purification Kit (Code: AG21003).

If there are multiple or diffuse bands, use SteadyPure DNA Gel Extraction Kit (Code: AG21005) or another equivalent product to recover the desired fragment for subsequent cloning and sequencing.

IV. Cloning and Transformation

1) Cloning: The 3' end of the amplification product from the L-Exp kit carries an A base and can be directly cloned into a T vector. DNA ligation is recommended using our DNA Ligation Kit (Code: AG11801) or other equivalent products. Please refer to the corresponding product manual for detailed procedures.

2) Transformation: Conventional competent cells can be used. Our E. coli HST08 competent cells (Code: AG11804) are suitable. Please follow the instructions in the product manual for detailed steps.
3) Positive Screening: Pick single colonies for colony PCR screening. Our 2X Accurate Taq Premix (with dye) (Code: AG11009) can be used. For 5′ RACE products, it is recommended to pick 8–10 single clones to obtain the longest 5′ sequence.
4) Sequencing Analysis: Confirm that the target gene has been inserted into the vector by performing Sanger sequencing. Sequencing primers can be either universal primers for the vector or custom-designed primers.

Appendix 1: Preparation of Single-Primer Control

Prepare the single-primer control PCR reaction on ice according to the table below. Gently mix using a pipette and place in the PCR instrument for amplification.

Components	UPM Single-Primer Control	GSP Single-Primer Control
5′ or 3′ RACE cDNA	2 μL	2.5 μL
10X Universal Primer Mix	5 μL	-
5′ or 3′ GSP Primer	-	1 μL
5× L-Exp Taq PCR Buffer (Mg^{2+} plus)	10 μL	10 μL
L-Exp Taq HS DNA Polymerase (5 U/μL)	0.5 μL	0.5 μL
dNTP Mix (10 mM each)	2 μL	2 μL
Nuclease-free water	Up to 50 μL	Up to 50 μL

Appendix 2: Troubleshooting

Problem	Possible Cause	Solution
5′RACE or 3′RACE amplification shows no target band	Low gene abundance	Try increasing the number of PCR cycles (do not exceed 40 cycles; for Touchdown PCR, do not exceed 50 cycles). If no product is obtained, increase the RNA input (1–4 μg) or use RNA with higher target gene content.
	Annealing temperature too high	Reduce annealing temperature in 2°C increments to find the optimal temperature.
	GSP primer unsuitable	Recheck primer design. Design multiple alternative primers according to GSP design requirements (primer Tm is critical).
	Complex template structure (e.g., secondary structure or high GC content)	Design GSP primers near the cDNA ends, or avoid high-GC regions.
	Known sequence may be incorrect	Verify the known sequence to ensure accuracy.
	Experimental errors	Use positive control reagents to confirm proper amplification.
5′RACE or 3′RACE amplification produces smear	No target transcript in RNA	Design PCR or qPCR primers for the target region to verify expression.
	Low target abundance in RNA	Increase RNA template (1–4 μg), increase cDNA input (reduce dilution), increase GSP primer amount (1–5 μl), or use nested GSP primers for multiple rounds of Nested PCR (up to 3 rounds).
	RNA degradation or contamination	Check RNA purity and integrity after extraction (agarose gel electrophoresis or Agilent analysis).
	PCR product is long	Increase extension time (add 1 min per additional kb); design primers near the ends of the target sequence.
	Template amount too high	Dilute RNA template (10 ng–1 μg) or dilute cDNA before RACE.
	Unsuitable PCR conditions	Reduce cycle number, increase annealing temperature, or use Touchdown PCR.
	Poor GSP specificity	Redesign primers and consider increasing Tm (≥65°C recommended).
5′RACE or 3′RACE shows multiple bands	Bands match known sequence but differ in length	Gene may have alternative splicing or multiple transcript variants; 5′RACE may have multiple transcription start sites or RNA degradation; 3′RACE may have multiple poly(A) sites.
	Bands cannot be aligned with known sequence	Non-specific amplification due to GSP primer design; try increasing annealing temperature or redesign primers to improve specificity.
No positive clone	Low recovery of amplified product	Perform multiple PCR reactions to enrich product concentration; scale up cloning system and increase target fragment input.

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