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5′/3′ RACE Kit, 2nd Generation

Version 14

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For the rapid amplification of either 5’ or 3’ cDNA ends

Formulation containing Transcriptor Reverse Transcriptase and recombinant Terminal Transferase

Cat. No. 03 353 621 001 Kit for 10 reactions

Store the kit at −15 to −25°C

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PROTOCOLS

PROTOCOLS

1. What this Product Does

Number of Tests

The kit is designed for 10 reactions for the rapid amplification of either 5' or 3' cDNA ends.

Kit Contents

Vial/Cap	Label	Contents / Function
1 purple	cDNA Synthesis Buffer (5x conc.)	• 100 µl • 250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithiothreitol, pH 8.5 (+20°C)
2 purple	Transcriptor Reverse Transcriptase	• 10 µl • 25 U/µl, in 200 mM potassium phosphate, 2 mM dithiothreitol, 0.2% (v/v) Triton X-100, 50% glycerol (v/v), pH 7.2
3 green	Deoxynucleotide Mixture	• 50 µl • mixture of dATP, dCTP, dGTP, dTTP 10 mM each, in Tris-HCl, pH 7.5 (+20°C)
4 green	dATP	• 30 µl • 2 mM, in Tris-HCl, pH 7.5 (+20°C)
5 green	Reaction Buffer (10x conc.)	• 1,000 µl • 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 (+20°C)
6 green	Terminal Transferase, recombinant	• 10 µl • 80 U/µl in 60 mM potassium phosphate (pH 7.2 at +4°C), 150 mM KCl, 1 mM 2-mercaptoethanol, 0.5% Triton X-100, 50% glycerol.
7 blue	Control neo-RNA	• 20 µl • 1 ng/µl, in double-distilled water
8 red	Oligo dT-Anchor Primer	• 40 µl • 37.5 µM, in double-distilled water
9 red	PCR Anchor Primer	• 40 µl • 12.5 µM, in double-distilled water
10 blue	Control Primer neo1/rev	• 40 µl • 12.5 µM, in double-distilled water
11 blue	Control Primer neo2/rev	• 40 µl, • 12.5 µM, in double-distilled water
12 blue	Control Primer neo3/for	• 40 µl • 12.5 µM, in double-distilled water

Storage and Stability

Store the kit at −15 to −25°C until the expiration date printed on the label.

Additional Equipment and Reagents Required

For PCR amplification of poly(A)-tailed first-strand cDNA, the following reagents are required:

- Blends of thermostable DNA polymerases with improved fidelity and performance, Expand High Fidelity PCR System* or Expand Long Template PCR System*

  Whether Expand High Fidelity or Expand Long Template PCR System should be used depends on the expected size of the PCR product and the amount of template cDNA present in the reaction. The final concentration of dNTP and $MgCl_2$ should be adjusted according to the protocols given in the individual Instructions for Use. If you want to use the Expand Long Template PCR System and you need to establish a new assay, it is advisable to test all three possible amplification systems to find the optimum reaction conditions.
- High Pure PCR Product Purification Kit*
- Thermal block cycler
- Protector RNase Inhibitor* (optional)
- Ethanol (pro analysis)
- Mineral oil

Application

The kit is used for analysis of mRNA structure and expression using RACE (rapid amplification of cDNA ends).

- Generation of full-length cDNAs
- Isolation and characterization of 5´or 3´ ends from low-copy RNA messages
- Amplification and further cloning of rare mRNAs
- Analysis in conjunction with exon-trapping methods
- Products of the RACE reaction can be directly sequenced without any further cloning step

2. How To Use this Product

2.1 Before You Begin

Handling Instructions

- To prevent degradation of RNA, autoclave all vessels and pipettes used for cDNA synthesis.
- Wear gloves when performing the experiment.
- Use Protector RNase Inhibitor* to prevent degradation of RNA during first-strand cDNA synthesis.
- The kit enables the transcription of poly(A)$^+$ RNA into first-strand cDNA. The Oligo(dT)-Anchor Primer effectively selects for polyadenylated RNA by initiating cDNA synthesis from the 5′ start site of the poly(A) tail.
- Allow all reagents required for reverse transcription to thaw completely.
- Mix well and centrifuge briefly to collect the solutions at the bottom of the vials.
- Keep the reagents on ice while performing the assay; store them at −15 to −25°C after the experiment.

Sequence Information

Vial/Cap	Sequence
Vial 8 (red): Oligo d(T)-Anchor Primer	5'-GACCACGCGTATCGATGTC- GACTTTTTTTTTTTTTTTV-3' *Mlu* I site *Cla* I site *Sal* I site, V= A, C, or G
Vial 9 (red): PCR Anchor Primer	5'-GACCACGCGTATCGATGTCGAC-3'
Vial 10 (blue): Control Primer neo1/rev	5'-CAGGCATCGCCATGGGTCAC-3' *Nco* I site
Vial 11 (blue): Control Primer neo2/rev	5'-GCTGCCTCGTCCTGCAGTTC-3' *Pst* I site
Vial 12 (blue): Control Primer neo3/for	5'-GATTGCACGCAGGTTCTCCG-3'

Primer Design for 5′ RACE

At least two antisense gene-specific primers are needed.

- Gene-specific primer SP1 is required to transcribe the mRNA into first-strand cDNA.
- A second, nested primer SP2 located upstream of SP1 is used for the first PCR amplification. For a second PCR round, use a further nested primer SP3.

5′ RACE Control Reaction

Perform a control reaction when working with the 5´/3´ RACE Kit the first time.

The kit contains a control system, including Control neo-RNA and three gene specific neo-primers. The Control neo-RNA is an *in vitro* transcribed RNA from the neomycin resistance gene that contains a 3' poly(A)$^+$ tail and is 1,000 bases in size.

- To your sample RNA, add the Control neo-RNA to test whether the cDNA synthesis, the purification, the dA-tailing and the following PCR amplification are working. Handle your RNA preparation and our nuclease-free Control neo-RNA together in one tube to check for presence of contaminating nucleases. Determine the sensitivity of the 5'/3' RACE Kit by using dilutions of the control RNA.
- Transcribe the Control neo-RNA into a 655 bp first-strand cDNA using the Control Primer neo 1/rev. To check the efficiency of the cDNA synthesis, the cDNA is amplified using the Control Primer neo 2/rev and Control Primer neo 3/for, obtaining a 157 bp PCR product. To check the purification efficiency, perform this control PCR before and after the purification step.
- Efficiency of the dA-tailing reaction of the purified cDNA is checked by a control PCR using Oligo(dT)-Anchor Primer and neo2/rev Primer. Specific amplification of the dA-tailed control cDNA results in a prominent 293 bp fragment. Alternatively, this PCR assay may be used to optimize PCR parameters.

Reaction	Primer	Resulting PCR Product
cDNA synthesis	neo1/rev	655 bp
Control of cDNA synthesis:	• neo3/for • neo2/rev	157 bp
Amplification of dA-tailed cDNA	• neo2/rev • Oligo (dT)-Anchor	293 bp

Primer Design for 3′ RACE

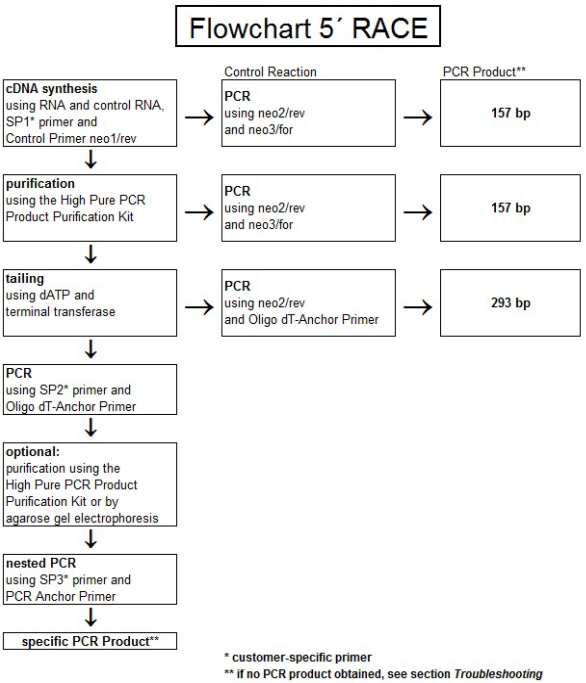
A gene-specific forward primer SP5 is required. Efficient and specific PCR amplification is highly dependent on effective primer design. Primer design is aimed at obtaining a balance between two goals: specificity and efficiency of amplification. Therefore, the potential for secondary structure and dimer formation should be minimized. The desirable primer length is 20 to 25 bp, with a GC content of 50 to 60%. For further information, see Saiki (4) and Frohmann (5).

3′ RACE Control Reaction

First-strand cDNA synthesis is initiated at the poly(A)$^+$ tail of the neo-RNA using the Oligo(dT)-Anchor Primer, obtaining a 1,040 bp cDNA. The first-strand cDNA can be directly amplified by PCR with the PCR Anchor Primer and the neo3/for Primer, resulting in a 1,026 bp PCR product.

Reaction	Primer	Resulting PCR Product
cDNA synthesis	Oligo(dT)-Anchor Primer	1,040 bp
Amplification of cDNA	• neo3/for • PCR Anchor Primer	1,026 bp

2.2 Experimental Overview



3. Experimental Protocol

3.1 Experimental Protocol for 5′ RACE

First-Strand cDNA Synthesis

Starting from total RNA or poly(A)$^+$ RNA, the kit enables the transcription of specific mRNA sequences into first-strand cDNA. In general, total RNA is used as starting sample material, but the usage of poly(A)$^+$ RNA may be advantageous for decreasing background or to enrich very rare messages. Transcriptor Reverse Transcriptase is provided in the kit, because of its increased heat stability compared to other reverse transcriptases, as well as its ability to reverse transcribe mRNA up to 14 kb in length. Therefore, the incubation temperature can be raised to +55°C to encourage reverse transcription to proceed through regions of difficult secondary RNA structure or high GC content. Applying such stringent reaction conditions will result in highly efficient cDNA synthesis.

⚠ Do not interrupt the protocol or store the cDNA because single-stranded cDNA is much more fragile than dsDNA. Directly proceed with cDNA purification.

❶ • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
cDNA Synthesis Buffer (Vial 1)	4 µl
Deoxynucleotide Mixture (Vial 3)	2 µl
cDNA synthesis primer SP1 (12.5 µM)	1 µl
poly(A)$^+$ RNA or total RNA 0.2 – 2 µg	x µl
For control reaction: Control Primer neo1/rev (Vial 10) Control neo-RNA (Vial 7)	 1 µl 1 µl
Transcriptor Reverse Transcriptase (vial 2)	1 µl
Double-distilled water	x µl
Total Volume	**20 µl**

• Mix the reaction mixture and spin down briefly.

❷ Incubate for 60 min at +55°C.

❸ Incubate another 5 min at +85°C.

❹ Briefly spin down the mixture.

❺ Remove 1 µl for later PCR control reaction.

⚠ Do not interrupt the protocol or store the cDNA because single-stranded cDNA is much more fragile than dsDNA. Directly proceed with cDNA purification.

For the following protocol, use the reagents from the High Pure PCR Product Purification Kit.

⚠ It is important to use this special protocol and not the protocol from the Instructions for Use of the High Pure PCR Product Purification Kit. Add 40 ml ethanol p. a. to the Wash Buffer of the pack size of 50 purifications of the High Pure PCR Product Purification Kit. The Binding Buffer (green cap) contains guanidine thiocyanate, which is an irritant. Wear gloves and follow laboratory safety conditions during handling.

❶ Add 100 µl Binding Buffer (green cap) to 20 µl of the first-strand cDNA reaction and mix well.

❷ Combine the High Pure Filter Tube and the Collection Tube and pipet sample into the upper reservoir.

❸ Centrifuge for 30 sec at 6,000 to 8,000 × *g* in an Eppendorf centrifuge.

❹
- Remove the Filter Tube from the Collection Tube.
- Discard the flow through liquid in the Collection Tube.
- Reinsert the Filter Tube into the same Collection Tube.

❺ Add 500 µl Wash Buffer to the upper reservoir of the Filter Tube.

❻ Centrifuge for 30 sec at 6,000 to 8,000 × *g* in an Eppendorf centrifuge.

❼ Make sure that the Filter Tube has no contact with the surface of the Wash Buffer flow through.
- Remove the Filter Tube from the Collection Tube.
- Discard the flow through liquid in the Collection Tube.
- Reinsert the Filter Tube into the same Collection Tube.

❽ Add 200 µl Wash Buffer to the upper reservoir of the Filter Tube.

❾ Centrifuge at least 2 min at maximum speed (approximately 13,000 × *g*) in an Eppendorf centrifuge.

🔍 This additional washing step with reduced buffer volume ensure optimal purity and the complete removal of residual wash buffer from the glass fiber fleece.

❿
- Remove the Filter Tube from the Collection Tube.
- Discard the Collection Tube with the flow through.

⓫
- Insert the Filter Tube into a sterile 1.5 ml microcentrifuge tube.
- Add 50 µl Elution Buffer (Vial 3) to the Filter.
- Centrifuge for 30 sec at 6,000 to 8,000 × *g* in an Eppendorf centrifuge.

⓬
- The microcentrifuge tube contains the eluted cDNA.
- Remove 1 µl of the purified cDNA from the microcentrifuge tube for later PCR control reaction.
- Use the purified cDNA directly for poly(A) tailing by Terminal Transferase.

⚠ Do not interrupt the protocol or store the cDNA because single-stranded cDNA is much more fragile than dsDNA.

Quantification of PCR Products

The concentration and purity of PCR product can be determined by spectrophotometric measurement at 260 nm and 280 nm.

⚠ In rare cases, glass fibers from the filter column may co-elute together with the cDNA; this can disturb subsequent UV absorbance measurements. Therefore, centrifuge the tube with the eluted cDNA for 1 to 2 min at high speed and carefully pipet from the surface.

Poly(A) Tailing of First-Strand cDNA

The following protocol describes the addition of a homopolymeric A-tail to the 3′ end of first-strand cDNA using recombinant Terminal Transferase and dATP.

❶ • Pipet the following into a sterile microcentrifuge tube on ice.

Component	Volume
Purified cDNA sample	19 µl
Reaction Buffer, 10x conc. (Vial 5)	2.5 µl
2 mM dATP (Vial 4)	2.5 µl

• Mix the reaction mixture and spin down briefly.

❷ • Incubate for 3 min at +94°C.
• Chill on ice.

❸ • Briefly spin down the mixture.
• Add 1 µl of Terminal Transferase rec. (80 U/µl, Vial 6).
• Mix and incubate at +37°C for 20 min.

🔍 The incubation time may be increased up to 30 min.

❹ • Incubate at +70°C for 10 min to heat inactivate the Terminal Transferase.
• Briefly spin down the mixture and place the tube on ice.

PCR Amplification of dA-Tailed cDNA

In the following table, the amplification of dA-tailed cDNA using the Expand High Fidelity PCR System* in a first and second optional (nested) PCR is described. The tailed cDNA can be directly amplified by PCR without prior purification or dilution.

🔍 If you want to use the Expand Long Template PCR System, follow the detailed instructions given in the Instructions for Use. for a 50 µl amplification reaction. Always use the primer and template concentrations given in section *Experimental Protocol*.

The optimal reaction conditions depend on the template/primer pair and must be determined individually. Use an annealing temperature close to the effective melting temperature of the primers.

❶ • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
dA-tailed cDNA	5 µl
Oligo dT-Anchor Primer (Vial 8)	1 µl
Specific primer SP2 (12.5 µM)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	36.25 µl
Total Volume	**50 µl**

• Mix the reaction mixture and spin down briefly.

❷ • Overlay with 50 µl mineral oil if necessary.
• Place the reaction mix in a thermal block cycler and start PCR.

❸ An example for temperature cycling conditions for the Perkin Elmer Cetus DNA Thermal Cycler (Models 9600 and 2400) is given below

	Temp.	Time	Cycle No.
Initial denaturation	94°C	2 min	1
Denaturation Annealing Elongation	94°C 55°C[1)] 72°C	15 sec 30 sec 40 sec	10
Denaturation Annealing Elongation	94°C 55°C[1)] 72°C	15 sec 30 sec 40 sec[2)]	25
Final elongation	72°C	7 min	1

[1)] Annealing temperature depends on the melting temperature of the primer used.
[2)] Elongate each successive cycle by additional 20 sec (*i.e.*, elongation time of cycle no. 11 is 40 sec; cycle no. 12, 60 sec; cycle no. 13, 80 sec; *etc.*).

❹

If	Then
you end up with enough PCR product,	store at +2 to +8°C
you used a cDNA for rare messages, you need a second PCR round to obtain a visible PCR product;	go ahead with Steps 5 to 10 and add a second PCR round (nested PCR).

❺ Dilute 10 µl of the amplification product from the first round to 1:20 in double-distilled water.

❻ Amplify 1 µl of the diluted material using the PCR Anchor Primer and a nested gene-specific primer 3 in a second PCR. If there is insufficient sequence information to design a nested primer, it is useful to reamplify gel-purified, size-selected PCR products using the PCR Anchor Primer and the original SP2 primer.

❼ Second PCR round (nested PCR), if necessary.
• Pipet the following into a sterile microfuge tube on ice:

Component	Volume
Diluted or undiluted PCR product	1 µl
PCR Anchor Primer (Vial 9)	1 µl
Specific primer SP3 (12.5 µM)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	40.25 µl
Total Volume	**50 µl**

• Mix well.

❽ Overlay with 50 µl mineral oil if necessary.

❾ Place the sample in a thermal block cycler and start PCR.

An example for temperature and cycle conditions is mentioned above. Depending on the melting temperature of the gene-specific primer SP3, increase the annealing temperature up to +60 to +65°C.

❿ Use 20 µl of both the first and second PCR product for analysis on a 1%, ethidium-bromide stained agarose gel with a corresponding DNA molecular weight marker.

In general, only one major band is generated, but occasionally, a background smear or a few minor bands might be visible. To verify that the correct fragment has been amplified, perform Southern blot hybridization analysis. If specific hybridization is not observed, see section *Troubleshooting*.

PCR Control Reaction

If you use the Control neo-RNA in the cDNA synthesis, perform the following PCR control reactions.

Control of First-Strand

Use 1 µl of the cDNA synthesis reaction assay (both before and after purification) as template for the PCR reaction.

1. • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
cDNA (purified or not purified)	1 µl
Control Primer neo2/rev (Vial 11)	1 µl
Control Primer neo3/for (Vial 12)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	40.25 µl
Total Volume	**50 µl**

   • Mix and spin down briefly.

2. • Overlay with mineral oil if necessary.
   • Place the reaction mix in a thermal block cycler and start PCR.

3. Use 20 µl of both the first and second PCR product for analysis on a 1% ethidium-bromide stained agarose gel with a corresponding molecular weight standard.

   If the cDNA synthesis and purification step have been successful, a strong PCR product band of 157 bp should be visible. If this is not the case, see section *Troubleshooting*.

Control PCR Amplification of the dA-Tailed First-Strand cDNA

Amplify 1 µl dA-tailed cDNA by using the PCR Oligo dT-Anchor Primer and the Control Primer neo2/rev.

1 • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
dA-Tailed DNA	1 µl
Oligo dT Anchor Primer (Vial 8)	1 µl
Control Primer neo2/rev (Vial 11)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	40.25 µl
Total Volume	**50 µl**

• Mix and spin down briefly.

2 • Overlay with mineral oil if necessary.
• Place the reaction mix in a thermal block cycler and start PCR.

3 Use 20 µl of both the first and second PCR product for analysis on a 1% ethidium bromide-stained agarose gel with a corresponding molecular weight standard.

If the dA-tailing reaction has been successful, a strong band of 293 bp should be visible after amplification.

3.2 Experimental Protocol for 3′ RACE

First-Strand cDNA Synthesis

❶ • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
cDNA Synthesis Buffer (Vial 1)	4 µl
Deoxynucleotide Mixture (Vial 3)	2 µl
Oligo dT-Anchor Primer (Vial 8)	1 µl
poly(A)$^+$ RNA or total RNA	0.5 to 2 µg
[for control reaction: Control neo-RNA (Vial 7)]	[1 µl]
Transcriptor Reverse Transcriptase (Vial 2)	1 µl
Double-distilled water	x µl
Total Volume	**20 µl**

• Mix and spin down briefly.

❷ Incubate for 60 min at +55°C.

❸ Incubate additionally for 5 min at +85°C.

❹ Briefly spin down the mixture.

PCR Amplification of cDNA

The cDNA can be directly amplified by PCR without prior purification. Use 1 µl of the cDNA reaction mix, the PCR Anchor Primer and a gene-specific primer SP5 in the PCR reaction. The optimal reaction conditions depend on the template/primer pair and must be determined individually. Use an annealing temperature from +60 to +65°C.

Whether Expand High Fidelity or Expand Long Template PCR System should be used depends on the expected size of the PCR product and the amount of template cDNA present in the reaction. The final concentration of dNTP and $MgCl_2$ should be adjusted according to the protocols given in the individual instructions for use. If you want to use the Expand Long Template PCR System and you need to establish a new assay, it is advisable to test all three possible amplification systems to find the optimum reaction conditions.

❶ • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
cDNA product	1 µl
PCR Anchor Primer (Vial 9)	1 µl
Specific primer SP5 (12.5 µM)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	40.25 µl
Total Volume	**50 µl**

• Mix and spin down briefly.

❷ • Overlay with 50 µl mineral oil if necessary.
• Place the reaction mix in a thermal block cycler and start PCR.

❸ Use 20 µl of the PCR amplification product for analysis on a 1% ethidium bromide-stained agarose gel with a corresponding DNA molecular weight marker.

3.2 Experimental Protocol for 3′ RACE, continued

PCR Control Reaction

If you have used the Control neo-RNA in the cDNA synthesis reaction, use the following PCR control reaction. Use 1 µl of the cDNA synthesis reaction assay, the provided neo3/for primer and the PCR Anchor Primer in the PCR reaction.

❶ • Pipet the following into a sterile microcentrifuge tube on ice:

Component	Volume
cDNA product	1 µl
PCR Anchor Primer (Vial 9)	1 µl
Control Primer neo3/for (Vial 12)	1 µl
Deoxynucleotide Mixture (Vial 3)	1 µl
Expand High Fidelity Enzyme Mix	0.75 µl
Expand High Fidelity Buffer (10×) with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5 µl
Double-distilled water	40.25 µl
Total Volume	**50 µl**

• Mix and spin down briefly..

❷ • Overlay with 50 µl mineral oil if necessary.
• Place the reaction mix in a thermal block cycler and start PCR.

❸ Use 20 µl of the PCR amplification product for analysis on a 1%, ethidium bromide-stained agarose gel with a corresponding DNA molecular weight marker.

If the 3' RACE amplification has been successful, a strong band of 1,026 bp should be visible. If this is not the case, see section *Troubleshooting*.

4. Troubleshooting

cDNA Synthesis

	Possible Cause	Recommendation
Low or no product	Reagents contaminated	Verify that your PCR system works well performing a common PCR reaction using a DNA template and primers to ensure the integrity of the reagents.
	RNA is degraded or of poor quality.	Electrophorese the RNA of interest in a 1% formaldehyde minigel and examine integrity of the 18S and 28S ribosomal bands. If the RNA is degraded or of poor quality, isolate a new total RNA (6).
	RNase contamination	• Successful cDNA synthesis demands RNase-free handling at all times. Wear gloves to avoid contamination of the kit components, use sterilized pipettes and tubes. For more details, see (6, 7). • If necessary, use Protector RNase Inhibitor* during first-strand cDNA synthesis.
	Reverse transcription is inhibited by contamination.	Ensure that the RNA preparation is free of agents that inhibit reverse transcription, such as phenol, lithium chloride and SDS (8).
	RNA preparation is contaminated with genomic DNA.	Make sure that the RNA preparation is free of contaminating genomic DNA. If necessary, perform a control experiment without the cDNA synthesis step; any obtained PCR products result from amplification of genomic DNA.
	Purification steps not efficient	If you do not obtain a strong 157 bp PCR product using the purified control cDNA as template, make sure that the purification procedure is correct.
	Reagents contaminated	Verify that your PCR system works well performing a common PCR reaction using a DNA template and primers to ensure the integrity of the reagents.
	Concentration of the specific product too low for detection by ethidium bromide staining	• Perform a Southern blot analysis of the PCR product using internal sequences as probe to identify specific product bands. • Try a second PCR round using a nested primer SP4 and the PCR Anchor Primer.

	Possible Cause	Recommendation
	Purification steps not efficient	• Use an agarose gel-purified PCR product as template in the second PCR for more specific amplification products. • Use the High Pure PCR Product Purification Kit between the first and second PCR round for more specific amplification products.
Low or no product	Nonspecific products are being amplified.	Raise the annealing temperature gradually until nonspecific products are no longer observed.
	Improper cDNA denaturation.	Check if the denaturation step prior to the tailing reaction was performed properly according the protocol: Incubate for 3 min at +94°C. Chill on ice.
	Insufficient incubation time for poly(A) tailing.	The incubation period for the tailing reaction can be increased to 30 min.
	Improper storage of the High Pure PCR Product Purification Kit.	If the High Pure PCR Product Purification Kit is not stored at +15 to +25°C but at +2 to +8°C, precipitation within the Binding Buffer may occur. Such precipitates of buffer components may be carried over to the final cDNA eluate and inhibit the subsequent Terminale Transferase reaction.
	Incorrect cDNA purification protocol.	Follow the purification protocol in the Instructions for Use of the 5'/3' RACE, not the High Pure PCR Product Purification Kit. It is crucial that centrifugation after the last washing step (prior to elution) is performed at maximum speed of 13,000 × *g* and for a mininum of 2 min. The Filter Tube should be completely dry before elution of bound cDNA. Otherwise, residual ethanol inhibits the tailing reaction. All other centriguation steps should be performed at 6,000 to 8,000 × *g* and here 30 sec duration is sufficient.
$A_{260\ nm}$ reading of nucleic acid eluate too high.	Glass fibers, which might co-elute with nucleic acid and disturb absorbance measurement.	1. Remove High Pure Filter Tube from tube containing eluted sample and spin sample for 1 min at maximum speed. 2. Use an aliquot of the supernatant. Do not disturb the glass fibers at the bottom of the original tube. 3. It may be useful to make a correction by subtracting the value measured at 320 nm from the value measured at 260 nm.

	Possible Cause	Recommendation
Stretch of dTs in the 5´ region of the final PCR product	Wrong annealing conditions	If the annealing conditions during the first PCR amplification are not optimal (*i.e.*, the anchor primer is not completely annealed and might bind within the 5´poly(A) tail) and a proofreading polymerase (*e.g.,* Expand High Fidelity PCR System or Expand Long Template PCR System) is used, the 3´ – 5´exonuclease activity might remove a non-annealed 3´ non-T anchor base. This would lead to priming of PCR from within the poly(A) tail and the introduction of long 5´ T stretches into the PCR product. Stringent annealing conditions are crucial for success of the reaction.
	Insufficient purification of cDNA.	Correct purification of the first-strand cDNA is extremely important to remove any residual unincorporated nucleotides. Otherwise, in the 5´ tailing reaction, other nucleotides than A could be incorporated, which would lead to internal binding of the Oligo-d(T) Anchor Primer to the 5´ poly(A) tail.

3' RACE

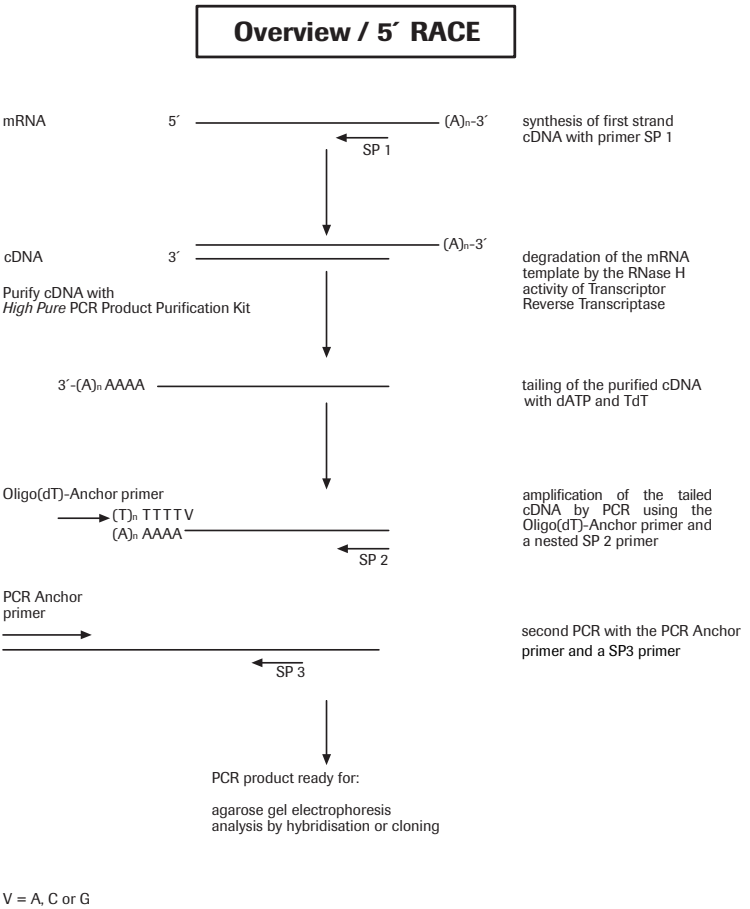
	Possible Cause	Recommendation
No PCR amplification product using the control system or no specific PCR amplification product with your own RNA/primer system.	Reagents contaminated	Verify that your PCR system in general works well by performing a common PCR reaction using a DNA template and primers to ensure the integrity of the reagents.
	RNA degraded or of poor quality	Electrophorese the RNA in a 1% formaldehyde minigel and examine integrity of the 18S and 28S ribosomal bands. If the RNA is degraded or of poor quality, isolate a new total RNA (6).
	RNase contamination	Successful cDNA synthesis demands RNase-free handling at all times. • Wear gloves to avoid contamination of the kit components and use sterilized pipettes and tubes. For more details, see (6, 7). • If necessary, use Protector RNase-Inhibitor* during first-strand cDNA synthesis.
	Reverse transcription is inhibited by contamination.	Ensure that the RNA preparation is free of agents that inhibit reverse transcription, such as phenol, lithium chloride and SDS (8).
	Contamination with genomic DNA	Make sure that the RNA preparation is free of contaminating genomic DNA. If necessary, perform a control experiment without the cDNA synthesis step; any obtained PCR products result from amplification of genomic DNA.
Only low yields of specific products are observed.	Concentration of the specific product too low for detection by ethidium bromide staining.	• Perform a Southern blot analysis of the PCR product using internal sequences as probe to identify specific product bands. • Try a second PCR round using a nested primer SP6 and the PCR Anchor Primer.
	Non-specific products are being amplified.	Raise the annealing temperature gradually until non-specific products are no longer observed.

5. Additional Information on this Product

How this Product Works

Generating a full-length cDNA is of critical importance in studies on gene structure and expression. An intact, full-length cDNA including the very 5´ end is rarely recovered from cDNA libraries, despite time-consuming cDNA library screening. Often, the 5´ end of the cDNA strand is missing because of the inability of the reverse transcriptases to read through an entire gene sequence; this is a problem particularly in the case of extremely large gene transcripts. Therefore, methods have been developed to amplify DNA sequences from a messenger RNA (mRNA) template between a defined internal site and unknown sequences of either the 3´ or the 5´ end of the mRNA. These methods are often referred to as RACE (rapid amplification of cDNA ends), "anchored" PCR (1), or "one-sided" PCR (2), and were first described by Frohmann *et al.* (3).

5′ RACE Overview



5' RACE allows the amplification of unknown sequences at the 5' end of the mRNA.

Test Principle

① First-strand cDNA synthesis
First-strand cDNA is synthesized from total or poly(A)$^+$ RNA using a gene-specific primer SP1, Transcriptor Reverse Transcriptase, and the Deoxynucleotide Mixture. Transcriptor Reverse Transcriptase is used because of its greater heat stability compared to the other reverse transcriptases and its ability to reverse transcribe up to 14 kb long mRNA. Therefore, the incubation temperature for first-strand cDNA synthsis can be raised up to +55°C to encourage reverse transcription to proceed through regions of difficult secondary RNA structure.

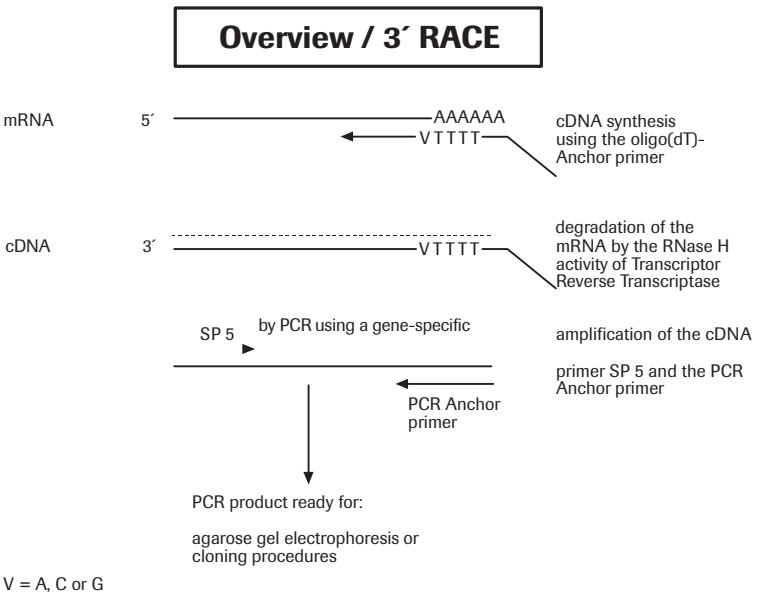
② Purification
The first-strand cDNA is purified from unincorporated nucleotides and primers using the High Pure PCR Product Purification Kit*.

③ Addition of homopolymeric A-tail
Terminal transferase is used to add a homopolymeric A-tail to the 3′ end of the cDNA. Since vertebrate coding sequences and 5′ untranslated RNA regions tend to be biased toward G/C residues, the use of a poly(A)-tail decreases the likelihood of inappropriate truncation by the Oligo dT-Anchor Primer (3). Additionally, poly(A)-tail is used because A/T binding is weaker than G/C binding; therefore, longer stretches of A residues are required before the Oligo dT-Anchor Primer will bind to an internal site and truncate the amplification product.

④ First PCR amplification
Tailed cDNA is then amplified by PCR using a gene-specific primer SP2 and the Oligo dT-Anchor Primer. The Oligo(dT)-Anchor Primer is a mixture of oligonucleotides carrying a non-T nucleotide (*i.e.,* A, C, or G) at the 3′ end following the dT-stretch. By this means the Oligo(dT)-Anchor Primer is forced to bind to the (5′) start site of the poly(A)-tail. Thus, the actual length of the poly(A)-tail has no influence on priming.

⑤ Second PCR amplification
The obtained cDNA is further amplified by a second PCR using a nested, gene-specific primer SP3 and the PCR Anchor Primer. As a result, the obtained 5′ RACE products can be cloned into an appropriate vector for subsequent characterization procedures, which may include sequencing and restriction mapping.

3′ RACE Overview



3′ RACE takes advantage of the natural poly(A)-tail of mRNAs as a priming site for PCR amplification.

A Control neo-RNA and three control primers (neo1/rev, neo2/rev, and neo3/for) are included in the kit to verify performance of the first-strand cDNA synthesis, tailing reaction, and following amplification.

Test Principle

①	First-strand cDNA synthesis is initiated at the poly(A)-tail of mRNA using the Oligo(dT)-Anchor Primer.
②	After converting mRNA into cDNA, the following amplification is then directly performed without a further purification step using the PCR Anchor Primer and a user-designed gene-specific primer (SP5).

References

1 Loh,Y. *et al.* (1989) *Science* **243**, 217.
2 Ohara *et al.* (1989) *Proc. Natl. Acad. Sci USA* **86**, 5673.
3 Frohmann, M. (1994) *PCR Methods and Applications* **4,** 540–558.
4 Saiki, R. (1990) PCR Protocols: A Guide to Methods and Applications, pp. 13–20.
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6 Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156.
7 Molecular Cloning, A Laboratory Manual (1989, Nolan, C, ed.) 2nd edition, pp 7.3–7.8 and 10.27–10.37.
8 Current Protocols in Molecular Biology (1987, Ausubel *et al.* eds.) Vol 1, pp 3.8 and 4, Massachusetts General Hospital, Havard Medical School.
9 David, M. *et al.* (2003) Critical role of the transcriptional repressor neuron-restrictive silencer factor in the specific control of connexinin insulin-producing cell lines. *J. Biol. Chem.* **278,** 53082-9.
10 Benachour, A. *et al.* (2005) The *enterococcus faecalis* SigV protein is an extracytoplasmic function sigma factor contributing to survival following heat, acid, and ethanol treatments. *J. Bacteriol.* **187,** 1022-35.
11 Golovine, K. *et al.* (2003) Three different promoters control expression of the aromatase cytochrome P450 gene (Cyp19) in mouse gonads and brain. *Biol Reprod* **68,** 978-84.
12 Mouchel, N. *et al.* (2003) Alternative 5' exons of the CFTR gene show developmental regulation. *Hum. Mol. Genet.* **12**, 759-69.
13 Nie, G.Y. *et al.* (2003) A novel serine protease of the mammalian HtrA family is up-regulated in mouse uterus coinciding with placentation. *Mol. Hum. Reprod.* **9,** 279-90.

Quality Control

- 3´ RACE: Transcription of the Control neo-RNA using the Oligo(dT) Anchor Primer. Amplification of cDNA using the anchor and the neo3/for primers, obtaining a 1,026 bp PCR product.
- 5´ RACE: Transcription of the Control neo-RNA using neo1/rev primer. Amplification of cDNA without prior tailing using the neo3/for and neo2/rev primers, obtaining a 157 bp PCR product. Amplification of cDNA after tailing using the PCR Anchor and neo2/rev primers, obtaining a 293 bp PCR product.

6. Supplementary Information

6.1 Conventions

Text Conventions To make information consistent and memorable, the following text conventions are used in this document:

Text Convention	Use
Numbered stages labeled ①, ②, *etc.*	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ❶, ❷, *etc.*	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Diagnostics.

Symbols In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

6.2 Changes to Previous Version

- Editorial changes.

6.3 Ordering Information

	Product	Pack Size	Cat. No.
Kits	High Pure PCR Product Purification Kit	1 kit (50 purifications) 1 kit (250 purifications)	11 732 668 001 11 732 676 001
	High Pure RNA Isolation Kit	1 kit (50 isolations)	11 828 665 001
	High Pure RNA Tissue Kit	1 kit (50 isolations)	12 033 674 001
	mRNA Isolation Kit	1 kit	11 741 985 001
	mRNA Isolation Kit for Blood/ Bone Marrow	1 kit	11 934 333 001
	Rapid DNA Ligation Kit	1 kit (40 reactions)	11 635 379 001
Single reagents	Protector RNase Inhibitor	10,000 U 2,000 U	03 335 402 001 03 335 399 001
	Transcriptor Reverse Transcriptase	250 U 500 U 2,000 U	03 531 317 001 03 531 295 001 03 531 287 001
	Terminale Transferase, recombinant	8,000 U 24,000 U	03 333 566 001 03 333 574 001
	Expand High Fidelity PCR System	100 U 2 × 250 U 10 × 250 U	11 732 641 001 11 732 650 001 11 759 078 001
	Expand Long Template PCR System	150 U 720 U 3,600 U	11 681 834 001 11 681 842 001 11 759 060 001
	Proteinase K, recombinant, PCR Grade	1.25 ml 5 ml 25 ml	03 115 887 001 03 115 828 001 03 115 844 001
	Agarose MP	100 g 500 g	11 388 983 001 11 388 991 001
	DNA Molecular Weight Marker VIII	50 µg (1 A_{260} unit)	11 336 045 001
	DNA Molecular Weight Marker IX	50 µg (1 A_{260} unit)	11 449 460 001
	DNA Molecular Weight Marker XIII	50 µg (1 A_{260} unit)	11 721 925 001
	DNA Molecular Weight Marker XIV	50 µg (1 A_{260} unit)	11 721 933 001

PCR Nucleotide Selection

Product	Pack Size	Cat. No.
Deoxynucleoside Triphosphate Set	4 × 250 µl 4 × 1,250 µl	11 969 064 001 03 622 614 001
PCR Nucleotide Mix	200 µl 10 × 200 µl	11 581 295 001 11 814 362 001
PCR Nucleotide Mix^{PLUS}	2 × 100 µl	11 888 412 001

6.4 Disclaimer of License

For patent license limitations for individual products please refer to: List of biochemical reagent products

6.5 Trademarks

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6.6 Regulatory Disclaimer

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