For life science research only.
Not for use in diagnostic procedures.

5′/3′ RACE Kit, 2nd Generation

Version: 16
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For the rapid amplification of either 5′ or 3′ cDNA ends.
Formulation contains Transcriptor Reverse Transcriptase and recombinant Terminal Transferase.

Cat. No. 03 353 621 001 1 kit
10 reactions

Store the kit at −15 to − 25°C.

1. General Information

1.1. Contents

Vial / bottle	Cap	Label	Function / description	Content
1	purple	5′/3′ RACE Kit, 2nd Generation, cDNA synthesis buffer, 5x conc.	250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, pH 8.5 (+20°C).	1 vial, 100 µL
2	purple	5′/3′ RACE Kit, 2nd Generation, Transcriptor RT	25 U/µL in 200 mM potassium phosphate, 2 mM DTT, 0.2% (v/v) Triton X-100, 50% glycerol (v/v), pH 7.2.	1 vial, 10 µL
3	green	5′/3′ RACE Kit, 2nd Generation, Deoxynucleotide mixture	Mixture of 10 mM each dATP, dCTP, dGTP, dTTP in Tris-HCl, pH 7.5 (+20°C).	1 vial, 50 µL
4	green	5′/3′ RACE Kit, 2nd Generation, dATP	2 mM in Tris-HCl, pH 7.5 (+20°C).	1 vial, 30 µL
5	green	5′/3′ RACE Kit, 2nd Generation, Reaction buffer, 10x conc.	100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 (+20°C).	1 vial, 1,000 µL
6	green	5′/3′ RACE Kit, 2nd Generation, Terminal Transferase	80 U/µL in 60 mM potassium phosphate, pH 7.2 +4°C, 150 mM KCl, 1 mM 2-mercaptoethanol, 0.1% Tween 20, 50% glycerol.	1 vial, 10 µL
7	blue	5′/3′ RACE Kit, 2nd Generation, Control neo-RNA	1 ng/µL in double-distilled water.	1 vial 20 µL
8	red	5′/3′ RACE Kit, 2nd Generation, Oligo dT-anchor primer	▪ 37.5 µM in double-distilled water. ▪ 5′-GACCACGCGTATCGATGTCGACT TTTTTTTTTTTTTTV-3′ Mlu I site Cla I site Sal I site, V = A, C, or G	1 vial, 40 µL
9	red	5′/3′ RACE Kit, 2nd Generation, PCR anchor primer	▪ 12.5 µM in double-distilled water. ▪ 5′-GACCACGCGTATCGATGTCGAC-3′	1 vial, 40 µL
10	blue	5′/3′ RACE Kit, 2nd Generation, Control primer neo1/rev. primer	▪ 12.5 µM in double-distilled water. ▪ 5′-CAGGCATCGCCATGGGTCAC-3′ Nco I site	1 vial, 40 µL
11	blue	5′/3′ RACE Kit, 2nd Generation, Control primer neo2/rev. primer	▪ 12.5 µM in double-distilled water. ▪ 5′-GCTGCCTCGTCCTGCAGTTC-3′ Pst I site	1 vial, 40 µL
12	blue	5′/3′ RACE Kit, 2nd Generation, Control primer neo3/for primer	▪ 12.5 µM in double-distilled water. ▪ 5′-GATTGCACGCAGGTTCTCCG-3′	1 vial, 40 µL

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to − 25°C, the kit is stable through the expiry date printed on the label.

Vial / bottle	Cap	Label	Storage
1	purple	cDNA synthesis buffer, 5x conc.	Store at −15 to − 25°C.
2	purple	Transcriptor RT	
3	green	Deoxynucleotide mixture	
4	green	dATP	
5	green	Reaction buffer, 10x conc.	
6	green	Terminal Transferase	
7	blue	Control neo-RNA	
8	red	Oligo dT-anchor primer	
9	red	PCR anchor primer	
10	blue	Control primer neo1/rev. primer	
11	blue	Control primer neo2/rev. primer	
12	blue	Control primer neo3/for primer	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction tubes for preparing PCR mixes and dilutions
- PCR reaction vessels, such as 0.2 mL thin-walled PCR tubes or plates
- Standard benchtop microcentrifuge
- Thermal block cycler

For primer design for 5′ RACE

- Gene-specific primer SP1
- Nested primer SP2
- Nested primer SP3

For primer design for 3′ RACE

- Gene-specific forward primer SP5

For first-strand cDNA synthesis

- Template RNA
- Double-distilled water
- High Pure PCR Product Purification Kit*

For PCR amplification of poly(A)-tailed first-strand cDNA

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

  ⚠ ***The decision to use one of these two kits 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, test all three possible amplification systems to find the optimum reaction conditions.***
- High Pure PCR Product Purification Kit*
- Protector RNase Inhibitor* (optional)
- Ethanol
- Mineral oil

1.4. Application

The 5′/3′ Race Kit is used for the 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

Sample Materials

For template RNA, use 0.2 to 2.0 µg total RNA or poly(A)$^+$ RNA.

Control Reactions

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 3 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.

① To your sample RNA, add the Control neo-mRNA 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 the presence of contaminating nucleases.
– Determine the sensitivity of the 5′/3′ RACE Kit using dilutions of the control RNA.

② Transcribe the Control neo-RNA into a 655 bp first-strand cDNA using the Control primer neo 1/rev.

③ 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.

④ To check the purification efficiency, perform this control PCR before and after the purification step.

⑤ 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 [bp]
cDNA synthesis	neo1/rev.	655
Control of cDNA synthesis	▪ neo3/for ▪ neo2/rev.	157
Amplification of dA-tailed cDNA	▪ neo2/rev. ▪ Oligo dT-anchor	293

3′ RACE control reaction

① 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.

② 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 [bp]
cDNA synthesis	Oligo dT-anchor	1,040
Amplification of cDNA	▪ neo3/for ▪ PCR anchor	1,026

Primers

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.

Primer design for 3′ RACE

- A gene-specific forward primer SP5 is required.
- Efficient and specific PCR amplification is highly dependent of 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; the GC content 50 to 60%.

General Considerations

Precautions

- 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; after the experiment, store at −15 to −25°C.

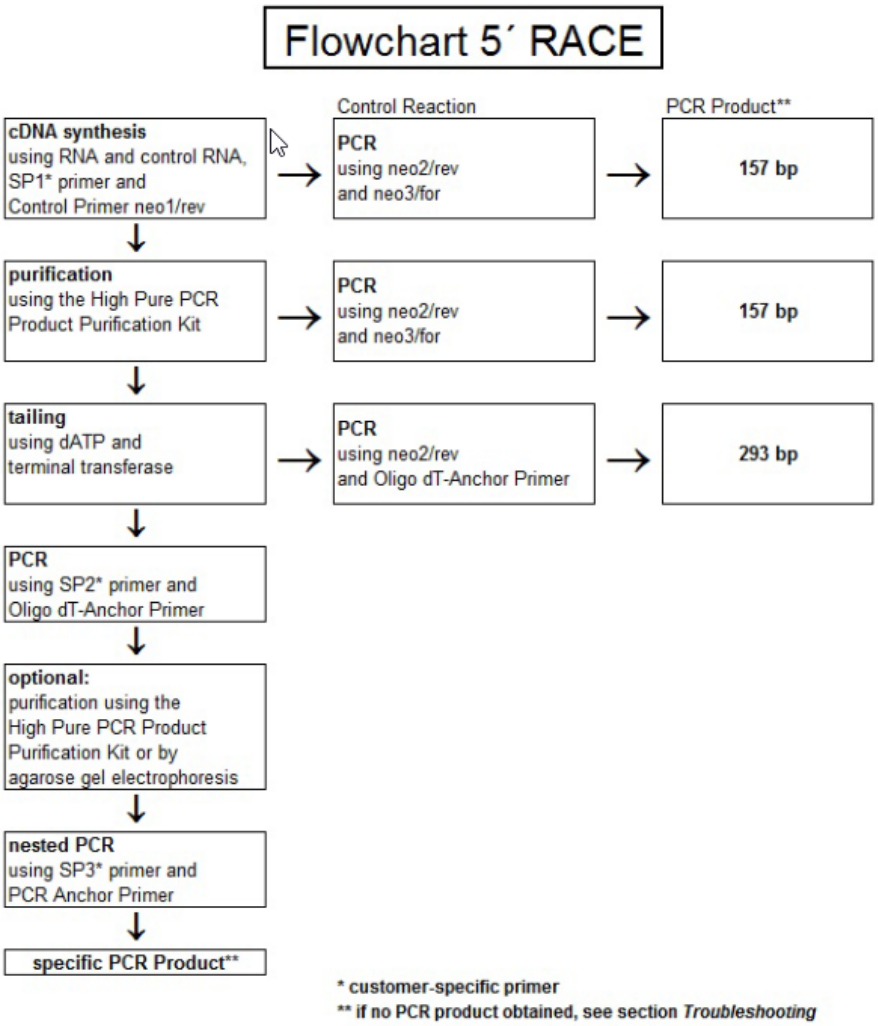
Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

2.2. Protocols

5′ RACE experimental protocol



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 RT (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.***

1. Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
cDNA synthesis buffer, 5x conc. (Vial 1)	4
Deoxynucleotide mixture (Vial 3)	2
cDNA synthesis primer SP1 (12.5 µM)	1
poly(A)$^+$ RNA or total RNA (0.2 – 2 µg)	X
For control reaction: Control primer neo1/rev. (Vial 10) Control neo-RNA (Vial 7)	 1 1
Transcriptor RT (Vial 2)	1
Double-distilled water	X
Total Volume	**20**

– Mix the reaction mixture and spin down briefly.

2. Incubate for 60 minutes at +55°C.

3. Incubate another 5 minutes at +85°C.

4. Briefly spin down the mixture.

5. Remove 1 µL for the 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.***

cDNA purification

Use the High Pure PCR Product Purification Kit* for the cDNA purification.

⚠ ***Use only this protocol, not the protocol from the Instructions for Use of the High Pure PCR Product Purification Kit. Add 40 mL ethanol to the Wash Buffer from the 50 purifications pack size 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.***

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

2. Combine the High Pure Filter Tube and the Collection Tube and pipette sample into the upper reservoir.

3. Centrifuge for 30 seconds at 6,000 to 8,000 × *g*.

4. 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.

5. Add 500 µL Wash Buffer to the upper reservoir of the Filter Tube.

6. Centrifuge for 30 seconds at 6,000 to 8,000 × *g*.

7. Make sure that the Filter Tube has no contact with the surface of the Wash Buffer flow through.

8. 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.

9 Add 200 µL Wash Buffer to the upper reservoir of the Filter Tube.

10 Centrifuge at least 2 minutes at maximum speed, approximately 13,000 × *g*.

i This additional washing step with reduced buffer volume ensures optimal purity and completely removes residual Wash Buffer from the glass fiber fleece.

11 Remove the Filter Tube from the Collection Tube.
– Discard the Collection Tube with the flow through.

12 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 seconds at 6,000 to 8,000 × *g*.

13 The microcentrifuge tube contains the eluted cDNA.
– Remove 1 µL of the purified cDNA from the microcentrifuge tube for the 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 minutes at high speed and carefully pipette 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.

1 Pipette the following reagents into a sterile microcentrifuge tube on ice.

Reagent	Volume [µL]
Purified cDNA sample	19
Reaction buffer, 10x conc. (Vial 5)	2.5
2 mM dATP (Vial 4)	2.5
Final Volume	**24**

– Mix the reaction mixture and spin down briefly.

2 Incubate for 3 minutes at +94°C.
– Chill on ice.

3 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 minutes.

i The incubation time may be increased up to 30 minutes.

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

PCR amplification of dA-tailed cDNA

The following protocol describes the amplification of dA-tailed cDNA using the Expand High Fidelity PCR System* in a first and second optional (nested) PCR. 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 in the Instructions for Use for a 50 µL amplification reaction. Always use the primer and template concentrations given in section,* **5′ RACE 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.

1 Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
dA-tailed cDNA	5
Oligo dT-anchor primer (Vial 8)	1
Specific primer SP2 (12.5 µM)	1
Deoxynucleotide mixture (Vial 3)	1
Enzyme High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	36.25
Total Volume	**50**

– Mix the reaction mixture and spin down briefly.

2 Overlay with 50 µL mineral oil if necessary.
– Place the reaction mix in a thermal block cycler and start PCR.

The following thermal profiles are an example. Different thermal cyclers may require different profiles.

Step	Temperature [°C]	Time	Number of Cycles
Pre-Incubation	94	2 min	1
Denaturation Annealing Elongation	94 55(1) 72	15 sec 30 sec 40 sec	10
Denaturation Annealing Elongation	94 55(1) 72	15 sec 30 sec 40 sec + 20 sec cycle elongation for each successive cycle(2)	25
Final Elongation	72	7 min	1

3 If you end up with sufficient PCR product, store at +2 to +8°C.
– If you used a cDNA for rare messages, you need a second PCR round to obtain a visible PCR product; Proceed to Steps 4 to 8 and add a second PCR round (nested PCR).

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

5 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.

6 For the second PCR round (nested PCR), pipette the following reagents into a sterile microfuge tube on ice:

Reagent	Volume [µL]
Diluted or undiluted PCR product	1
PCR anchor primer (Vial 9)	1
Specific primer SP3 (12.5 µM)	1
Deoxynucleotide mixture (Vial 3)	1
Expand High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	40.25
Final Volume	**50**

– Mix the reaction mixture and spin down briefly.

7 Overlay with 50 µL mineral oil if necessary.
– Place the reaction mix in a thermal block cycler and start PCR.

Temperature and cycling conditions are shown in Step 2 above. Depending on the melting temperature of the gene-specific primer SP3, increase the annealing temperature up to +60 to +65°C.

8 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**.

(1) Annealing temperature depends on the melting temperature of the primers used.
(2) For example, cycle 11 is 20 seconds longer than cycle 10. Cycle 12 is 40 second longer than cycle 10. Cycle 13 is 60 seconds longer than cycle 10, etc.

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 Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
Purified or not purified cDNA sample	1
Control primer neo2/rev. (Vial 11)	1
Control primer neo3/for (Vial 12)	1
Deoxynucleotide mixture (Vial 3)	1
Expand High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	40.25
Final Volume	**50**

– 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 marker.

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 Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
dA-tailed DNA	1
Oligo dT-anchor primer (Vial 8)	1
Control primer neo2/rev. (Vial 11)	1
Deoxynucleotide mixture (Vial 3)	1
Enzyme High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	40.25
Total Volume	**50**

– Mix the reaction mixture 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 marker.

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

3′ RACE experimental protocol

First-strand cDNA synthesis

1. Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
cDNA synthesis buffer, 5x conc. (Vial 1)	4
Deoxynucleotide mixture (Vial 3)	2
Oligo dT-anchor primer (Vial 8)	1
poly(A)$^+$ RNA or total RNA (0.5 – 2 µg)	X
For control reaction: Control neo-RNA (Vial 7)	1
Transcriptor RT (Vial 2)	1
Double-distilled water	X
Total Volume	**20**

– Mix the reaction mixture and spin down briefly.

2. Incubate for 60 minutes at +55°C.

3. Incubate another 5 minutes at +85°C.

4. 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.

1. Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
cDNA product	1
PCR anchor primer (Vial 9)	1
Specific primer SP5 (12.5 µM)	1
Deoxynucleotide mixture (Vial 3)	1
Expand High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	40.25
Final Volume	**50**

– Mix the reaction mixture 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 the PCR amplification product for analysis on a 1% ethidium bromide-stained agarose gel with a corresponding molecular weight marker.

PCR control reaction

If you use the Control neo-RNA in the cDNA synthesis, perform the following PCR control reaction. Use 1 µL of the cDNA synthesis reaction, the supplied Control primer neo3/for, and the PCR anchor primer in the PCR reaction.

1. Pipette the following reagents into a sterile microcentrifuge tube on ice:

Reagent	Volume [µL]
cDNA product	1
PCR anchor primer (Vial 9)	1
Control primer neo3/for (Vial 12)	1
Deoxynucleotide mixture (Vial 3)	1
Expand High Fidelity Enzyme Mix	0.75
Expand High Fidelity Buffer, 10x conc. with 15 mM $MgCl_2$ (supplied with the Expand High Fidelity PCR System)	5
Double-distilled water	40.25
Final Volume	**50**

– 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 the PCR amplification product for analysis on a 1% ethidium bromide-stained agarose gel with a corresponding molecular weight marker.

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

3. Troubleshooting

cDNA synthesis

Observation	Possible cause	Recommendation
Low or no product.	Reagents are contaminated.	To ensure the integrity of the reagents, verify that your PCR system works well; perform a common PCR reaction using a DNA template and primers.
	RNA is degraded or of poor quality.	Electrophorese the RNA of interest in a 1% formaldehyde minigel and examine the integrity of the 18S and 28S ribosomal bands. If the RNA is degraded or of poor quality, isolate a new total RNA.
	RNase contamination present.	Successful cDNA synthesis demands RNase-free handling at all times. ⚠ ***Wear gloves to avoid contamination of the kit components; use sterilized pipettes and tubes.***
		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.
	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.
	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.
	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.
		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.
	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 minutes at +94°C, and chill on ice.
	Insufficient incubation time for poly(A) tailing.	Increase incubation period for the tailing reaction to 30 minutes.
	Improper storage of the High Pure PCR Product Purification Kit.	If the kit is stored incorrectly at +2 to +8°C instead of +15 to +25°C, precipitation within the Binding Buffer may occur. These precipitates in the Binding Buffer may be carried over to the final cDNA eluate and inhibit the subsequent Terminal 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. ⚠ ***Centrifugation after the last washing step (prior to elution) must be performed at maximum speed of 13,000 × g and for a minimum of 2 minutes. The Filter Tube should be completely dry before elution of bound cDNA.*** Otherwise, residual ethanol inhibits the tailing reaction. All other centrifugation steps should be performed at 6,000 to 8,000 × *g*; 30 seconds duration is sufficient.

$A_{260\ nm}$ reading of nucleic acid eluate too high.	Glass fibers could co-elute with nucleic acid and disturb absorbance measurement.	▪ Remove High Pure Filter Tube from tube containing eluted sample and spin sample for 1 minute at maximum speed. ▪ Use an aliquot of the supernatant; do not disturb the glass fibers at the bottom of the original tube. ▪ It may be useful to make a correction by subtracting the value measured at 320 nm from the value measured at 260 nm.
Stretch of dTs in 5′ region of the final PCR product.	Incorrect annealing conditions.	Stringent annealing conditions are crucial for success of the reaction. If the annealing conditions during the first PCR amplification are not optimal, that is, the anchor primer is not completely annealed and could bind within the 5′ poly(A) tail, and a proofreading polymerase, such as 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.
	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 dT-anchor primer to the 5′ poly(A) tail.

3′ RACE

Observation	Possible cause	Recommendation
No PCR amplification product using the control system, or no specific PCR amplification product with your RNA/primer system.	Reagents are contaminated.	To ensure the integrity of the reagents, verify that your PCR system works well; perform a common PCR reaction using a DNA template and primers.
	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.
	RNase contamination present.	Successful cDNA synthesis demands RNase-free handling at all times. ⚠ ***Wear gloves to avoid contamination of the kit components; use sterilized pipettes and tubes.***
		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.
	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.
	Nonspecific products are amplified	Raise the annealing temperature gradually until nonspecific products are no longer observed.

4. Additional Information on this Product

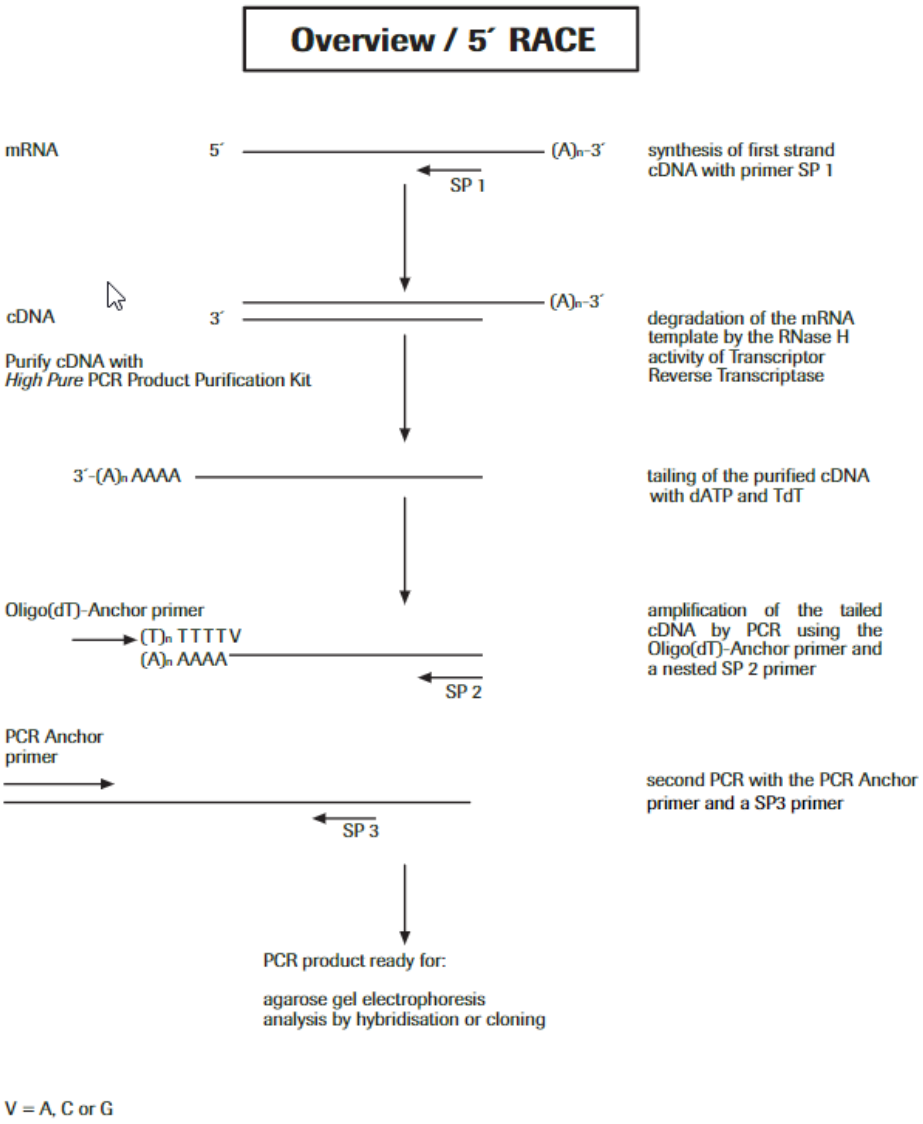
4.1. Test Principle

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, or one-sided PCR.

5′ RACE overview



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

5′ RACE 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 RT, and the Deoxynucleotide mixture. Transcriptor RT 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 synthesis can be raised up to +55°C to encourage reverse transcription to proceed through regions of difficult secondary RNA structure.

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2 Purification

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

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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. Additionally, the 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.

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4 First PCR amplification

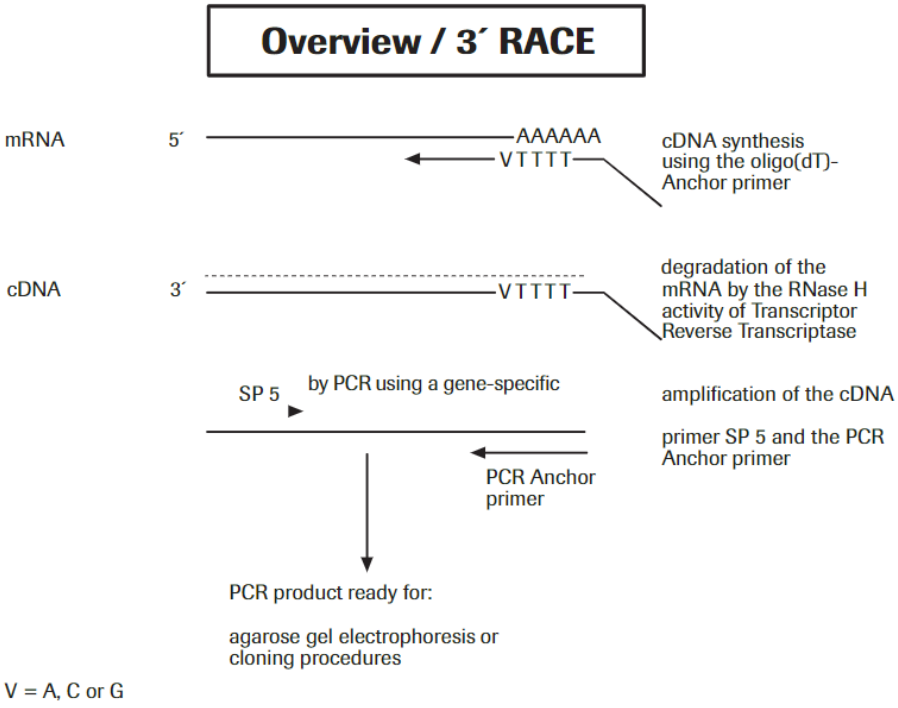
– Tailed cDNA is then amplified by PCR using a gene-specific primer SP2 and the Oligo dT-anchor primer. This primer is a mixture of oligonucleotides carrying a non-T nucleotide, that is, A, C, or G at the 3′ end following the dT-stretch. By this means, the Oligo dT-anchor primer is forced to bind to 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



A Control neo-RNA and 3 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.

3′ RACE 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 an additional purification step using the PCR anchor primer and a user-designed gene-specific primer SP5.

4.2. Quality Control

For lot-specific certificates of analysis, see section, **Contact and Support**.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

Editorial changes.
Updated section 1.1 “Contents”: 0.5% Triton X-100 is replaced with 0.1% Tween 20.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
High Pure PCR Product Purification Kit	1 kit, up to 50 purifications	11 732 668 001
	1 kit, up to 250 purifications	11 732 676 001
Expand High Fidelity PCR System	100 U, 1 x 100 U 40 reactions in a final volume of 50 µL	11 732 641 001
	500 U, 2 x 250 U 200 reactions in a final volume of 50 µL	11 732 650 001
	2,500 U, 10 x 250 U 1,000 reactions in a final volume of 50 µL	11 759 078 001
Expand Long Template PCR System	150 U, 1 x 150 U 38 reactions in a final volume of 50 µL	11 681 834 001
	720 U, 2 x 360 U 190 reactions in a final volume of 50 µL	11 681 842 001
	3,600 U, 10 x 360 U 950 reactions in a final volume of 50 µL	11 759 060 001
Protector RNase Inhibitor	2,000 U, >40 U/µL	03 335 399 001
	10,000 U, 5 x 2,000 U	03 335 402 001

5.4. Trademarks

EXPAND is a trademark of Roche.
All other product names and trademarks are the property of their respective owners.

5.5. License Disclaimer

For patent license limitations for individual products please refer to:
Product Disclaimers.

5.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

5.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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