For general laboratory use. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Expand Long Range, dNTPack

Deoxynucleoside-triphosphate : DNA deoxynucleotidyltransferase, E.C. *2.7.7.7*

Cat. No. 04 829 034 001 175 U
Cat. No. 04 829 042 001 700 U
Cat. No. 04 829 069 001 3500 U

Version July 2006

Store the kit at −15 to −25°C

1. What this Product Does

Number of Tests

The kit is designed for

- approx. 50 reactions (Cat. No. 04 829 034 001)
- approx. 200 reactions (Cat. No. 04 829 042 001)
- approx. 1000 reactions (Cat. No. 04 829 069 001)

with a final reaction volume of 50 µl each.

Contents

Vial	Label	Content A) Cat. No. 04 829 034 001 B) Cat. No. 04 829 042 001 C) Cat. No. 04 829 069 001
1	Expand Long Range Enzyme mix	A) 35 µl (175 U) B) 140 µl (700 U) C) 5 × 140 µl (5 × 700 U)
2	Expand Long Range buffer with $MgCl_2$, 5× conc.	With 12.5 mM $MgCl_2$ A) 1 ml (175 U pack size) B) 2 × 1 ml (700 U pack size) C) 10 × 1 ml (3500 U pack size)
3	Expand Long Range buffer without $MgCl_2$, 5× conc.	A) 1 ml (175 U pack size) B) 2 × 1 ml (700 U pack size) C) 10 × 1 ml (3500 U pack size)
4	25 mM $MgCl_2$	A) 1 ml (175 U pack size) B) 1 ml (700 U pack size) C) 5 × 1 ml (3500 U pack size)
5	100 % DMSO	A) 1 × 1 ml (175 U pack size) B) 1 × 1 ml (700 U pack size) C) 5 × 1 ml (3500 U pack size)
6	10 mM dNTP-Mix	A) 1 × 200 µl (175 U pack size) B) 3 × 200 µl (700 U pack size) C) 13 × 200 µl (3500 U pack size) Ⓢ contains dATP, dCTP, dGTP, and dTTP at 10 mM each.

Storage and Stability

- If stored at –15 to –25ºC, the kit is stable through the expiration date printed on the label.
  - Ⓢ The product is shipped on dry ice.
  - Ⓢ Enzyme storage buffer: 20 mM Tris-HCl, pH 7.5 (25°C), 100 mM KCl, 1 mM Dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Nonidet P40 (v/v), 0.5% Tween 20 (v/v), 50% glycerol (v/v)
- Once the kit is opened, store all kit components at -15 to -25°C.

Application

The Expand Long Range dNTPack is optimized to efficiently amplify large genomic DNA fragments from 5 kb to 25 kb in combination with a threefold higher fidelity than Taq DNA polymerase.

The Expand Long Range dNTPack is ideally suited for genome mapping and sequencing, contig construction, characterization of cloned sequences in lambda phages or cosmids, and eukaryotic-gene cloning and analysis. It allows the rapid identification and cloning of complete genes from genomic DNA using cDNA sequence information as a starting point.

Additional Equipment and Reagents Required

Additional equipment and reagents required to perform PCR reactions with the Expand Long Range, dNTPack, but not provided, include:

- general laboratory equipment
  - nuclease-free, aerosol-resistant pipette tips
  - pipettes with disposable, positive-displacement tips
  - sterile reaction tubes for preparing PCR mixes and dilutions
  - standard benchtop microcentrifuge
- for the PCR reaction
  - thermal block cycler (*e.g.,* the Applied Biosystems GeneAmp PCR System 9700; the Biometra T3 Thermocycler, the Eppendorff Mastercycler Gradient, or the MJ Research DNA Engine Tetrad 2)
  - PCR primers
  - template DNA
  - PCR reaction vessels (thin-walled PCR tubes or plates are recommended)
  - Water, PCR-grade*

**available from Roche Applied Science*

Enzyme Characteristics

Volume activity	5 U/µl
Standard enzyme concentration	3.5 U per 50 µl reaction
Proofreading activity	Yes
Error rate	Expand Long Range dNTPack has approx. 3-fold higher fidelity of DNA synthesis, compared to Taq DNA Polymerase
Optimal elongation temperature	68°C
Optimal denaturation temperature	92°C
Final Mg^{2+} concentration	2.5 mM
PCR product size	From 5 kb to 25 kb (when using human genomic DNA as template)
PCR cloning	Expand Long Range dNTPack generated PCR products can be used both for TA cloning or blunt-end ligation.
Incorporation of modified nucleotides	Accepts modified nucleotides like DIG-dUTP, Biotin-dUTP, or Fluorescein-dUTP.
Carryover prevention (*i.e.,* incorporation of dUTP)	Expand Long Range dNTPack is not compatible with carryover prevention using the dUTP/Uracil DNA-glycosylase procedure.

2. How to Use this Product

2.1 Before You Begin

General

The optimal conditions, such as incubation times and temperatures, concentrations of enzyme, template DNA, Mg^{2+}- and DMSO concentration, depend on the template and primers used and must be determined independently for each application. To ensure optimal reaction efficiency, for every new assay you may need to adjust some of the PCR conditions.

As a starting point for developing your assays, use the following guidelines:

- Usually optimal enzyme concentration ranges from 2.5 to 5 U per assay. The optimal concentration for standard applications is 3.5 U. For special applications it may be necessary to increase the enzyme concentration up to 5 U per assay (*e.g.,* for multiplex PCR).
- For every new assay, you should first determine the optimal DMSO concentration. Depending on the GC-content, the secondary structure and other amplicon-specific parameters the addition of DMSO improves the yield in long range PCR. Recommended starting concentration is 3% DMSO. The optimal concentration may vary from 0 up to 12%.
  In some cases the addition of additives (*e.g.*, 7-deaza-dGTP*, PEG (1000 or 6000), Tween 20*, glycerol, T4 Gene 32 Protein*) may improve the PCR performance.
- Keep denaturation steps as short as possible and denaturation temperature as low as possible.
  Sometimes fragments with high GC-content need higher denaturation temperatures. The enzyme mix is compatible with denaturation temperatures up to 96°C; however, always keep in mind that the yield increases when denaturation temperature is decreased.
- For templates with a very low GC-content, the reduction of the elongation temperature can sometimes help to get an amplification product.
- The quality of the template has a tremendous effect on the success of the PCR. Template DNA should be prepared carefully, applying gentle methods that do not shear the template in order to yield high molecular weight DNA of high purity.

  ⚠ Never vortex complex genomic DNA. Always store complex genomic DNA at +2 to +8°C. Avoid repeated freeze-thaw steps.

Use any template DNA (*e.g.,* genomic or plasmid DNA, cDNA) suitable for PCR in terms of purity, concentration, and absence of inhibitors. For reproducible isolation of nucleic acids use:

- either the MagNA Pure LC Instrument or the MagNA Pure Compact Instrument together with the MagNA Pure LC® DNA Isolation Kit I or the MagNA Pure Compact Nucleic Acid Isolation Kit I (for automated isolation) or
- the High Pure PCR Template Preparation Kit (for manual isolation).

  Human genomic DNA and a human tPA Control Primer Set – especially tested with the Expand Long Range dNTPack - are available from Roche Applied Science as a positive control. For details, see the Roche Applied Science catalog or homepage, www.roche-applied-science.com.

- Template concentration: use 1 to 500 ng of complex genomic DNA or 1 pg to 100 ng of plasmid DNA/cDNA or bacterial DNA.

  In initial experiments to determine the optimum amount of cDNA template, we recommend running undiluted, 1:10 diluted, and 1:100 diluted cDNA template in parallel. Adding too much cDNA may inhibit the PCR.

  The optimal buffer for the template DNA is either sterile double-distilled water or PCR-grade water* or 5-10 mM Tris (pH 7-8). Avoid dissolving the template in TE buffer because EDTA chelates Mg^{2+}.

- Primer design is crucial for the performance of long-range PCR. Follow these general guidelines:
  - primers should be between 22-34 nucleotides long
  - the GC-content should be in the range of 45 to 65%
  - optimally there should be 2 G's or C's at the 3´-end of the primer
  - avoid high similarity (>80%) to other sequences in the genome
  - check primers for possible primer-dimer formation

If there are difficulties to amplify the sequence of interest, sometimes designing different primers or using different primer pairs, helps to overcome the problem.

- Use 0.2 ml thin-walled PCR tubes.

Negative Control

Please be aware that even with long PCR products cross-contamination can occur. To detect DNA contamination, always include a negative control in each run. To prepare this control, replace template DNA with PCR-grade water. Sometimes a contamination will not lead to a prominent band, but will be visible as a smear in the lane of the agarose gel.

Reaction Volume

You can apply the Expand Long Range dNTPack for various reaction volumes. Please refer to the recommendations of the supplier of the thermal block cycler instrument for suitable volumes and tubes/plates.

2.2 Procedure

Preparation of the PCR mix

Prepare individual PCR reactions of *e.g.,* 50 µl in suitable reaction tubes on ice by adding the required components in the order listed in the table below.

If you want to set up more than one reaction, prepare a Master Mix. Multiply the volumes given below for one single reaction by the number of reactions you want to perform and add one extra reaction to compensate for pipette deviation errors.

⚠ After pipetting the last reaction component, start the reaction immediately. Do not store complete reaction mixes on ice.

Step	Action
❶	• Always thaw and equilibrate the required buffers and DMSO at 37°C for 10 min before use. If precipitates have formed during storage, incubate at 37°C until they are completely dissolved. • Vortex thoroughly.
❷	For maximum recovery of the contents, briefly spin vials in a microcentrifuge before opening.
❸	Mix template DNA carefully to avoid shearing. Do not vortex!
❹	Keep all components on ice, except DMSO. Keep DMSO at room temperature.
❺	Set up the PCR mix in a sterile microfuge tube on ice:

Component	Volume	Final conc.
Water, PCR-grade	add to 50 µl	
5× Expand Long Range Buffer with 12.5 mM $MgCl_2$	10 µl	1×
PCR Nucleotide Mix (dATP, dCTP, dGTP, dTTP at 10 mM each)	2.5 µl	500 µM
Forward primer	variable	0.3 µM
Reverse primer	variable	0.3 µM
DMSO	0 – 6 µl	0 – 12 %
Expand Long Range Enzyme mix (5 U/µl)	0.7 µl	3.5 U
Template	variable	up to 500 ng
Total Volume	**50 µl**	

Step	Action
❻	• Mix the solution carefully by pipetting up and down. Do not vortex. • Pipet 50 µl of the PCR mix into each PCR reaction vessel or well of a PCR multiwell plate (depending on your block cycler PCR instrument).
❼	According to the instructions supplied with your thermal block cycler, prepare the tubes or multiwell plates for PCR (*e.g.,* add mineral oil to a tube or seal the plate with self-adhesive foil).

Performing PCR

The standard temperature profile given in the table below was tested for the Applied Biosystems GeneAmp PCR System 9700, the Biometra T3 Thermocycler, the Eppendorff Mastercycler Gradient, and the MJ Research DNA Engine Tetrad 2. Other thermal block cyclers may require different profiles. Follow the instruction manual provided by your instrument supplier,

Step	Temp.	Time	Cycles
Denaturation	92°C [a]	2 min	1×
Denaturation Annealing Elongation	92°C [a] 45 to 65°C [b] 68°C	10 s 15 s 60 s / kb [c]	10×
Denaturation Annealing Elongation	92°C [a] 45 to 65°C [b] 68°C	10 s 15 s 60 s / kb [c] + 20 s cycle elongation for each successive cycle	15× - 25×
Final Elongation	68°C	up to 7 min	1×
Cooling	8°C	∞	

[a] The optimal denaturation temperature depends on the GC-content of the amplified fragment. Sometimes denaturation temperatures up to 96°C may be necessary to amplify a fragment with high secondary structure.
[b] The optimal annealing temperature depends on the melting temperature of the primers and the target sequence used.
[c] Elongation time depends on amplicon length. The recommended elongation time is 1 min per kb.

3. Troubleshooting

	Possible Cause	Recommendation
Little or no PCR product	Difficult template	• Titrate DMSO. • Depending on the GC-content, it may be necessary to raise the denaturation-/annealing temperature to improve the yield. • For fragments with a very low GC-content, reduction of the elongation temperature down to 58°C can help to obtain a product.
	Poor DNA template quality	• Check quality and concentration of template: • Analyze an aliquot on an agarose gel (0.5%, 60V, 5h) to check for possible degradation. • Include a control reaction using a known template under established PCR conditions. • Check or repeat purification of template.
	$MgCl_2$ concentration too low	Increase the $MgCl_2$ concentration in 0.25 mM steps from 1.75 mM up to 3.5 mM.
	Cycle conditions not optimal	• Reduce annealing temperature. • Increase number of cycles.
	Primer design not optimal	Design alternative primers.
Multiple bands or background smear	Annealing temperature too low	• Increase annealing temperature. (Maximum annealing temperature is 68°C.) • Determine the optimal annealing temperature by using a gradient.
	Contamination with old PCR product	• Replace all reagents • Set up PCR reactions in an area separate from that used for PCR product analysis (*e.g.* flow box)
Multiple bands or background smear	Primer quality or storage problems	• If you are using an established primer pair, check their performance under established PCR conditions (with a control template). • Make sure primers are not degraded. • Always store primer aliquoted at –15 to –25°C.
	Primer design or concentration not optimal	• Review primer design. • Both primers must be present in the reaction at the same concentration. • Primers should have similar melting temperatures.
	Influence of the DNA template	• Use serial dilutions of template. • Check quality of the DNA • Check purity of the DNA
PCR products in negative controls	Carryover contamination	• Replace all reagents, especially water. • Use aerosol-resistant pipette tips. • Set up PCR reactions in an area separate from that used for PCR product analysis.
Specific problems in RT-PCR	No product, additional bands, background smear	• The volume of cDNA template (from reverse transcriptase reaction) should not exceed 10% of the final PCR reaction volume. • Increase $MgCl_2$ in 0.25 mM steps. • Follow troubleshooting suggestions above.

4. Additional Information on this Product

How this Product Works

Taq DNA Polymerase, the most common PCR enzyme, is able to amplify up to 15 kb from λDNA templates and up to 3 kb from complex genomic templates. Barnes et al (1, 2) first observed that using a mixture of Taq DNA Polymerase and a thermostable DNA polymerase with proofreading (3´-5´ exonuclease) activity could overcome limitations in the length of fragments amplified: the proofreading enzyme can remove misincorporated nucleotides from the 3´-end of the growing DNA strand. Such misincorporations would otherwise cause the Taq DNA polymerase to fall off the DNA strand and lead to generation of truncated PCR products. Based on these findings, Roche Applied Science developed the Expand PCR Systems, which combine the processivity of Taq DNA Polymerase with the accuracy of a proofreading polymerase.

Expand Long Range dNTPack is a unique enzyme mix that contains thermostable Taq DNA polymerase and a thermostable DNA polymerase with proofreading activity (3). This powerful polymerase mixture produces a high yield of PCR product from genomic DNA. Expand Long Range dNTPack is optimized to efficiently amplify large genomic DNA fragments up to 25 kb long (or up to 40 kb from λDNA templates). Due to the inherent 3´-5´ exonuclease activity of the proofreading polymerase, Expand Long Range dNTPack copies DNA threefold more accurately than Taq DNA polymerase (4).

Quality Control

Each lot of Expand Long Range dNTPack is function tested in PCR. Routinely, Expand Long Range dNTPack is used to amplify a human genomic DNA with primers that are specific for 5 kb, 9 kb, 15 kb, and 25 kb fragments. The enzyme mix is tested for the absence of any contaminating activities, including endo- and exonucleases and nicking activity.

References

1 Barnes, W.M. (1994). PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl. Acad. Sci. USA* **91,** 2216–2220.

2 Cheng, S. et al. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA* **91,** 5694–5699.

3 Hopfner, K.P. et al. (1999). Crystal structure of a thermostable type B DNA polymerase from *Thermococcus gorgonarius*. *Proc. Natl Acad. Sci. USA* **96**, 3600-3605.

4 Frey, B. & Suppmann, B. (1995). Demonstration of the Expand PCR System's Greater Fidelity and Higher Yields with a lacI-based PCR Fidelity Assay. *Biochemica* **2,** 8-9.

5. Supplementary Information

5.1 Conventions

Text Conventions

To make information consistent and memorable, the following text conventions are used in this package insert:

Text Convention	Use
Numbered Instructions labeled ①, ②,etc.	Steps 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 Applied Science

Symbols

In this package insert 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.

5.2 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, http://www.roche-applied-science.com, and our Special Interest Sites including:

- Amplification – Innovative Tools for PCR: http://www.roche-applied-science.com/pcr
- DNA & RNA preparation – Versatile Tools for Nucleic Acid Purification: http://www.roche-applied-science.com/napure
- The MagNA Pure System family for automated nucleic acid isolation: http://www.magnapure.com

Product	Pack Size	Cat. No.
Water, PCR Grade	25 ml (25 vials of 1 ml) 25 ml (1 vial of 25 ml) 100 ml (4 vials of 25 ml)	03 315 932 001 03 315 959 001 03 315 843 001
Tween 20	5 × 10 ml	11 332 465 001
T4 Gene 32 Protein	100 µg 500 µg	10 972 983 001 10 972 991 001
7-Deaza-2'-deoxy-guanosine-5'-triphosphate	2 µmol (200 µl)	10 988 537 001

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Contact and Support

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

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed. Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

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