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Not for use in diagnostic procedures.

Expand 20 kb^{PLUS} PCR System, dNTPack

With additional ready-to-use 10 mM PCR Grade Nucleotide Mix

Cat. No. 04 743 814 001 200 U

Version 05
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Store the kit at −15 to −25°C

1. What this Product Does

Number of PCR Reactions

The kit is designed for approximately 40 reactions with a final reaction volume of 50 µl each.

Kit Contents

Vial	Label	Contents
1	Expand 20 kb ^{PLUS} Enzyme mix	40 µl Enzyme storage buffer: 20 mM Tris-HCl, pH 7.5 (+25°C), 100 mM KCl, 10 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Nonidet P40 (v/v), 0.5% Tween 20 (v/v), 50% glycerol (v/v))
2	Expand 20 kb ^{PLUS} reaction buffer	1 ml 10 × conc. with 27.5 mM $MgCl_2$
3	$MgCl_2$ 25 mM Stock Solution	1 ml
4	Human Genomic DNA	12.5 µl (0.2 mg/ml) in 10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA Store at +2 to +8 °C
5	Human ß-globin control primer forward (HßG forw.)	10 µl (158 ng/µl) in double-distilled water 5´-CAC AAG GGC TAC TGG TTG CCG ATT-3´
6	Human ß-globin control primer reverse (HßG rev.)	10 µl (198 ng/µl) in double-distilled water 5´-AGC TTC CCA ACG TGA TCG CCT TTC TCC CAT-3´
7	PCR Grade Nucleotide Mix	1 × 200 µl Ready-to-use 10 mM dNTP solution

Storage and Stability

Store the kit components excluding the human genomic DNA at −15 to −25°C. When properly stored, the kit is stable through the expiration date printed on the label.

⚠ Always thaw and equilibrate all buffers at +37°C to +56°C before use. Vortex thoroughly. If crystals have formed, incubate at +37°C to +56°C until they are dissolved.

⚠ The supplied human control DNA must be stored at +2 to +8 °C since multiple freezing and thawing will degrade the DNA.

Additional Equipment and Reagents Required

- Template DNA, gene-specific PCR primer pair
- Water, PCR Grade*
- Thermal block cycler (*e.g.*, Applied Biosystems GeneAmp PCR System 9600)
- 0.2 ml thin-walled PCR tubes
- Sterile reaction tubes for preparing master mixes and dilutions

Application

Polymerase chain reaction (PCR).
This PCR system is an improvement of the Barnes Technology (1,2,3) and shows good performance for the amplification of fragments longer than 20 kb.

The Expand 20 kb^{PLUS} PCR System is composed of a unique enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. This powerful polymerase mixture and associated buffer system is designed to give a high yield of PCR product when fragments longer than 20 kb need to be amplified.

The Expand 20 kb^{PLUS} PCR System contains human control DNA and human control ß-globin primers which allow the amplification of a 23 kb fragment.

These reagents may serve as a control reaction but can also be used to test the quality of human template DNA's and/or the respective primer pairs.

Enzyme Properties

Volume activity	5 U/µl
Optimal enzyme concentration	varies from 2.5 to 7.5 U per 50 µl reaction
Standard enzyme concentration	5 U (1 µl) per 50 µl reaction
Optimal elongation temperature	+68°C
Standard Mg^{2+} concentration	2.75 mM (as $MgCl_2$) when using 500 µM dNTP's each.
PCR product size	~ 28 kb
PCR Cloning	T/A cloning
Incorporation of dUTP	no
Repair of mismatched primers at 3' end	yes, due to the 3'-5' exonuclease activity of the proofreading polymerase

* *available from Roche Diagnostics*

2. How To Use this Product

2.1 Before You Begin

General Considerations

The optimal conditions (incubation times and temperatures, concentrations of enzyme, template DNA, Mg^{2+} concentration) depend on the system used and must be determined for each system. In particular, to ensure optimal reaction efficiency, you should titrate the Mg^{2+} concentration and the amount of enzyme used per assay. In addition, increasing the cycle number may improve the yield of amplified DNA.

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

Amount of Enzyme

- Optimal enzyme concentration range from 2.5 to 7.5 U per assay.
- The recommended starting concentration is 5 U (1 µl).

dNTP/Mg^{2+} Concentration

The combination of 2.75 mM $MgCl_2$ (concentration of the supplied buffer) with 500 µM dNTPs (each) is recommended. Nevertheless in some cases titration of Mg ions (adding additional Mg ions) may be necessary to obtain optimal results.

Dilution Buffer

- The optimal buffer for dilution of the template DNA is either double-distilled water or 5 to 10 mM Tris (pH 7-8).

  Avoid dissolving the template in TE buffer because EDTA chelates Mg^{2+}.

Primers

- The potential for secondary structure and dimer formation should be minimized.
  Typical primers for long PCR amplifications have a length of 22 to 34 nucleotides with balanced melting temperatures > +60°C.
- Such primers permit the use of higher temperatures to enhance reactions specificity. This can be critical as the amplification of long targets will be compromised by preferential amplification of shorter non-specific fragments. The design of primers suitable for the amplification of > 20 kb fragments is very critical. The following forward primer TGC TGC TCT GTG CAT CCG AGT G can be used, in combination with the enclosed HbG reverse primer to amplify a 29.8 kb fragment from the human globin gene. The annealing temperature is +60°C.

Hot Start

- Do not use AmpliWax because of resulting difficulties with volume reduction.

Cloning

- The obtained PCR fragments have mainly a 3'-single A overhang.

Sample Material

Template DNA, *e.g.*, human genomic DNA*

- The quality (length and purity) of the template influence dramatically the performance of PCR. Therefore, it is recommended to check the length of the DNA by agarose gel electrophoresis. DNA fragments should be longer than 50 kb. A recommended procedure to get high molecular weight DNA is described in ref. 6 and 7.
- Keep denaturation steps as short as possible and denaturation temperature as low as possible (*i.e.,* +92°C).

  If possible, linearize circular templates.

2.2 Preparation of the Reaction Mixes

For a larger number of reactions, we recommend that you prepare two reaction mixes. This circumvents the need of "Hot Start" and avoids that the enzyme interacts with primers and template in the absence of dNTPs which could lead to partial degradation of primer and template through the 3´-5´ exonuclease activity of Tgo DNA Polymerase.

It is also recommended to prepare a Master Mix for setting up multiple reactions. The Master Mix typically contains all of the components needed for all PCR tests to be performed at a volume 10% greater than that required for the total number of PCR assays.

2.3 Procedure

Please refer to the following table.

The use of 50 µl reaction volumes is recommended. Smaller volumes, *i.e.,* 30 µl are possible. Avoid evaporation by using mineral oil or a thermal block cycler with heated lid.

1
- Thaw the components and place them on ice.
- Mix briefly and centrifuge all reagents before starting.

2 Prepare a 10× conc. solution of each respective PCR primer.

If you are using, *e.g.,* a final concentration of 0.4 µM for each primer, the 10× conc. solution would contain a 4 µM concentration of the respective primer.

3
- Prepare two mixes or reagents in sterile microfuge tubes (on ice):
- **Mix 1** (for one reaction):

Reagent	Vol.	Control	Final conc.
Water, PCR Grade or double-distilled water	add up to 25 µl	19.3 µl	
PCR Grade Nucleotide Mix	2.5 µl	2.5 µl	500 µM (of each dNTP)
Upstream primer	5 µl	–	0.4 µM
Downstream primer	5 µl	–	0.4 µM
β-globin control forward primer	–	1 µl	
β-globin control reverse primer	–	1 µl	
Template DNA	variable	-	250 to 500 ng genomic DNA
Human genomic DNA	-	1.2 µl	250 ng
Final volume	**25 µl**	**25 µl**	

- **Mix 2** (for one reaction):

Reagent	Volume	Control	Final conc.
Water, PCR Grade or double-distilled water	19 µl	19 µl	
Expand 20 kb^{plus} reaction buffer, 10×	5 µl	5 µl	1×
Expand 20 kb^{plus} enzyme mix	1 µl	1 µl	5 U
Final volume	**25 µl**	**25 µl**	

4
- Combine Mix 1 and Mix 2 in a thin-walled PCR tube (on ice).
- Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect sample at the bottom of the tube.
- Continue to thermal cycling immediately.

2.4 Thermal Cycling

1. Place samples in the thermal block cycler, and start cycling using one of the thermal profiles below.

	Temperature	Time	Cycles
Initial Denaturation	92°C	2 min	1×
Denaturation	92°C	10 sec	10×
Annealing	variable (62°C[a] for control reaction)	30 sec	
Elongation	68°C (for control reaction)	18 min[b]	
Denaturation	92°C	10 sec	20[c]×
Annealing	variable (62°C[a] for control reaction)	30 sec	
Elongation	68°C (for control reaction)	18 min[b] + 10 sec cycle elongation for each successive cycle[c]	
Final Elongation	68°C	7 min	1×
Cooling	4°C	unlimited time	

[a] Optimal annealing temperature depends on the melting temperature of the primers and the system used. Appropriate primers should have annealing temperatures > +60°C.

[b] Elongation time depends on fragment length. we recommend the following times:

PCR fragmenth length (kb):	15	20	25	30	35	40	45
Elongation Time (min):	11	14	17	20	23	27	30

Do not forget to extend the elongation time for each new cycle.

[c] The number of cycles depends on the amount of template (copies of target) DNA used. For human genomic DNA, we get good results with 250 ng of template using 30 cycles (in total). However, an increase of the cycle number up to 35 or 40 may increase the yield of the amplified DNA.

2. After cycling, the samples may be frozen for later use. Possible further procedures:

- Check the PCR product on an agarose gel for size and specificity using an appropriate size marker*.
- Purify the PCR product with the High Pure PCR Product Purification Kit *.

The obtained PCR fragments have mainly a 3'-single A overhang.

The thermal profiles were developed for the Applied Biosystems GeneAmp PCR System 9600. Other thermal block cyclers may require different profiles.

3. Troubleshooting

	Possible Cause	Recommendation
Little or no PCR product	DNA template problems	Check quality and concentration of template: • Use highest purified template (phenolisation). • Analyze an aliquot on an agarose gel to check for possible degradation. • Make a control reaction on template with an established primer pair or PCR system. • Check or repeat purification of template. • Circular templates should be linearized if possible.
	$MgCl_2$ concentration too low	Increase the $MgCl_2$ concentration in 0.25 mM steps (solution supplied).
	Cycle conditions not optimal	• Check annealing temperature and denaturation temperatures. If necessary decrease annealing temperature. • Increase cycle number. • Make sure that the final elongation step was carried out.
	Primer design not optimal	• Design alternative primers. • Both primers should have nearly the same melting temperatures.
	Primer concentration not optimal	• Both primers must have the same concentration. • Titrate primer concentration.
	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 primers at –15 to –25°C and as a stock solutions.
Multiple bands or background smear	Annealing temperature too low	Increase annealing temperature, never exceed +68°C.
	Primer design or concentration not optimal	• Review primer design. • Titrate primer concentration (0.1 to 0.6 µM). • Both primers must be present in the reaction at the same concentration. • Perform nested PCR with nested primers.
	Enzyme concentration too high	Reduce amount of enzyme
	DNA template problems	Use serial dilutions of template.
	Cycle conditions not optimal	Reduce the number of cycles (not recommended for human or similar complex DNA).

4. Additional Information on this Product

Quality Control

Each lot of Expand 20 kb^{PLUS} PCR System, dNTPack is function tested in PCR. Routinely, the Expand 20 kb^{PLUS} PCR System is used in combination with human genomic DNA and specific human ß-globin primers to amplify a 23 kb PCR fragment.

References

1 Barnes, W.M. (1994) *Proc. Natl. Acad. Sci USA* **91**, 2216-2220
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3 Frey, B. & Suppmann B. (1995) *Biochemica* **2**; 8-9
4 Lindahl, T. (1993) *Nature* **362**, 709-715
5 Lindahl, T. *et al.* (1972) *Biochemistry* **11**, 3611-3618
6 Cheng, S. *et al.* (1995) *PCR Methods and Applications* **4**, 294-298
7 Huder, J. B. *et. al.* (2002); Identification and Characterization of Two Closely Related Unclassifiable Endogenous Retroviruses in Pythons (Python molurus and Python curtus) *J. Virol.* **76,** 7607-7615.
8 Simonic,T. *et.al.* (2000) cDNA cloning of turtle prion protein. *FEBS Letters* **469,** 33-38.

For general information on PCR, please see the following (available on our website):
9 PCR Special Interest Site: http://www.roche-applied-science.com/PCR
10 PCR Applications Manual, 3rd Edition
11 PCR Product Family Flyer
12 Lab FAQS "Find a Quick Solution"

5. Supplementary Information

5.1 Conventions

Text Conventions

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

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 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.

5.2 Ordering Information

	Product	Pack Size	Cat. No.
DNA Purification	High Pure PCR Template Preparation Kit	100 purifications	11 796 828 001
	High Pure PCR Prod. Purification Kit	50 purifications 250 purifications	11 732 668 001 11 732 676 001
Additional Reagents	Digoxigenin-11-dUTP (alkali-labile)	25 µl (25 nmol) 125 µl (125 nmol)	11 573 152 910 11 573 179 910
	Digoxigenin-11-dUTP (alkali-stable)	25 µl (25 nmol) 125 µl (125 nmol) 5 × 125 µl (5 × 125 nmol)	11 093 088 910 11 558 706 910 11 570 013 910
	Biotin-16-dUTP	50 µl (50 nmol)	11 093 070 910
	Fluorescein-12-dUTP	25 µl (25 nmol)	11 373 242 910
	Water, PCR Grade	25 ml (25 ×1 ml) 25 ml (1 × 25 ml) 100 ml (4 × 25 ml)	03 315 932 001 03 315 959 001 03 315 843 001

Changes to Previous Version

Editorial changes

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