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CERTAMP KIT FOR LONG AMPLIFICATIONS

Ref.	FORMAT	CONTENT
10.332	250 rxns	CERTAMP Kit for Long Amplifications
10.333	500 rxns	CERTAMP Kit for Long Amplifications

Store at -20ºC

Notice to users: Some of the applications which may be performed with this product are covered by applicable patents in certain countries. The purchase of this product does not include or provide a license to perform patented applications. Users may be required to obtain a license depending on the country and/or application.

Ed 21 –January 2010

1. GENERAL INFORMATION

CERTAMP kit for Long Amplifications has been designed for the optimisation and improvement of amplification reactions of long DNA fragments (> 5 Kb). An exclusive combination of proof-reading and non proof-reading polymerases, cofactors and especial buffers, provides robust amplification of long targets/allows high fidelity and excellent yield in long amplifications.

RESEARCH USE ONLY

The use of non proof-reading DNA polymerases results in higher processivity (or synthesis rate) and may introduce miss-incorporations during the synthesis. For amplicons longer than 3 Kb, this is an important issue, having fatal consequences on fidelity and yield of the final product.

However the use of DNA polymerases with proof-reading activity gives a minor error rate (an order of magnitud less than conventional polymerases), provides less processivity and they need more time to amplify the same products. So, the mix of *proof-reading* y non *proof-reading* polymerases of the CERTAMP Kit for Long Amplifications (Certamp *Long Enzyme Mix*), guarantees maximum processivity while the error rate is kept to a minimum. This mix was able to amplify up to 15 Kb from genomic DNA, and up to 40 Kb from lambda DNA.

This robust enzyme system also minimizes background, provides greater product yields, and requires less optimization than other "long and accurate" polymerases. Additionally, the kit includes 10X Certamp Long Buffer $MgCl_2$ FREE that has been optimised for high yields and increased fidelity.

Aplications of Certamp Kit for Long Amplifications:

- Amplification of long DNA fragments (up to 40 Kb)
- Amplification of eukaryotic genomic DNA (up to 15 Kb)
- Cloning: PCR product can be used in T/A cloning as well as blunt-ended cloning.
- The kit is not recommended for sequencing uses.

2. STORAGE CONDITIONS

Store all components of the CERTAMP Kit for Long Amplifications at -20ºC, in a **constant temperature freezer** (do not use frost-free freezers). Under these conditions the kit remains stable for **24 months**. Repeated freeze-thaw cycles do not reduce performance of the product.

3. KIT REAGENTS

Certamp Long Enzyme Mix (1 U/µl)-Mix of *proof-reading* and non *proof-reading*. DNA polymerases. ***Store at –20ºC***.

10X Certamp Long Buffer $MgCl_2$ FREE- The buffer has been formulated for optimal PCR fidelity and yield when performing long PCR amplifications. This buffer has adjuncts and stabilizers which allow an easy optimisation of long amplification reactions. ***Store at –20ºC***.

10X Reaction Buffer $MgCl_2$ FREE- 75 mM Tris HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$. An alternative reaction buffer for conventional PCR amplifications (fragments < 5 Kb). ***Store at –20ºC***.

50 mM $MgCl_2$ Solution-None of the reaction buffers include the Mg^{2+} ion in their composition. Magnesium concentration should be optimised for each amplification reaction. ***Store at –20ºC***.

4. ALTERNATIVE PROTOCOLS

Optimal reaction conditions should be optimal for each experiment. Biotools recommends two different user protocols of CERTAMP Kit for Long Amplifications, according to the individual target system to be amplified.

I. *Protocol 1 –10X Reaction Buffer $MgCl_2$ FREE*

This protocol has been optimised for conventional PCR fragments (***≤ 5 Kb***).

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see table bellow). The inclusion of positive and negative amplification controls is recommended.

2.- Thaw and preserve reagents in ice during their manipulation. Reaction tubes should be stored in ice before thermal cycling.

Master Mix 1

Reactive	Amount per 25 µl reaction
CERTAMP LONG ENZYME MIX	1 µl
10X REACTION BUFFER $MgCl_2$ FREE	2.5 µl
50 mM $MgCl_2$ Solution	1 µl
dNTPs 10 mM each (Ref. 20.031)	0.5 µl
Primers	10-20 pmol of each primer
Distilled water	Up to 25 µl

3.- Aliquot the Master Mix into PCR tubes and add DNA template (1 pg to 500 ng).

4.- Perform PCR using optimised cycling conditions.

5.- Analyse the PCR amplification products on agarose gel.

II. *Protocol 2 – 10X Certamp Long Buffer $MgCl_2$ FREE*

This protocol has been optimised for long PCR fragments (***> 5 Kb***).

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see table bellow). The inclusion of positive and negative amplification controls is recommended.

2.- Thaw and preserve reagents in ice during its manipulation. Reaction tubes should be stored in ice before thermal cycling.

Master Mix 2

Reactive	Amount per 25 µl reaction
CERTAMP LONG ENZYME MIX	1 µl
10X Certamp Long Buffer $MgCl_2$ FREE	2.5 µl
50 mM $MgCl_2$ Solution	1 µl
dNTPs 10 mM each (Ref. 20.031)	0.5 µl
Primers	10-20 pmol of each
Distilled water	Up to 25 µl

3.- Aliquot the Master Mix into PCR tubes and add template DNA (1 pg to 500 ng).

4.- Perform PCR using optimised cycling conditions.

5.- Analyse the PCR amplification products on agarose gel.

III. *Amplification of Lambda DNA*

This protocol has been optimised for the amplification of λ fragments up to 20 Kb using CERTAMP Kit for Long Amplifications. Although this PCR cycling may be suitable for amplifications of up to 5 Kb, specific thermal cycling parameters should be optimised for each experiment.

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see table bellow). The inclusion of positive and negative amplification controls is recommended.

2.- Thaw and preserve reagents in ice during its manipulation. Reaction tubes should be stored in ice before thermal cycling.

Master Mix

Reactive	Amount per 25 µl reaction
CERTAMP LONG ENZYME MIX	1 µl
10X Certamp Long Buffer $MgCl_2$ FREE	2.50 µl
50 mM $MgCl_2$ Solution	1.75 µl
dNTPs 10 mM each (Ref. 20.031)	0.94 µl
Specific primers	20 pmol of each
Distilled water	Up to 25 µl

3.- Aliquot **13 µl** of Master Mix into PCR tubes and template DNA (10 ng).

4.- Perform PCR using optimised cycling conditions (see **Note 1**).

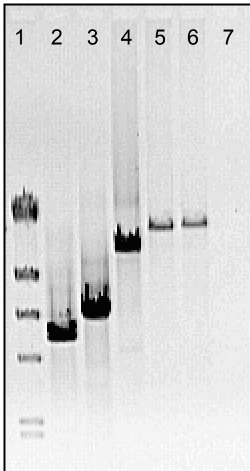
PCR Cycling Parameters:

Cycle Step	Nº cycles	Temperature	Duration
Initial denaturing	1	96ºC	5 min
Denaturing Annealing Extension	10	96ºC 58ºC 72ºC	1 min 30 sec 1 min + 1 sec/cycle
Denaturing Annealing	20	96ºC 68ºC	30 sec 23 min
Final Extension	1	72ºC	10 min
Cooling	∞	4ºC	∞

Note 1: The first 10 cycles are intended to create partial amplification fragments that will serve as primers for the following amplification steps. The following 20 cycles unify annealing and extension steps at 68 ºC, which improve the yield of Lambda PCR products.

5.- Analyse the PCR amplification products on 0.5 % (w/v) agarose gel.

Amplification of λ DNA using
Certamp Kit for Long Amplifications



Lane 1: Lambda/*Hind III* marker (Ref. 31.011)
Lane 2: Amplification of λ DNA (5 Kb)
Lane 3: Amplification of λ DNA (7 Kb)
Lane 4: Amplification of λ DNA (13 Kb)
Lane 5: Amplification of λ DNA (19 Kb)
Lane 6: Amplification of λ DNA (20 Kb)
Lane 7: Negative control (without DNA) using specific primers for the 20 Kb amplification

5. TROUBLESHOOTING

Problem	Cause	Solution
No product or low yield	Missing reagent or pipetting error	Check the concentration and storage conditions of dNTPs, primers, etc. Repeat the PCR and make sure that no pipetting errors or missing reagents occur.
	Problems with the template DNA	Check the concentration and quality of the starting material. If the template is difficult, e.g. rich in GC sequences, we recommend adding DMSO to the master mix. Repeat the PCR with a new dilution of template or with a new DNA purification
	Problems with the primers	Revise the primer design and primers condition. Avoid any designs prone to the formation of primer dimmers. Repeat PCR with different primer concentration from 0.1-0.5 µM in 0.1 increments. Check primer degradation on a denaturing polyacrylamide gel.
	Concentration of $MgCl_2$	Repeat the PCR with different Mg2+ concentration from 1.5-4 mM in 0.25 increments
	Low enzyme-mix concentration	Increase the enzyme- mix concentration in 0.2 U increments
	PCR cycling non optimal	Check the following parameters of the amplification program: **Denaturation-** increase the temperature and length of initial denaturation. **Annealing-** optimise the annealing temperature and time. To increase the specificity, perform a touchdown or step-down PCR cycling. **Extension time-** in case the extension time is short increase the extension time by increments of 30 sec. **Number of cycles-** i perform additional cycles by increments of 5 cycles. Verify or include a **final elongation** step.
Nonspecific amplification products or background smear	Annealing temperature too low	Increase the annealing temperature in increments of 1ºC.
	Primer design	Review the primer design and primer condition. Both primers must have the same concentration. Titrate concentration of primers. Repeat PCR with different primer concentration from 0.1-0.5 µM in 0.1 increments. Check primer degradation on a denaturing polyacrylamide gel.
	Too much DNA template	Use a serial dilution of the template
	Carryover contamination	Always prepare a negative control PCR (no template). If the NC shows a PCR product or a smear exchange all reagents..
	Enzyme-mix concentration too high	Optimise the concentration of enzyme-mix in your experiment
	PCR cycling non optimal	Use *touchdown or stepdown* thermocycler. Reduce the number of cycles
	Concentration of $MgCl_2$	Repeat the PCR with different Mg2+ concentrations from 1.5-4 mM in 0.25 increments
PCR products in negative control	Carryover contamination	Repeat the PCR exchanging all the reagents.

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