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CERTAMP KIT FOR LONG AMPLIFICATIONS

PRODUCT	FORMAT	REF.
Certamp kit for Complex Amplifications	250 rxn	3B085
Certamp kit for Complex Amplifications	500 rxn	3B086

1. GENERAL INFORMATION

CERTAMP kit for Complex Amplifications has been designed for the optimisation and improvement of amplification reactions of low-copy-number targets in complex DNA backgrounds. An exclusive combination of proof-reading and non proof-reading polymerases, cofactors and an especial buffer, provides robust amplification of complex PCR.

RESEARCH USE ONLY

Genomic DNA, templates rich in GC or with secondary structure are difficult to amplify by standard PCR techniques. The main reason of the difficulty is that when conventional polymerases (without proof-reading activity) misincorporate a nucleotide, they tend to stall and either fall off the template resulting in a truncated product or eventually continue in the product sequence. As a result, these enzymes have fairly high error rates and produce relatively low yields of largely incomplete products. Secondary structure or GC-rich templates cause the polymerase to fall off the template before completely duplicating the target sequence.

Certamp Kit for Complex Amplifications is able to overcome the deficiencies of conventional polymerases largely through the combination of proof-reading and non proof-reading enzymes. The proof-reading polymerase provides 3' to 5' exonuclease activity that removes misincorporated bases, thus allowing the conventional polymerase, which has higher synthesis rates than the proof-reading ones, to continue copying the target DNA. The presence of both enzymes significantly improves fidelity and processivity, which results in high yields of highly accurate, full-length products. Polymerase mixes allow highly sensitive and accurate amplification of virtually any target, regardless of size or complexity.

This robust enzyme system also minimizes background, provides greater product yields, and requires less optimization than other long and accurate polymerases. Certamp Kit for Complex Amplifications was also able to amplify targets with as little as 0.5 pg of template. Additionally, 10X Certamp Complex Buffer $MgCl_2$ FREE provided with the kit has been optimised for high yields and increased fidelity.

Aplications of Certamp Kit for Complex Amplifications:

- Amplification of genomic DNA
- Amplification of GC-rich templates
- Amplification of DNA targets with extensive secondary structure
- Amplification from low amounts of templates (≥ 0.5 pg)
- 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 Complex 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 Complex Enzyme Mix (1 U/µl)**-**Mix of *proof-reading* and non *proof-reading* DNA polymerases. ***Store at –20ºC***.

10X Certamp Complex Buffer $MgCl_2$ FREE - The buffer has been formulated for optimal PCR fidelity and yield when performing complex PCR. This buffer has adjuncts and stabilizers which allow an easy optimisation of complex amplification reactions. This buffer has been optimised for high yields and increased fidelity in PCR thechniques. ***Store at –20ºC***.

10X Reaction Buffer $MgCl_2$ FREE- 750 mM Tris HCl (pH 9.0), 500 mM KCl, 200 mM $(NH_4)_2SO_4$. An alternative reaction buffer for conventional PCR amplifications. ***Store at –20ºC***.

50 mM $MgCl_2$ Solution-The reaction buffers provided with this kit have no Mg^{2+} ion in its 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. 3B recommends two different user protocols of CERTAMP Kit for Complex 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 amplifications.

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see Table 1). 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.

Table 1: ***Master Mix 1***

Reactive	Amount per 25 µl reaction
CERTAMP COMPLEX 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.

4.- Perform PCR using optimised cycling conditions.

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

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

This protocol has been optimised for complex targets.

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see Table 2). The inclusion of positive and negative amplification controls is recommended.

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

Table 2: ***Master Mix 2***

Reactive	Amount per 25 µl reaction
CERTAMP COMPLEX ENZYME MIX	1 µl
10X Certamp Complex 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 (0.5 pg to 500 ng).

4.- Perform PCR using optimised cycling conditions.

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

III. Amplification of difficult template (human DNA) using Certamp Kit for Complex Amplifications

CERTAMP kit for Complex Amplifications gives good results in amplification of human DNA.

1.- Prepare a Master Mix for the appropriate number of samples to be amplified (see Table 3). The inclusion of positive and negative amplification controls is recommended.

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

Table 3: ***Master Mix***

Reactive	Amount per 25 µl reaction
CERTAMP COMPLEX ENZYME MIX	1 µl
10X Certamp Complex Buffer $MgCl_2$ FREE	2.5 µl
50 mM $MgCl_2$ Solution	1 µl
dNTPs 10 mM each (Ref. 20.031)	0.5 µl
Specific primers	0.5 µl (20 µM of each primer)
Distilled water	Up to 25 µl

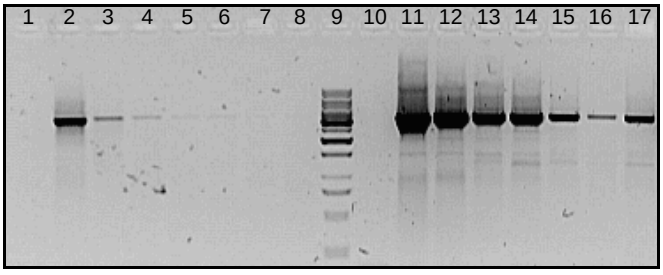
3.- Aliquot the Master Mix into PCR tubes and add DNA template (0.5 pg to 500 ng).

4.- Perform amplification using the following PCR cycling:

CYCLE STEP	Nº CYCLES	TEMPERATURE	DURATION
Initial Denaturalisation	1	94ºC	3 min
Denaturalisation Annealing Extension	35	94ºC 55ºC 72ºC	30 sec 1 min 3 min
Final Extension	1	72ºC	10 min
Cooling	∞	4ºC	∞

5.- Analyse the PCR amplification products on 1-1.5 % agarose gel. The specific product has a size of 3 Kb.

Amplification of different amounts of human DNA using 3B DNA Polymerase and Certamp Kit for Complex Amplifications



3B DNA Polymerase *Certamp kit for Complex Amplifications*

Lanes 1, 10: Negative controls (without DNA)
Lanes 2, 11: 100 pg of template DNA
Lanes 3, 12: 15 pg of template DNA
Lanes 4, 13: 7.5 pg of template DNA
Lanes 5, 14: 3.75 pg of template DNA
Lanes 6, 15: 1.875 pg of template DNA
Lanes 7, 16: 0.937 pg of template DNA
Lanes 8, 17: 0.47 pg of template DNA
Lane 9: 1 Kb Ladder marker (Ref. 31.005)

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 primer 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- mixture 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.