



Encyclo Plus PCR kit

Cat #PK101

User Manual

This product is intended for research use only

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I. Kit components and storage conditions

Component	Amount
50X Encyclo polymerase mix	100 μ l
10X Encyclo buffer	600 μ l
5X Encyclo Red buffer	1.2 ml
2X Encyclo GC buffer	2 x 1.5 ml
50X dNTP mix (10 mM for each)	120 μ l
Sterile PCR water	3 x 1.5 ml

Shipping/Storage: Encyclo Polymerase mix is shipped on dry ice or at -20°C . All other components of Encyclo Plus PCR kit can be shipped at ambient temperature. Once arrived, the kit must be kept at -20°C .

II. Product description

Encyclo Plus PCR kit is intended for most PCR and primer extension applications. It is especially recommended for cDNA amplification due to optimal combination of high fidelity and processivity provided by Encyclo polymerase mix.

The kit includes all components necessary for PCR: Encyclo polymerase mix, reaction buffers, a mix of high purity deoxyribonucleotides and sterile PCR water.

Evrogen Encyclo polymerase mix produces high yields of PCR products from a wide variety of templates and is suitable for difficult templates, long PCR (up to 15 kb) and cloning.

The kit includes three reaction buffers optimized for superior performance of the Encyclo polymerase mix in different applications

Encyclo polymerase mix features

- High processive 5'>3' DNA polymerase activity
- Proofreading 3'>5' exonuclease activity
- Automatic hot start
- TA cloning compatibility

II. A. Buffers

- 1) Encyclo Plus buffer has been developed to provide successful amplification of long DNA templates and is also suitable for real-time PCR with intercalated dyes SYBR Green I and Eva Green.
- 2) Encyclo Red buffer contains red and yellow dyes, which do not interfere with the performance of Encyclo polymerase, and components increasing the density of the samples for direct loading on agarose gels. It is convenient to use this buffer when PCR products are analyzed by gel-electrophoresis, since the additional use of a gel-loading buffer is not required.
In 1% agarose gel with 1xTAE buffer the red dye co-migrates with approximately 1000 bp DNA, and yellow dye co-migrates with the front with 20-30 bp.
- 3) Encyclo GC buffer is recommended for efficient amplification of GC-rich (e.g. >65%GC) templates. Upon freezing, a precipitate may be formed in the buffer. If this occurs warm the buffer at 50 °C before use and vortex until no visible precipitate is left in the solution.

The following changes should be made in a standard PCR program when Encyclo GC buffer is used:

- 1) Increase the initial denaturation stage to 2 min.
- 2) Decrease the annealing temperature T_m for 2-5°C with respect to the calculated value.
- 3) Increase the number of PCR cycles by 2-7.

II. B. Product Use Limitations

Encyclo Plus PCR kit is intended for research purposes only.

III. Guidelines for PCR amplification

III. A. General considerations

III. A.1. Avoid cross contamination

Minute amounts of contaminating DNA can lead to nonspecific amplification even in the absence of an added DNA template.

We recommend setting up PCRs in a dedicated lab area separately from that used for DNA preparation or analysis of PCR products.

Use PCR pipette tips containing hydrophobic filters to minimize cross contamination.

It is strongly recommended to include a negative control (in which sterile water is used instead of DNA template) in every experiment.

III. A.2. Include positive control

Always perform a positive control to ensure that each component is functional.

III. A.3. Use PCR master mix

Use of a PCR master mix reduces tube-to-tube variations in multiple PCR. The master mix typically contains all components needed for PCR except for those varying from one reaction to another. For example, if multiple templates are being tested with the same primers, include the primers in the master mix. If one template is being tested with multiple primer sets, include the template in the master mix. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays including positive and negative controls. The master mix should be thoroughly mixed before use.

III. A.4. Use careful pipetting technique

When small volumes of reagents are used in PCR experiments, careful pipetting technique is crucial to avoid tube to tube variations. Always be sure that no extra solution is on the outside of a pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture, deliver the solution, and rinse the pipette tip by pipetting up and down several times.

III Guidelines for PCR amplification

III. A.5. Do not use manual hot start

Encyclo polymerase mix provides automatic hot start. Manual hot start is not required.

III. B. PCR protocol

- 1) Prepare a PCR master mix by combining the following reagents in the order shown in **Table 1**.

Table 1. Setting up PCR master mix

Component*	PCR with Encyclo Plus buffer	PCR with Encyclo Red buffer	PCR with Encyclo GC buffer	Final concentration
Sterile water	to 25 μ l	to 25 μ l	to 25 μ l	–
10X Encyclo Plus buffer	2.5 μ l	–	–	1X
5X Encyclo Red buffer	–	5 μ l	–	1X
2X Encyclo GC buffer***	–	–	12.5 μ l	1X
50X dNTP mix	0.5 μ l	0.5 μ l	0.5 μ l	1X (0.2 mM each)
Upstream Primer**	variable	variable	variable	0.2-0.5 μ M
Downstream Primer**	variable	variable	variable	0.2-0.5 μ M
DNA template**	variable	variable	variable	1pg-200ng/25 μ l
50X Encyclo polymerase mix	0.5 μ l	0.5 μ l	0.5 μ l	1X
Total volume	25 μ l	25 μ l	25 μ l	–

* The recipe is for one reaction and must be adjusted for multiple samples.

** These components should be added into a PCR master mix (when same components are used for all PCRs) or into PCR tubes after PCR master mix aliquoting (when different components are used in different PCRs).

*** Warm before use until no visible precipitate is left in the solution.

- 2) Mix by vortexing (without bubbling) and spin the tube briefly in a microcentrifuge.
- 3) Immediately before thermal cycling, aliquot the PCR master mix into an appropriate number of sterile 0.2-ml or 0.5-ml PCR tubes.
 - ▶ *Note: Thin wall PCR tubes are recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance.*
- 4) If required, add missing components, i.e. components that vary from one reaction to the other.
- 5) If your thermal cycler is not equipped with a heated cover, overlay each reaction with a drop of molecular biology grade mineral oil.
- 6) Perform PCR using the optimized cycling conditions as described in **the section III. C.**
- 7) If required, analyze the PCR amplification products using electrophoresis on a 1.0-4.0 % (w/v) agarose gel with etidium bromide (EtBr) staining.

▶ *Note: PCR products can be stored at -20°C .*

III. C. Cycling conditions

Use **Table 2** to determine PCR cycling parameters.

▶ *Note: Optimal cycling conditions, such as incubation times, temperatures, and the number of cycles may vary and must be individually determined. Optimization of PCR parameters allows achieving highest product yield and specificity.*

Table 2. PCR cycling parameters

Cycle step	Number of cycles	Temperature	Duration
Initial denaturation	1	92-95°C	1 min-3 min
Denaturation	10-35	92-95°C	5 sec - 1 min
Annealing		T _m	5 sec - 1 min
Extension		72°C	1 minute/1-1.5 kb
Final extension (optional)	1	T _m 72°C	5 sec - 1 min 2-10 min

III. C.1. Denaturation

Denaturation time and temperature depend at least in part on the ramp rate and temperature control mode of the thermal cycler. After an initial denaturation for up to 3 min at 92-95 °C, keep the denaturation as short as possible (for example 20 sec or less). This is particularly important for long PCR.

Initial denaturation for 3 min is recommended for complex genomic DNA, while shorter time (up to 2 min) should be used with simpler templates.

III. C.2. Primer annealing

Optimal primer annealing temperature depends on the primer structure. Typically annealing temperatures range between 55 °C and 72 °C. Simplified formula for estimating annealing temperature (T_m) is

$$T_m = 2\text{ °C} \times (A+T) + 4\text{ °C} \times (G+C)$$

Optimal annealing temperatures may be above or below the estimated T_m (for up to 5 °C). In many cases, use of an annealing temperature which is 5 °C above the calculated T_m, i.e. T_m + 5 °C, can sharply increase PCR specificity.

To achieve maximal reaction specificity, use primers designed to have a high annealing temperature (preferably 65 °C-68 °C).

Whenever possible, design primer pairs with similar T_m values. When two primers have different T_m, use the lowest one for PCR cycling.

III. C.3. Extension

Extension should be performed at 72 °C for the most PCR applications. A constant extension time can be used (one minute per 1.3-1.5 kb of expected extension product).

To enhance TA cloning and achieve complete DNA extension, it may be helpful to include an additional incubation step of 2-10 min at 72 °C at the end of cycling.

III. C.4. Number of PCR cycles

We recommend using the marginally possible number of PCR cycles since overcycling may yield a nonspecific PCR product. If necessary, undercycling can be easily rectified by placing the reaction tube back into the thermal cycler for a few more cycles (see Troubleshooting Guide).

A number of PCR cycles required to produce a certain amount of PCR product (e.g. 5-10 ng/ μ l) strongly depends on the initial number of target DNA molecules used for PCR amplification.

This dependence may be formulated as follows:

$$N=2^{(40-n)}$$

where "N" means a number of DNA molecules at the start of amplification and " n " means a number of PCR cycles required to amplify the product to yield the concentration of 5-10 ng/ μ l.

For example, for a 1 kb long DNA molecule (weight is about 10^{-18} g) the following rules are correct (when optimal PCR conditions are used):

Template amount at PCR start (50 μ l reaction)	Number of PCR cycles to amplify 5-10 ng/ μ l of DNA
1 molecule	40 cycles
1000 molecules	30 cycles
10^6 molecules (1 pg)	20 cycles
10^9 molecules (1 ng)	10 cycles

Please keep in mind that a PCR product visible on agarose/EtBr gel only after 40 or more PCR cycles is amplified from a single molecule and could result from a casual contamination.

IV. Troubleshooting Guide

The following general guidelines apply to most PCR reactions. However, no attempt has been made to address troubleshooting for all of the many applications for which the Encyclo PCR kit can be used.

IV. A. Low yield or no product observed

Possible cause	Solution
PCR component missing or degraded	Use a checklist when assembling reactions. Do not use buffers optimized for another polymerase. Check the concentrations and storage conditions of reagents including primers and template. Repeat PCR. If PCR still does not work, contact Evrogen technical support: customer-support@evrogen.com
Not enough PCR cycles	Increase the number of PCR cycles (3-5 additional cycles at a time).
Annealing temperature too high	Decrease the annealing temperature in increments of 2-4 °C.
Extension time too short	Increase the extension time in too short 30 sec increments.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt long try using a longer primer(s).
Too high or too low template concentration	Repeat PCR varying the concentration of DNA template.
Template DNA may be damaged	Check template integrity by agarose/EtBr gel electrophoresis. If necessary, repurify your template using methods that minimize DNA nicking.
DNA template contains high percentage of GC basepairs	Use 2X Encyclo GC buffer for PCR amplification

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Possible cause	Solution
Template DNA may comprise components inhibiting PCR	Repurify your template.
Too little enzyme	In rare cases, the PCR yield can be improved by increasing the concentration of the enzyme mix. However, increasing the concentration >2X is likely to lead to higher background levels
DNA template is dissolved in a buffer with high concentration of EDTA	If the concentration of EDTA in the cDNA sample is more than 5 mM, this can reduce the efficacy of PCR by decreasing the concentration of Mg ²⁺ in the reaction buffer

IV. B. Multiple PCR products or smear observed

Possible cause	Solution
Too many cycles	Reducing the cycle number may PCR eliminate non-specific bands and smear.
Annealing temperature too low	Increase the annealing temperature in increments of 2-3 °C.
Denaturation temperature too low	Increase the denaturation temperature in increments of 1 °C.
Extension time too long	Decrease the extension time in 1 min increments..
Suboptimal primer	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt long try using a longer primer(s). If the original primer(s) had a GC content of less than 45 %, try to design a primer with a GC content of 45-60 %.
Contamination	Contamination most often results in extra bands or smearing. It is important to include a negative control (i.e., a control using sterile water instead of the DNA template) in every PCR experiment to determine if the PCR reagents, pipettes or PCR reaction tubes are contaminated with previously amplified targets. Also, when performing PCR directly on phage plaques or bacterial colonies, failure to isolate single plaques or colonies will also produce multiple bands.
Too much templates	Try a lower concentration of DNA template in the PCR reaction.
Poor template	Check template integrity by agarose/EtBr gel electrophoresis. If necessary, repurify your template.

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Possible cause	Solution
Too much enzyme	If smearing is observed, first try optimizing the cycle parameters as described above, then try reducing the enzyme concentration to 0.5X Encyclo polymerase mix.

V. References

Chester N., Marshak D.R. (1993) Dimethyl sulfoxide mediated primer T_m reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction. *Anal. Biochem.* 209(2): 284-290.

Roux K. H. (1995) Optimization and troubleshooting in PCR. *PCR Methods Appl.* 4: 5185-5194.

For note...

Endnotes

The Product is intended for research use only.

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Evrogen JSC

Miklukho-Maklaya str, 16/10,

117997, Moscow, Russia

Tel: +7(495)988-4084

Fax: +7(495)988-4085

www.evrogen.com