DATA SHEET

Jena Bioscience

Pfu-X Core Kit

Kit for high accuracy PCR and Ligase-free cloning

Cat. No.	Amount
PCR-237S	100 units
PCR-237L	500 units

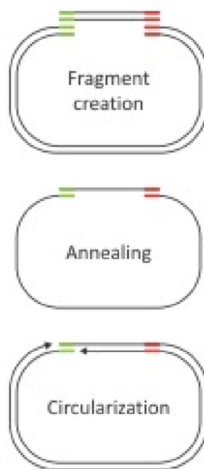

Fig. 1: Functionality of Ligase-free Cloning

Unit Definition: One unit is defined as the amount of the enzyme required to catalyze the incorporation of 10 nmol of dNTP into an acid-insoluble form in 30 minutes at 74 °C.

For *in vitro* use only!

Shipping: shipped on gel packs

Storage Conditions: store at -20 °C

Additional Storage Conditions: avoid freeze/thaw cycles

Shelf Life: 12 months

Form: liquid

Concentration: 2.5 units/µl

Description:

Pfu-X Core Kit contains all reagents required for PCR (except template and primer) in one box combining simple handling with high flexibility. The premium quality polymerase, ultrapure dNTPs and the optimized complete reaction buffer ensure superior amplification results.

Pfu-X Polymerase is the ideal choice for applications where the efficient amplification of DNA with highest fidelity is required.

The enzyme is a genetically engineered Pfu DNA polymerase, showing a 2-fold higher accuracy and an increased processivity, resulting in shorter elongation times.

The enzyme catalyzes the polymerization of nucleotides into duplex DNA in 5'→3' direction but does not possess a 5'→3' exonuclease replacement activity. Its inherent 3'→5' exonuclease proofreading activity results in a greatly increased fidelity of DNA synthesis compared to Taq polymerase. Pfu-X Polymerase-generated PCR fragments are blunt-ended.

The enzyme is highly purified and free of bacterial DNA.

Fidelity of the enzyme:

Pfu-X Polymerase is characterized by a 50-fold higher fidelity compared to Taq polymerase and a 2-fold higher fidelity compared to standard Pfu polymerase.

$ER_{\text{Pfu-X Polymerase}} = 0.25 \times 10^{-6}$

The error rate (ER) of a PCR reaction is calculated using the equation ER = MF/(bp x d), where MF is the mutation frequency, bp is the number of base pairs of the fragment and d is the number of doublings

(2^d = amount of product / amount of template).

Content:

Component	PCR-237S	PCR-237L
Pfu-X Polymerase 2.5 units/µl in storage buffer* **red cap**	40 µl 100 units	200 µl 500 units
dNTP Mix 10 mM each dATP, dCTP, dGTP, dTTP **white cap**	100 µl	500 µl
Pfu-X Buffer 10x conc. **green cap**	500 µl	2 x 1.2 ml
PCR-grade Water **white cap**	2 x 1,2 ml	2 x 6 ml

* (50 % Glycerol, 50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT 0.1 % Tween 20, 0.1 % Nonidet P-40)

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Recommended 50 µl PCR assay:

5 µl	10x Pfu-X Buffer	green cap
1 µl	dNTP Mix	white cap
0.4 µM	each Primer	-
1 - 100 ng	template DNA	-
0.5 µl (1.25 units)	Pfu-X Pol	red cap
Fill up to 50 µl	PCR-grade water	-

Please note that it is essential to add the polymerase as last component.

Recommended cycling conditions:

Three-step standard protocol

initial denaturation	95 °C	2 min	1x
denaturation	95 °C	20 sec	25-30x
annealing[1]	50 - 68 °C	30 sec	25-30x
elongation[2]	68 °C	1 min/kb	25-30x
final elongation	68 °C	1 min/kb	1x

Two-step protocol for amplification of longer fragments (>3 kb)

Please note that for performing two-step cycling a sufficiently high primer T_m is necessary. If T_m of primers is below 65 °C or two-step PCR does not yield a sufficient product quality the three-step cycling protocol is recommended.

initial denaturation	95 °C	2 min	1x
denaturation	95 °C	20 sec	25-30x
annealing/ elongation[1,2]	68 °C	30 sec/kb	25-30x
final elongation	68 °C	30 sec/kb	1x

[1]The annealing temperature depends on the melting temperature of the primers used.

[2]The elongation time depends on the length of the fragments to be amplified. A time of 1 min/kb is recommended.

For optimal specificity and amplification an individual optimization of the recommended parameters may be necessary for each new template DNA and/or primer pair.

Ligase-free Cloning

Ligase-free Cloning is based on a cloning technique invented by Quan and Tian in 2009. It offers a number of advantages over conventional cloning methods. The system:

- works with any vector that can be linearized
- allows efficient cloning even into blunt end vectors
- allows directed cloning into single-cut vectors
- allows fast and easy preparation of vector and insert with no or only few purification steps
- does not require to dephosphorylate the vector
- allows the use of any restriction enzyme that linearizes the vector even if its recognition site(s) are present in the insert
- does not need a ligation step
- does not add additional sequences to the plasmid or the insert

Principle (see Fig. 1)

Ligase-free Cloning is based on generation of inserts with homologous ends to the linearized vector.
In a circularization reaction, vector and insert anneal due to their homologous ends.
Using a specially selected DNA polymerase, the resulting single-stranded plasmids are recircularized.
These plasmids can directly be used for transformation. They still have two nicks each, which will be repaired by *E. coli*'s endogenous DNA repair system and thus do not have to be ligated in vitro.

Supplements (to be provided by user)
PCR purification kit
Gel extraction kit
Competent *E. coli* cells

Protocol

1. Vector preparation
Linearization with more than one enzyme will result in a higher percentage of positive clones. If the used restriction sites are not reconstructed after cloning and not present in the target fragment, the inactivation or purification steps can be omitted. Make sure the vector is linearized completely to reduce background in the transformation step (chapter 5).

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1.1 With enzymes that can be heat inactivated:

- Linearize 500 ng of vector in 20 µl total reaction volume in Pfu-X Buffer, use at least 2 units of enzyme per µg of plasmid DNA
- Refer to Table 1 for optimal buffer concentrations for each enzyme
- Heat inactivate restriction enzymes

1.2 With enzymes that cannot be heat inactivated:

- Linearize 2 µg of vector in 40 µl total reaction volume in Pfu-X Buffer, use at least 2 units of enzyme per µg of plasmid DNA
- Refer to Table 1 for optimal buffer concentrations for each enzyme
- Remove enzymes with a DNA purification kit

Table 1: Optimal final Pfu-X Buffer concentrations for individual restriction enzymes

Enzyme	Pfu-X Buffer	Enzyme	Pfu-X Buffer	Enzyme	Pfu-X Buffer
ApaI	1x	HpaI	1x	PvuII	1x
ApaLI	0.9x	KpnI	1x	SalI	2x
AsuII	1x	MluI	1x	ScaI	1x
BamHI	1x	MspCI	2x	SlaI	1x
BglII	1x	NaeI	1x	SmaI	1x
BseAI	1x	NcoI	1x	SnaBI	1x
BssHII	1x	NdeI	1x	SpeI	1x
CspAI	1x	NheI	1x	SphI	2x
DraI	1x	NotI	1x	SseBI	1x
EcoRI	1x	NruI	1x	SspI	1x
EcoRV	1x	PstI	1x	SstI	1x
HindIII	1x	PvuI	1x	XbaI	1x

2. Primer design

- Primers need a 5' extension with the same sequences as the ends of the linearized vector
- Melting temperature of the overlap should be in the range of 60-70 °C
- When linearizing the plasmid with enzymes that create sticky ends, use the strand with the overhang for calculations
- HPLC purification of the primers is recommended

Example: Vector was cut with NcoI and NotI. Vector is shown in lower case, primers in capitals, with the overlap in bold. Denote the primer sequence for amplification of the insert.

Forward primer:
CCTTGCCACCAGATCTGCCATGNNNNNN...
...gtgccttgccaccagatctgc
...cacggaacggtggtctagacggtac

Reverse primer:
ggccgccctcctcctcctttcttgttc...
cgggaggaggaggaaagaacaag...
...NNNNNN**CCGGCGGGAGGAGGAGGAAAGA**

3. Insert preparation

3.1 Standard preparation

- Perform PCR in 1x Pfu-X Buffer with 0.2 mM dNTPs and 1 µl of Pfu-X Polymerase per 100 µl of reaction volume and a final primer concentration of 1 µM for 25-30 cycles
- Set elongation temperature to 68 °C and elongation time to 1.5 min per 1000 bp
- Isolate fragment from agarose gel

3.2 Quick preparation

This works only if the PCR template does not have the same antibiotic resistance as the target vector, or if the PCR template is removed by Dpn I digestion.

- Perform PCR in 1x Pfu-X Buffer with 0.2 mM dNTP Mix and 1 µl of Pfu-X Polymerase per 100 µl of reaction volume and a final primer concentration of 125 nM for 25-30 cycles
- Further purification of the insert is not necessary

4. Circularization reaction

4.1 Components

Prepare the following reaction (20 µl volume):

- 100 ng of linearized vector
- 10-fold molar excess of insert
- 1x Pfu-X Buffer (note that fragment and/or vector may already contain buffer, add only that amount of buffer to reach 1x final concentration)
- 0.2 mM dNTP Mix
- 0.5 units Pfu-X Polymerase
- Add PCR-grade Water to a final volume of 20 µl

As a control, perform the same reaction without adding any insert.

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4.2 Reaction conditions

- Step 1: 30 sec 94 °C
- Step 2: 30 sec 50 °C (depends on annealing temperature of the overlaps, 50 °C will work in most cases)
- Step 3: x min 68 °C (depends on vector or fragment size, whichever is larger, set to 1.5 min / 1000 bp)
- 10 cycles

5. Transformation

- Transform competent *E. coli* cells, using your standard transformation protocol, with 5 µl of the circularization reaction or the control
- Transformation with the reaction should yield 100-1000 colonies, of which 95-99 % are positive (less if the vector has not been cut efficiently)
- Transformation of the control usually gives 1-50 colonies and shows the amount of background from negative clones with non-linearized vector

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Related Products:
Ready-to-Use Mixes / direct gel loading
Ready-to-Use Mixes
Thermophilic Polymerases
Deoxynucleotides (dNTPs)
Supplements
Primers and Oligonucleotides
DNA Ladders

Selected References:
Quan *et al.* (2009) Circular polymerase extension cloning of complex gene libraries and pathways. *PLoS One.* **4**:e6441.