Xpert TA Easy Cloning Kit

GC05.0010 (10 rxns) | GC05.0020 (20 rxns)

(FOR RESEARCH ONLY)

Product:

The Xpert TA Easy cloning kit is designed for the direct ligation of PCR fragments containing 3´-A overhangs (generated using non-proofreading DNA polymerases such as Taq or blends thereof) into a ready-to-use stable linearized vector. The vector is prepared by digestion of pGRS TA Easy cloning vector with *Eco*RV and the subsequent addition of a single thymidine at each of the 3´-ends. This system offers greater efficiency than most TA vectors available (with over 700 positive colonies under optimal conditions) and with a low background (less than 4%) due to reduced self-ligation.

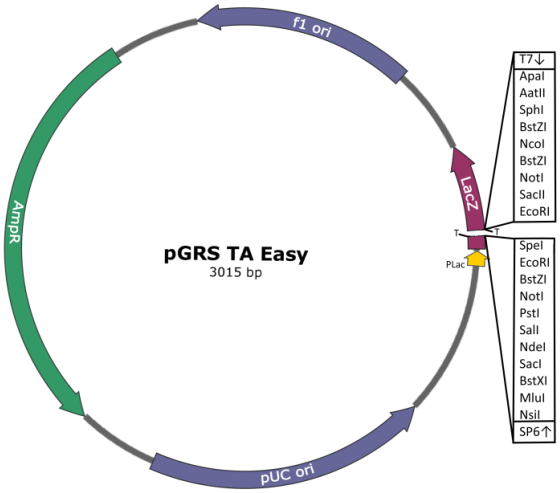
The pGRS TA Easy vector (3015bp) contains the β-lactamase gene resulting in ampicillin resistance for selection purposes. The PCR amplicon is inserted between pUC/M13 primers, which facilitates DNA sequencing. Flanking Lac, and dual opposed SP6/T7 promoters ensure the possibility for correct *in vitro* transcription of the insert, independently of its orientation. Flanking *Eco*RI and *Not*I recognition sites enable single enzyme digestion for subcloning.

Contents:

Product	GC05.0010	GC05.0020
pGRS TA Easy cloning vector (50ng/µL)	10 rxns	20 rxns
T4 DNA Ligase (5 Weiss U/µL)	50 U	100 U
T4 DNA Ligase Buffer (5X)	100 µl	200 µl
Control Insert (600bp)	5 µl	5 µl

Storage: Store at -20ºC.

Features:



T7 RNA Polymerase transcription initiation site	1
Multiple cloning region	10-117
SP6 RNA Polymerase transcription initiation site	137
SP6 RNA Polymerase promotor	138-156
pUC/M13 Reverse Primer binding site	174-190
LacZ start codon	197
Lac operator	198-214
β-lactamase coding region	1335-2195
Phage f1 region	2378-2833
pUC/M13 Forward Primer binding site	2974-2992
T7 RNA Polymerase promotor	2954-3015

Figure 1. Map of pGRS TA Easy. The complete vector sequence can be downloaded from our site www.grisp.pt

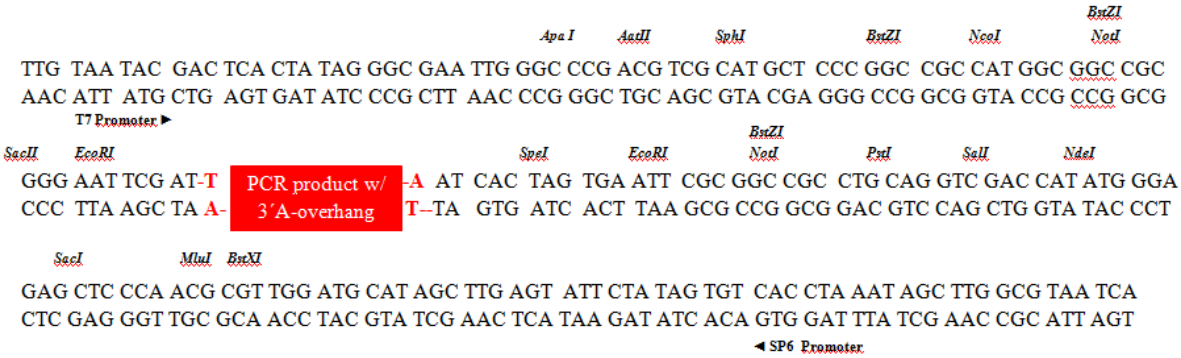


Figure 2. Cloning site of pGRS TA Easy. Inserts can be released with a single digest using either *Eco*RI or *Not*I or via double-digest

PROTOCOL

PCR: Use a PCR polymerase that produces 3´-A overhangs, e.g. "normal" Taq or blend. Include a final extension step of 5-10 minutes to ensure integrity of the amplicons. If the PCR is carried out with a high-fidelity DNA polymerase, an amplicon with blunt ends will be generated and the fragment cannot be cloned directly into the pGRS TA Easy vector. In that case, one could add 3´-A ends, using Klenow fragment or normal Taq, in an additional PCR step. For successful cloning, any primers can be used, phosphorylated or unphosphorylated, as well as with modified bases. For the cloning of large fragments (>2kb), however, the addition of PIG sequences (**GTTTCTT**) at 5´-ends of the primers is recommended as it increases the cloning efficiency greatly (nearly 10-fold in case of a PCR fragment of 3.5kb). The Xpert TA Easy cloning kit has been successfully used for the cloning of PCR amplicons of up to 10kb.

Purification: Although unpurified PCR product can be cloned directly into pGRS TA Easy vector, crude PCR reaction mixture often contains many unspecific fragments, which also will be cloned into the vector resulting in false positives. Moreover, if an plasmid harbouring the gene encoding β-lactamase was used as template, then it is very likely that many colonies will be transformed with that template plasmid, also resulting in false positives. It is therefore highly recommended to analyze PCR products by agarose gel electrophoresis before ligation to verify quality and quantity of the desired amplicon and to allow for easy purification. The band of interest can be easily and quickly purified from the agarose gel using a gel extraction kit, such as GRiSP´s PCR & Gel Band Purification kit.

►Exposure to short wavelength ultraviolet light (e.g. 254, 302, or 312nm) for 1-2 minutes (time required to visualize bands, when using ethidium bromide, and to cut out the desired band reduces the cloning efficiency up to 10.000 times due to the formation of pyrimidine dimers. We strongly recommend to use a safer DNA stain, such as GRiSP´s Xpert Green DNA Stain, which is not only safer in usage as it is not carcinogenic, but also allows for the visualization of DNA fragments using blue light, which does not affect cloning efficiency, instead of UV-light.

Ligation: Prior to use, briefly vortex components and centrifuge to be able to pipet contents from the bottom of the tubes. Set up the ligation reactions, including controls, using DNase-free tubes, as described in the table below. Mix the reactions by pipetting slowly.

Component	Control reaction	Background control	Cloning reaction
pGRS TA Easy cloning vector (50ng/µL)	1 µl	1 µl	1 µl
T4 DNA Ligase (5 Weiss U/µL)	1 µl	1 µl	1 µl
T4 DNA Ligase Buffer (5X)	2 µl	2 µl	2 µl
PCR product	-	-	* µl
Control insert (600bp)	1 µl	-	-
Molecular Biology grade water	up to 10 µl	up to 10 µl	up to 10 µl

***The optimal molar ratio of insert:vector is in the range of 3:1 to 5:1 (see below how to determine volume)**

Then incubate for 1 hour at 22 ºC, using a thermoblock, thermocycler or waterbath and then place on ice. Alternatively, incubate at room temperature, and then place on ice.

►Often a white precipitate is formed in the T4 DNA Ligase Buffer (5x). This does not result in any loss of performance and the Buffer should be used as is. However, the buffer contains ATP, which is sensitive to temperature fluctuations. It is therefore recommended to make aliquots, to avoid repeated freezing and thawing. For the same reason one should not heat the buffer in order to try to dissolve any precipitates, as ATP will be degraded.

►The optimal **molar ratio** of insert:vector is about 3:1 (ranging from 1:1 to 5:1). In order to determine the required volume of insert, one must first determine/estimate the DNA concentration of the purified PCR product. There are several options, such as visual comparison with a DNA mass standard on an agarose gel or using a Nanodrop™ spectrofotometer. In case of a 3:1 ratio, the amount of insert to be used, depending on its size (bp) is then determined as follows:

ng of insert = 3x [(50ng pGRS TA Easy) x size insert (bp) / (3015bp (size vector)]

Example: PCR product has a size of 1500bp and its concentration is 40ng/µl:

ng of insert = 3x [(50ng pGRS TA Easy) x 1500bp) / (3015bp (size vector)] = 74.6ng

and the required volume is 74.6ng /40ng/µl = 1.87 µl → 2 µl

Transformation:

Carry out the transformation as normal: add 10 µl of the ligation mixture (or control mixture) to 50µl of competent cells (already placed on ice). The use of competent cells with a transformation efficiency of at least $5x10^7$ cfu/µg is strongly recommended, especially in case of cloning of long PCR products (>2kb). Mix gently by tapping on the tube (do not pipet up and down) and incubate for 30 minutes on ice. Incubate for 45 seconds at 42ºC (heat-shock) and place the cells immediately back on ice for 2 minutes. Add 250µl of non-selective medium (e.g. SOC or LB) and incubate at 37ºC (with shaking) for 1 hour. Spread 100-200µl (or all) on pre-warmed selective plates (e.g. LA containing ampicillin for selection and IPTG/X-gal for blue white screening) and incubate at 37ºC, overnight. In case of expected higher transformation efficiency, spread 100µl of a 10 fold and a 100 fold diluted (with SOC or LB) cell suspension onto the selective plate to ensure well separated individual colonies. Include untransformed cells as a negative control. If colonies appear this might indicate contamination of the competent cells, or alternatively, degradation of antibiotics. It is also recommended to include a transformation with a control vector, such as pUC19, to ascertain cell competence.

►Transformation by electroporation should be carried out using manufacturer´s instructions.

Verification:

Analyze 5-10 white colonies, independently, by transferring half of the cells (keep remaining cells for future use, if desired) to 5µl of ultrapure water. Use 1µl of this mixture as template for a 25µl PCR reaction with pUC/M13 primers. Cycling conditions depend on the polymerase to be used, and extension time on the insert size. Confirm expected size by DNA agarose gel electrophoresis and analyze positive clones by RFLP and/or DNA Sequencing using either pUC/M13 primers. Control DNA is included in this kit, which can be amplified with pUC/M13 primers (not included).

pUC/M13 primers:
Forward: 5´-GTAAAACGACGGCCAGT-3´ (Tm=52ºC)
Reverse: 5´-AGGAAACAGCTATGACCATG-3´(Tm=58ºC)

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