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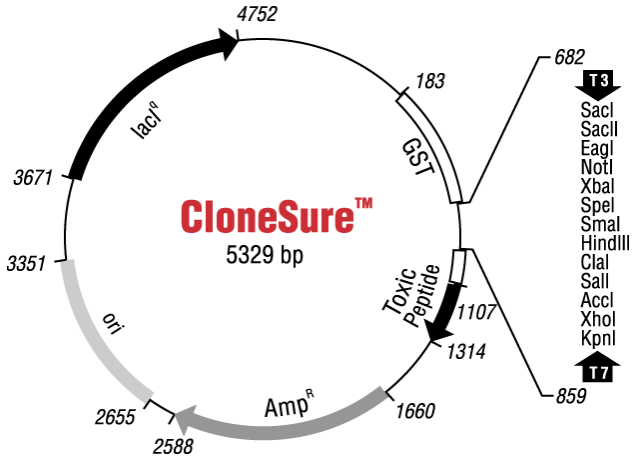
CloneSure™ pGATA Positive Selection Vector
Instruction Manual

Product: **CloneSure™ pGATA Positive Selection Vector** (10 µg, 0.5 µg/µl, enough for 100 ligations)
Product No: **PGATA10**
Procedure: **Background-Free Cloning (34.7)**
Kit Storage: **-20°C**
Stability: **Performance of this product is guaranteed for 2 years from date of purchase**

INTRODUCTION

CloneSure™ is a general cloning vector which contains a gene coding for a toxic peptide that inhibits bacterial growth when expression is induced with IPTG. Ligation of a DNA fragment into the multiple cloning site present in this vector disrupts the gene coding for the toxic peptide. The recombinant molecules are then non-toxic, while the vector, in the absence of insert, produces a toxic peptide. This constitutes a '**positive selection system'** for recombinant molecules.



BACKGROUND

Most prokaryotic cloning vectors that are in common use today provide a marker gene (ampicillin, kanamycin, tetracycline, etc.) to select against the growth of cells that do not contain the vector; however, cells will grow if they harbor the vector whether or not they contains the DNA fragment one is attempting to clone. To select the bacteria containing recombinant molecules, a second selection system has to be used that will discriminate cells with recombinant molecules from cells containing non-recombinant molecules. A type of positive selection system that has seen wide use is the blue-white color selection based on the ß-galactosidase enzyme that is available in pUC vectors and their derivatives. Since there is no growth selection against vector without insert in this case, the vector must be completely digested and dephosphorylated for cloning purposes. A second generation of positive selection systems is based on the inactivation of a toxic protein by the cloning process. With this new generation of vectors, bacterial cells containing vector without insert are killed by the toxic peptide and only cells containing recombinant molecules can grow on the selective media.

CloneSure™ is based on the toxic effect of the DNA binding domain of the mouse eukaryotic transcription factor GATA-1. This peptide consists of a zinc finger followed by a region rich in basic amino acids. Trudel *et al.* (1) have shown that the toxic peptide is able to bind to the bacterial origin of replication. The kinetics of the toxic effect is compatible with an inhibition of all new rounds of DNA replication. The major site of action is most probably at the initiation step of DNA replication. The existence of other sites of interaction cannot be excluded, but if they exist, their presence is masked by the very rapid arrest of the cell's growth.

DESCRIPTION

CloneSure™ is a 5329 bp vector. It contains a gene coding for a portion of the DNA binding domain of the transcription factor GATA-1 fused with a disrupted glutathione-S-transferase gene (GST). The sequence of GST was interrupted by the in-frame insertion of a multiple cloning site at the glutathione binding site. This fusion protein constitutes the toxic peptide. Expression of the toxic peptide is under the control of the very strong *tac* promoter. Induction is carried out with the common and inexpensive synthetic inducer isopropyl-ß-D-thiogalactoside (IPTG).

CloneSure™ also contains the strong $lacI^q$ repressor gene to reduce the leaky expression of the fused protein to a level that is not toxic for the bacteria. The multiple cloning site present in CloneSure™ contains 13 unique restriction sites (see restriction enzymes underlined in the following section) and it is flanked by T3 and T7 RNA polymerase promoters. Furthermore, CloneSure™ contains the ampicillin resistance gene and a high copy number origin of replication (coIEI type).

RESTRICTION SITES

The following catalog of restriction sites should only be used as a general reference. For precise and updated information we recommend you perform your own restriction list from the sequence. To receive the complete CloneSure™ transfer vector sequence contact technical support through info@purebiotechllc.com. This catalog of restriction sites was generated using DNA Star's Edit Seq (version Windows 3.89) and Map Draw (version Windows 3.08b). Only commercially available enzymes have been selected and displayed in the present lists.

<u>One Site</u>

Aat II (1605), <u>Acc I (804),</u> Acc65 I (824), AlwN I (3003), Bal I (465), Bbe I (4671), Bsa I (2458), BsaA I (1506), BsaM I (1178), BspM I (62), BssH II (4439), BstE II (4209), Bsu36 I (5121), <u>Cla I (795),</u> Csp45 I (655), EclHK I (2524), <u>Eco52 I (739),</u> EcoICR I (724), EcoN I (268), Ehe I (4669), <u>Hind III (788),</u> Hpa I (4534), Kas I (4667), <u>Kpn I (828),</u> Mlu I (4028), Nar I (4668), <u>Not I (739), Sac I (726), Sac II (733), Sal I (803),</u> <u>Sma I (766),</u> <u>Spe I (752),</u> Tth111 I (1499), Van91 I (3610), <u>Xba I (746), Xho I (809),</u> Xma I (764).

<u>Two Sites</u>

Apa I (822, 4239), Ava I (764, 809), BamH I (758,1107), Bcl I (869,4042), Bgl I (2406,5049), BsmF I (245, 5126), Bsp120 I (818,4235), BspLu11 I (496, 3412), Cfr10 I (2439, 3714), Drd I (1447, 3310), Dsa I (730, 5229), Eco57 I (1858, 2870), EcoR I (776, 1321), EcoR V (784, 4478), Esp3 I (1395, 4643), Fsp I (2301, 5039), Pst I (774,2282), Pvu I (2155,5020), Sap I (337, 3529), Sca I (1008, 2043), Ssp I (166, 1719), Xmn I (651, 1924).

<u>Three Sites</u>

Afl II (496, 3412, 4028), Ban II (726, 822, 4239), Bbs I (1541, 4174, 4513), Bcg I (322, 2020, 4320), BspH I (1579, 1684, 2692), BssS I (1548, 1855, 3239), Eco0109 I (290, 818, 1544), Nsp I (500, 1391, 3416), Psp1406 I (1922, 2295, 3690), Pvu II (4628, 4721, 4989), Xcm I (3884, 4400, 4418).

<u>No Sites</u>

Acc III, Age I, Asc I, Avr II, Bgl II, BsaB I, BseR I, BsiW I, BsrG I, Bst1107 I, Bst98 I, Csp I, Dra III, Eco47 III, Eco 72 I, Fse I, Mun I, Nae I, Nco I, Nde I, NgoM I, Nhe I, Nru I, Nsi I, Pac I, Pme I, Ppu10 I, PpuM I, PshA I, SanD I, SexA I, Sfi I, Sgf I, SgrA I, SnaB I, Sph I, Srf I, Sse8387 I, Stu I, Sty I, Swa I.

METHODS

In most standard cloning protocols, the vector termini are dephosphorylated after digestion to prevent religation of the vector. It is also necessary to ensure that the vector is completely digested. This is done by using a large excess of restriction enzyme and/or by purifying the digested form of the vector. To have sufficient vector DNA to perform all ligation experiments and controls, it is common to start with 10 to 100 µg of vector DNA. These steps are time-consuming, and dephosphorylation of the vector reduces the cloning efficiency considerably. It is therefore difficult to clone DNA fragments, which are available in limited quantities.

Use of the CloneSure™ pGATA positive selection vector streamlines your cloning strategy by alleviating the above requirements. The quality and quantity of your vector preparation is not critical. DNA from standard, or even 'dirty', minipreps can be used because partial digestion is not a problem. Since the only manipulation is to extract the restriction enzyme used to cut the vector, loss of starting material is reduced to a minimum and a cloning experiment can be done with less than 1µg of starting material.

Cloning of a DNA fragment into a single restriction site

Preparation of the vector for a cloning experiment

1. Digest CloneSure™ with the restriction enzyme of your choice within the multiple cloning site.

2. Extract the enzyme with phenol-chloroform or then precipitate DNA and redissolve at a concentration of 100 ng /µl.

The vector is now ready to use. For routine sub cloning, we do not recommend dephosphorylation or gel purification of the linear form.

Preparation of insert fragment

The following conditions must be met by your sequence to disrupt the toxic peptide:

- the sequence must contain a stop codon
  or
- the sequence must introduce a frame shift

There is no minimum requirement for fragment length as long as the above criteria are met.

The insert DNA fragment should be gel purified only if it was retrieved from another vector.

Ligation

We recommend the use of 100 ng of vector per reaction and a 2:1 molar ratio of insert to vector using standard ligation reaction conditions.

$$\text{Quantity of insert (ng) for a 2:1 insert:vector ratio} = \frac{\text{length of insert DNA fragment in bp} \times 100 \text{ ng (vector)} \times 2}{5329 \text{ (length of vector in bp)}}$$

If insufficient insert DNA is recovered for the ideal insert:vector ratio of 2:1, do not worry. The production of the desired recombinant molecules is obtained at a yield of greater than 50% even when the insert:vector ratio is 1:10.

> **NOTE:** An accepted standard control used with vectors without positive selection is to perform a ligation reaction with the vector alone. Since CloneSure™ is not dephosphorylated, a high number of colonies will be observed if you perform this control. This background is generated by the ligation of two molecules of CloneSure™ vector in reverse orientation followed by a gene rearrangement to generate a stable molecule. The ligation of the vector to itself is not a good control with a positive selection vector since the frequency of the mechanism described above is negligible when an appropriate amount of insert is present.

Transformation

Any transformation protocol can be used. Since the vector is not dephosphorylated, the number of colonies should be about ten times greater than obtained with a dephosphorylated vector. It is a good practice to prepare plate dilutions of your transformation reaction in order to obtain well-isolated colonies and avoid unnecessary and time consuming rounds of purification. There is no need to use a special bacterial strain since CloneSure™ contains the $lacI^q$ gene.

Selection

The selection medium consists of your currently used medium to which 80 to 150 μM IPTG is added. Add the IPTG before pouring the plate. Small variations in the optimal amount of IPTG have been observed between different strains of *E. coli*. If your application requires optimization of the GATA selection system with your bacterial strain, you can determine the optimal amount of IPTG by plating dilutions of an overnight culture on plates containing different concentrations of IPTG. We recommend trying 100 μM of IPTG as a starting point.

Typical Results

After ligation of 100 ng of CloneSure™ with an insert:vector ratio of 2:1 in a 10 μl reaction volume, transformation of DH5α competent cells (efficiency of 3×10^7 transformants/μg DNA) typically yields more than 10^4 colonies and greater than 90% recombinants.

Directional cloning

To perform a directional cloning, digest CloneSure™ with the two restriction enzymes of choice. Extract the enzymes with phenol-chloroform. Precipitate DNA and redissolve at a concentration of 100 ng/μl. The vector is now ready to use. There is no need to purify the vector from the small DNA fragment that was originally present between the two restriction sites. The re-insertion of this fragment will reconstitute the vector, and this molecule will be selected against. It is important to use an insert:vector ratio of 2:1 in order to obtain a good number of recombinant molecules.

Production of a cDNA library without background

A good cDNA library is characterized by a complete representation of the different mRNAs normally present in the cell line or tissue and the lowest possible background of vectors without insert. For this

reason, CloneSure™ can be used to produce excellent cDNA libraries. In this special situation where the positive clones-to-background ratio must be maximized, it would be advisable to ensure that the ratio of insert to vector is at least 2:1. Moreover, dephosphorylation of the vector also lowers the background to less than 5%, however the cloning efficiency is also reduced.

TROUBLESHOOTING

1. The positive selection system will not work with very short DNA fragments that do not introduce a stop codon or frame shift of the fusion protein. If you try to clone this kind of fragment, a very small number of colonies will grow. These represent mostly background of mutated vector molecules and not recombinants.

2. Degradation of vector termini by nucleases is the most probable source of problem if a high level of background is observed. Use autoclaved reagents and/or a new lot of phenol and/or enzyme to prepare the vector and perform the ligation.

3. The use of old and/or heat inactivated IPTG or limiting concentration of IPTG in the medium can result in a 'lawn' of cells on the plate.

4. The frequency of mutations in bacterial cells is low but bacterial cells containing vector molecules mutated in the GST-GATA gene will grow on the selective medium and produce background. If standard microbiological techniques are used to maintain the bacterial stock and to produce the plasmid, the number of mutated vectors will never generate background greater than 10%. Use of CloneSure™ bought from PureBiotech, LLC will guarantee consistent results with low background since each lot is rigorously tested for low background cloning efficiency.

Reference:

Trudel, P., Provost, S., Massie, B., Chartrand, P., and Wall, L. (1996) pGATA: a positive selection vector based on the toxicity of the transcription factor GATA-1 to bacteria. *BioTechniques* 20, 684-693.

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